Final Report (revision 1)
22.03.2025

REPORT

Clarification on the differences in content of *Withania somnifera* (ashwagandha) roots and leaves

Summary

[redacted]

[redacted] has not been involved in conducting any of the studies but bases its assessment entirely on data provided by the client as it is, including e.g. scientific articles, study reports, analysis results and, in some cases, preliminary reports, study summaries, or personal communications. Please note that [redacted] experts have used the data as received, without reviewing the raw data or conducting an in-depth quality analysis of the information. Therefore, the responsibility of the accuracy of the information lies with Ixoreal Biomed Inc.

[redacted]

Summary

In this summary, [redacted] reviewed the following information, namely:

Alkemist Labs 2025. Identification, Characterization, and Detection of Adulteration in Ashwagandha (*Withania somnifera*) Root and Leaf, using HPTLC and HPLC.

Chatterjee *et al*. 2010. Comprehensive metabolic fingerprinting of Withania somnifera leaf and root extracts.

Javad *et al*. 2021. Chemical Composition, Biological Activity, and Health-Promoting Effects of Withania somnifera for Pharma-Food Industry Applications.

Singh *et al*. 2023. Estimating the production of withaferin A and withanolide A in Withania somnifera (L.) dunal using aquaponics for sustainable development in hill agriculture.

Please see the enclosed attachments.

Ashwagandha (*Withania somnifera*) is a common herbal medicine used throughout the world. Extracts prepared from the leaves and/or roots of the plant are used in products sold to consumers, such as teas and food supplements. While concerns over the safety of ashwagandha has arisen, there currently has not been enough discussion of the differences of composition between root and leaf extracts, and the resulting differences in effects to consumers.

This summary presents the major chemical compositional differences of *Withania somnifera* leaves and roots.

Ashwagandha has numerous different chemical constituents, of which alkaloids, withanolides and withaferins are considered to be the biologically relevant compounds (Javad et al, 2021). Another compound, withanone, has been reported only in leaves of *W. somnifera* and has been shown to form DNA adducts under the conditions of limited antioxidants (Siddiqui et al, 2021, as reported in the Dutch risk assessment (RIVM letter report 2024-0029)). Withaferin A is known for its cytotoxic properties (Annex 1). The studies on the aerial parts of *W. somnifera* have demonstrated a reduction in primary and secondary spermatocytes, mature sperm count, and testes weight. In contrast, multiple studies in both humans and animals have shown that the root normalizes sperm concentration, count, motility, ejaculate volume, and serum testosterone levels (Annex 1).

An evaluation of the differences in components of ashwagandha roots and leaves using NMR and HPLC was conducted by Chatterjee et al. in 2010. The authors studied metabolic fingerprinting in roots versus leaves by using these methods rather than quantitative concentrations although some concentration data were also reported.

While the study listed numerous amounts of different constituents, most notable were the higher amounts of withanone and withaferins present in the leaves compared to the roots. Singh et al (2023) conducted studies on extracts of ashwagandha and noted drastic differences in the levels of withaferin A and withanolide A between the roots, stems, and leaves (Figure 1).

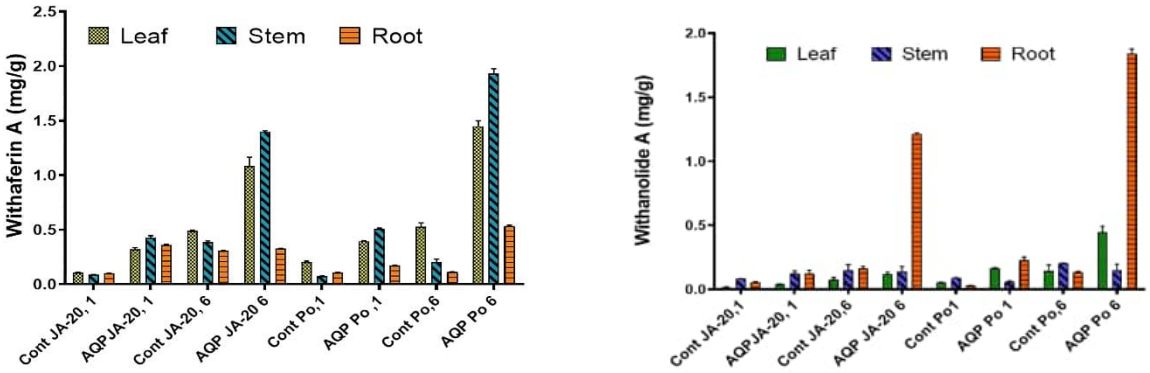


Figure 1. Differences in amounts of withaferin A and withanolide A between the roots, stems, and leaves of Ashwagandha.

A very recent private study commissioned by Ixoreal Biomed Inc to Alkemist Labs, Inc. (located in the USA) also confirmed the findings of Singh et al (2023). Both HPTLC and HPLC techniques were employed to analyse the compositional differences between roots and leaves. Both analytical methods underwent quality assurance evaluation, and a reference standard "USP Ashwagandha Root Powder Monograph" was used to identify and quantify key ashwagandha chemical markers in the HPLC analysis of root and leaf[1]. The amounts of withaferin A and withanolide A differed greatly between extracts of the leaf and the root (Figure 2). Additionally, a large difference was also observed in the amounts of total flavonol glycosides between the two plant parts (Figure 3). Similarly, the HPTLC analyses demonstrated a clear compositional difference between the roots and leaves.

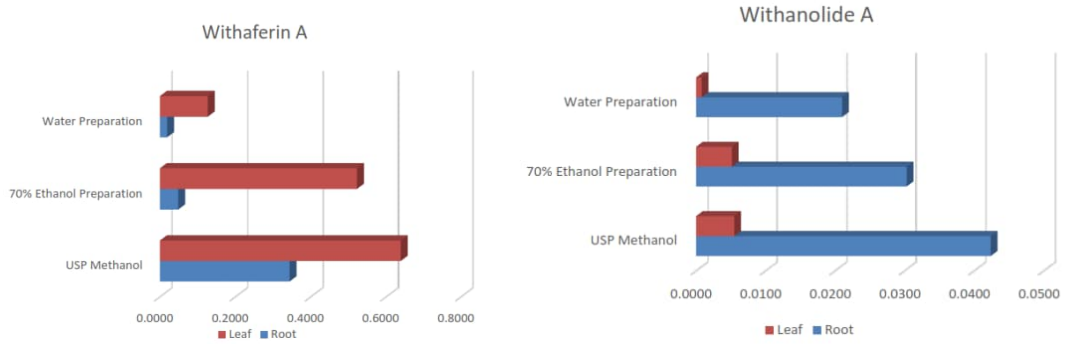


Figure 2. Differences in amounts of withaferin A and withanolide A between the roots and leaves of Ashwagandha.

[1] It was noted, however, that the Alkemist report did not report the units for the concentrations analysed. Nonetheless, the differences in the key chemical contents between roots and leaves can be clearly observed.

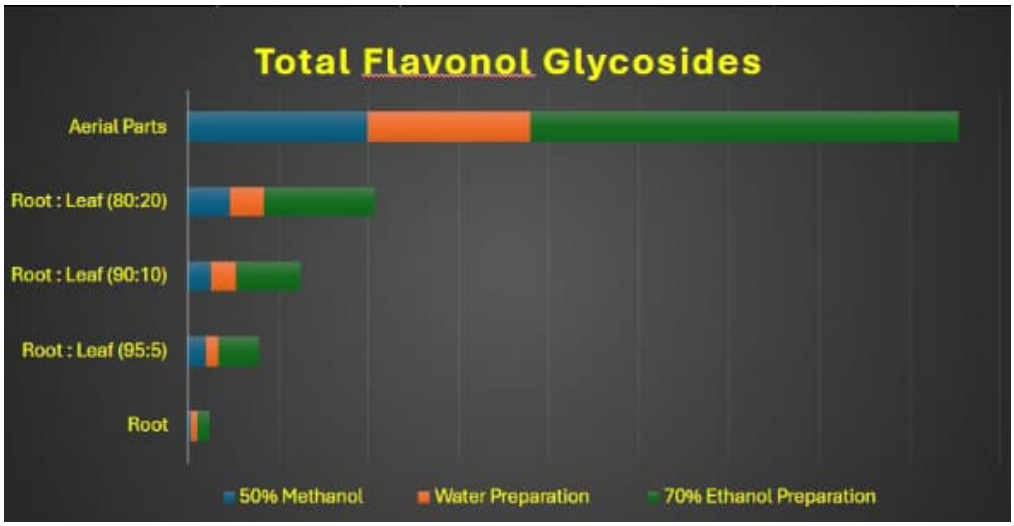


Figure 3. Differences in total flavonol glycosides between different extractions of Ashwagandha roots and leaves.

While the root and leaf are both part of the same plant, the data presented above show stark differences between the composition and amounts of chemical constituents in the extracts of each. Thus, it is imperative that the chemical composition of each plant part is well characterised and quantified. Based on the aforementioned data, it can be concluded that the chemical composition of roots and leaves are clearly very different and consequently, the toxicological profiles are also different. **Thus, the roots and leaves should be considered as two separate entities in risk assessment and any risk management.**

Annex 1. Presentation: Roots Vs Leaves Addressing the Concerns on Ashwagandha Safety. By Ixoreal Biomed Inc, 17th March 2025.

Annex 1

Roots Vs Leaves
Adressing the Concerns on Ashwagandha Safety

By Ixoreal Biomed Inc

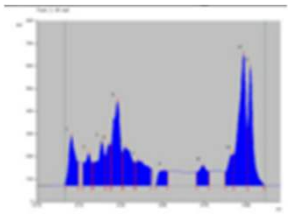
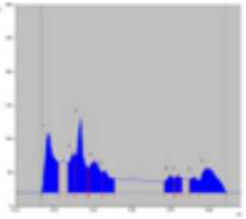
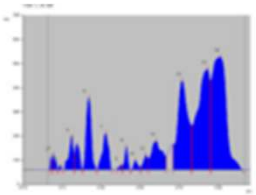
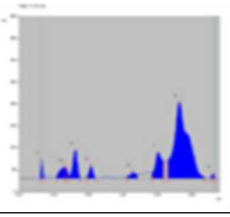
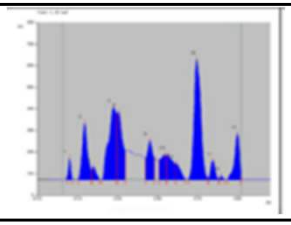
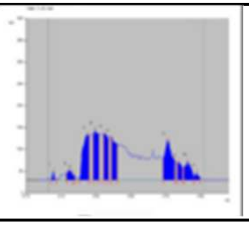
17th March 2025

Roots Vs Leaves – Regulators from Different Countries

Agencies	Comments
IP/IAP (India)	Root with total withanolides content >0.3% (IP) or >0.2% (IAP)
USP (DSC-USP 2015) (USA)	Roots/root powder; total withanolides >0.3%
B.P. (UK)	Roots containing >0.1% withanolides
Bfr (Germany)	Mainly root is used. Few preparation from leaves are used.
GIS (Poland)	Powdered root
RIVM (Netherlands)	Root powder
HoA (EU)	Containing root, herb preparation or extract thereof
DTU (Denmark)	Withanolides and alkaloids are present in other plant parts and hence considered to understand possible effects of the root

Roots Vs Leaves– Are these parts similar?

Qualitative Differences (Sahoo et al., 2024) – Many more peaks in leaves than roots

Leaf	Root
Chromatogram under UV-254 nM	
	
Chromatogram under 520 nM afer derivatization with anisaldehyde sulphuric acid	
	
Chromatogram under 520 nM afer derivatization with Dragendorff reagent	
	

Roots Vs Leaves– Are these parts similar?

Qualitative Differences (Javed et al., 2021)

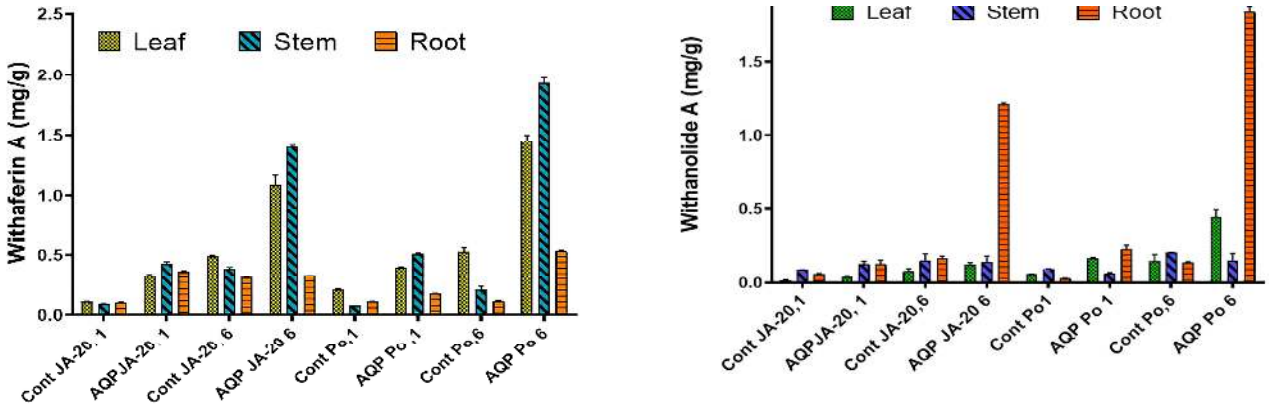
List of selected withanolides and other compounds identified from *Withania somnifera* (L.) Dunal.

Plant parts	Compounds	References
Roots	Withanolide A, withanolide B, 27-hydroxy withanolide B, withanolide D, withaferin A, 16β-acetoxy-6α, 7α-epoxy-5α–hydroxy-1-oxowitha-2, 17 (20), 24-trienolide, 5, 7α-epoxy-6α, 20α–dihydroxy-1-oxowitha-2, 24-dienolide	[44]
	Withanoside I, withanoside II, withanoside III, withanoside IV, withanoside V, withanoside VI, withanoside VII, withaferin A, physagulin D, coagulin Q	[45]
	Withasilolide A, withasilolide B, withasilolide C, withasilolide D, withasilolide E, withasilolide F	[47]
	Withanolide E, withanolide F, withanolide G, withanolide H, withanolide I, withanolide J, withanolide K, withanolide L, withanolide M	[48]
	Withanolide Q, withanolide R	[49]
	Withanolide E, withanolide F, withanolide S, withanolide P	[48]
	Withanolide T, withanolide U	[50]
	Glucosomniferanolide	[51]
Stem bark	Withasomnilide, withasomniferanolide, somniferanolide, somniferawithanolide, somniwithanolide	[52]
Leaves	Withanolide C, 4-deoxyphysalolactone	[46]
	(20R, 22R)-14α, 2OαF-dihydroxy-1-oxowitha-2, 5, 16, 24-tetraenolide	[53]
	Withaferin A	[54]
	24,25-Dihydrowithanolide A, withanolide A, withanone, withaferin A, 27-hydroxy withanone, and 17-hydroxy withaferin A, 27-deoxy-16-en-withaferin A, 2, 3-dihydro-3β-hydroxywithanone, 2,3-dihydro withanone-3β-O-sulfate	[55]
Fruits	24,25-Dihydrowithanolide VI, withanoside IV, withanoside V, withanoside VI, withanamide A, withanamide B, withanamide C, withanamide D, withanamide E, withanamide F, withanamide G, withanamide H, withanamide I	[56]

Roots Vs Leaves– Are these parts similar?

Quantitative Differences

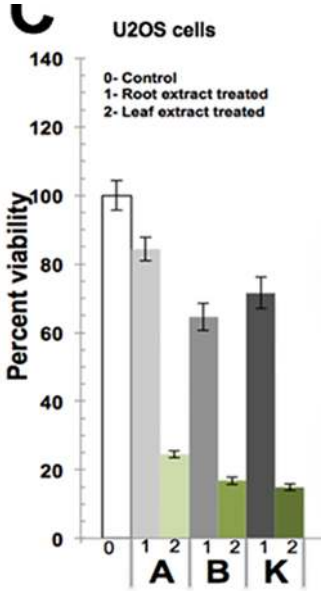
Substance	Roots	Leaves	Reference
Withaferin A (mg/g DW)	0.53	10.10	Kaul et al., 2016
	0.92	22.31	Chatterjee et al., 2010
Withanone (mg/g DW)	1.57	18.7	Kaul et al., 2016
	0.50	3.61	Chatterjee et al., 2010



Withaferin A and Withanolide A content in root, leaf and stem (Singh et al., 2023)

Roots Vs Leaves– Are these parts similar?

Bioactivity



Kaul et al., 2016
(1.0 mg/ml)

Plant Part	HepG2	L929
Leaf	43.48	78.77
Stem	45.6	90.55
Root	314.4	361.21

IC_{50} values (μg/ml) (Lingfa et al., 2023)

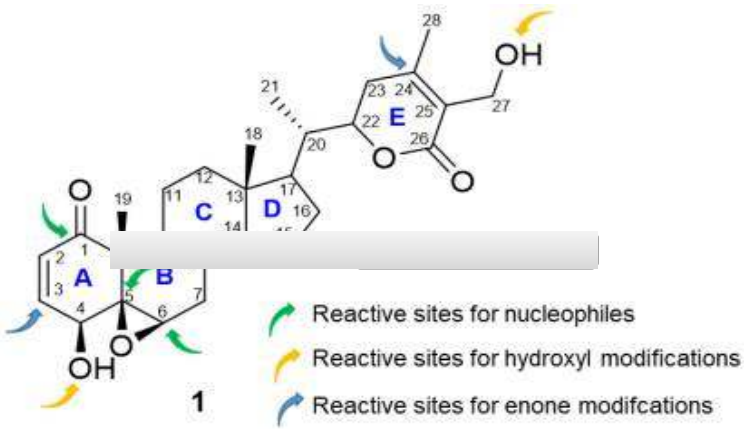
Roots Vs Aerial Parts– Are these parts similar?

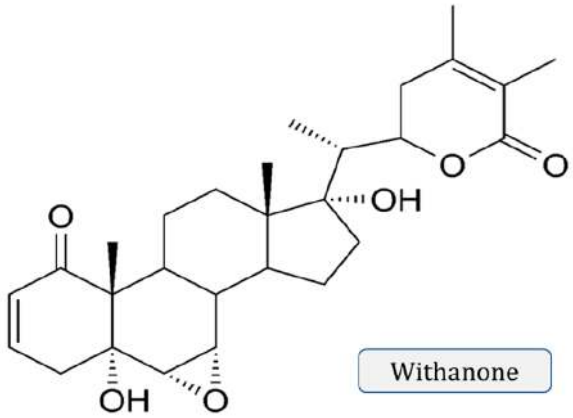
Bioactivity

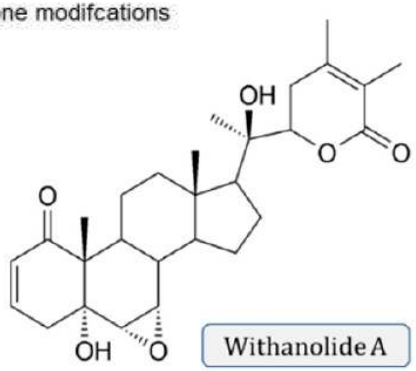
Plant Part	Effect on Sperms	Testing System	References
Roots	increase in sperm conc. Count, motility, ejaculate volume and serum test.	Rat Human	Kumar A et al., 2015; Ganu et al. 2010 Sahin et al., 2016 Ambiye et al, 2013 Sengupta et al., 2018
Fruit and Stem	Reduces primary and secondary spermatocytes, mature sperm, and testes weights	Rat In vitro	Mali PC, 2013 Singh et al., 2013,

Roots Vs Leaves – Are these parts similar?

Chemical Reactivity



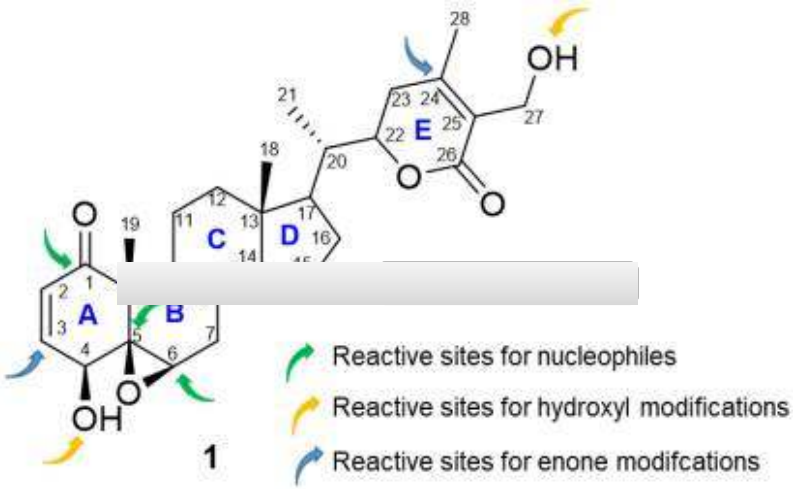


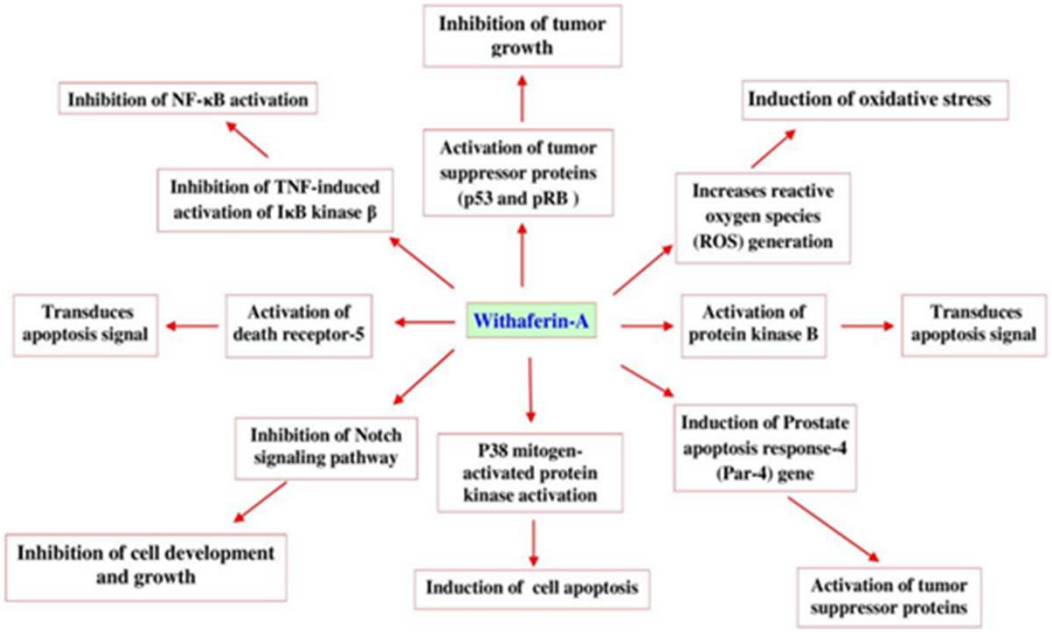


Withaferin A with oxidation at carbons 4, 5, and 6 is considered as an active type, especially as anticancer, whereas the withanolide A and withanone with oxidation at carbons 5, 6, and 7 rarely show any activity.

Roots Vs Leaves – Are these parts similar?

Chemical Reactivity

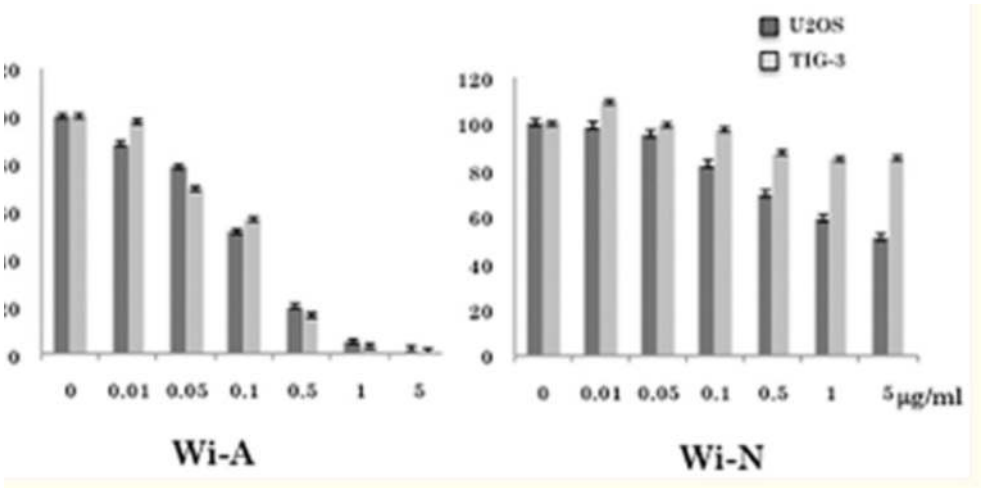




Kumar et al., 2015

Roots Vs Leaves – Are these parts similar?

Chemical Reactivity –Withanone is not cytotoxic but form adduct with amines and cause DNA damage



Withaferin A (Wi-A) and withanone (Wi-N) treated normal (TIG) and cancer cells U2OS) in MTT assay (Vaishnavi et al, 2012)

DNA damage by Withanone (Siddiqui et al., 2021)

- dG, dA, dC, and histones have primary amines; act as nucleophiles
- Withanone formed adducts with the nucleosides dG, dA, and dC and interfere with its biological property. This process is reversible and detoxified by GSH.
- Under limiting GSH levels it can cause DNA damage.
- This could be a potential mechanism for Ashwagandha-mediated liver damage.

Roots Vs Aerial Parts– Are these parts similar?

Conclusions

There is enough regulatory, qualitative and quantitative evidence which are backed by bioactivity data to prove that roots are the preferred part as food or food supplement

- Roots are rich in rejuvenating withanolides while leaves and other parts have much more Withaferin A and Withanone.
- Withaferin A has beneficial effects but is also cyto-toxic and is being developed as anticancer.
- In cancerous or compromised cells, Withaferin A has effects, different from normal cells.
- Withanone is highly reactive to form DNA adduct.

Thank you

Identification, Characterization, and Detection of Adulteration in Ashwagandha (*Withania somnifera*) Root and Leaf, using HPTLC and HPLC

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Ixoreal Summary Report

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1. Introduction

Ashwagandha (*Withania somnifera*) root is a traditional medicinal plant used for thousands of years in Ayurvedic medicine. The root has many diverse benefits for both physical and mental health. Ashwagandha root product with undeclared leaf material is a known issue in the herbal supplement industry. Advanced analytical methods like High-Performance Thin-Layer Chromatography (HPTLC) and High-Performance Liquid Chromatography (HPLC) are used to identify and characterize Ashwagandha root and hence, prevent adulteration with Ashwagandha leaf.

This report summarizes the Identification and Characterization of Ashwagandha (*Withania somnifera*) Root and Leaf by HPTLC and HPLC. It provides scientific evidence that not only can the root and leaf be identified and distinguished but also demonstrates the ability to detect adulteration at low levels. HPTLC is a sensitive technique used to generate a phytochemical constituent profile (fingerprint) and identify compounds that distinguish between the different plant parts. HPLC is another powerful technique used to analyze the chemical composition of ashwagandha products to identify and quantify specific compounds and provide supporting evidence/orthogonality for both methods used.

2. Study Design:

The Ashwagandha Identification study was performed as described below in Table 1 using the two (2) proposed methods listed in Table 2. Two (2) types of extracts, 1. aqueous extracts and 2. hydroethanolic extracts, were evaluated and compared using the two methods. Limit of detection of leaf in root using Method 2 (aerial) was performed. In addition to 100% root and 100% leaf materials, three (3) levels of leaf (5%, 10%, and 20%) in root were analyzed. The study parameters were conducted according to items listed below using experimental methods developed by the Alkemist Laboratory's Botanical Identity Department.

The following parameters were determined during this Ashwagandha study:

- **System Suitability:** System suitability is used to establish that the performance of the method is stable and has not changed for the given set of chromatographic conditions. This is determined based on the resolution of one or more reference substances or the sequence of bands in the chromatogram of the reference substances remain the same.
- **Sensitivity (True Positive Result):** The sensitivity measures how well the method identifies and classifies the botanical of interest when compared to authenticated botanical reference materials. Chromatographic fingerprints of test samples are compared with respect to number, position, color, and intensity of bands. Natural variability is considered by comparing with more than one representative botanical reference sample.

- **Specificity (True Negative Result):** The specificity of a method ensures that the botanical of interest is distinguishable from materials that truly are not the botanical of interest. Chromatographic fingerprints of test samples are compared with respect to number, position, color, and intensity of bands. Natural variability is considered by comparing with more than one representative non-botanical of interest reference sample.
- **Robustness Testing:** Refers to the ability of a method to tolerate variations of parameters without significant changes in the result. Botanical test materials were extracted in different solvents to confirm the performance of the HPTLC identification method.

Table 1: Ashwagandha Identification Study Summary

Validation Element	Experimental Design	Acceptance Criteria	Pass/Fail
System Suitability	The experiment is executed according to the selected test methods (see Table 2).	The profile is valid if the bands representing Withaferin A and Withanolide A are clearly distinguishable in the chromatogram of the reference standard, test sample, and botanical reference materials.	Pass
Sensitivity & Specificity	Test samples, botanical reference materials, and reference standards are chromatographed according to the method.	The method is specific if the profiles obtained with the test solutions representing Ashwagandha (*Withania somnifera*) root and leaf are not similar with respect to number and position of bands matching the chromatogram of the botanical reference materials. The color and intensity of the bands may or may not vary. Samples of other botanical identity yield different profiles.	Pass
Robustness: Extraction solvent	The methods are executed with aqueous and hydroethanolic extracts	The profiles obtained for the aqueous extracts and the hydroethanolic extracts are similar with respect to number, position, color, and intensity of bands. The R_f values for Withaferin A and Withanolide A are not significantly different if $\Delta R_f \leq 0.05$. The R_f values for Kaempferol 3-O-robinoside-7-O-glucoside are not significantly different if $\Delta R_f \leq 0.05$.	Pass
Limit of Detection (LOD):	Test LOD of aerial part in root at 5 levels: 0%, 5%, 10%, 20%, and 100% of aerial part in root for both aqueous and hydroethanolic extracts.	Confirm the presence or absence of flavonoid compounds in the 5 levels, with both qualitatively (HPTLC) and quantitatively (HPLC).	N/A

Table 2: HPTLC Methods

	Mobile Phase	Derivatization
Method 1 (for Root)	Toluene/Ethyl Acetate/Acetic Acid (5.5/4.5/0.3; v/v/v)	10% Sulfuric Acid Reagent
Method 2 (for Aerial)	Ethyl Acetate/Acetic Acid/Formic Acid/Ultra-Pure Water (10/1.1/1.1/2.6; v/v/v/v)	Natural Products Reagent Polyethylene Glycol Reagent 10% Sulfuric Acid Reagent

3. System Suitability:

The experiment is executed according to the selected test methods (see Table 2). Refer to the Appendix sections for the experimental design and HPTLC images described in the results section below.

3.1 *Root Material*

The bands for Withaferin A and Withanolide A are clearly distinguishable in the chromatograms for both *Withania somnifera* root aqueous and hydroethanolic materials in Method 1 (root). Withanolide A is present in only *Withania somnifera* root materials, as indicated by the green boxes in Images 1, 2. The absence of Withanolide A is marked by the red boxes indicative of aerial parts . The band for Withaferin A is present in both root and leaf materials and therefore is not a discriminating marker for plant part differentiation.

3.2 *Leaf Material*

The bands for the flavonoid compounds are clearly distinguishable in the chromatograms for both *Withania somnifera* root aqueous and hydroethanolic materials in Method 2 (aerial). Flavonoid compounds are visible in the ranges of Rf 0.1-0.2 and Rf 0.3-0.45, as indicated by the green boxes in Images 3 and 4. The absence of the flavonoid compounds is indicated by the red boxes.

3.3 *Acceptance Criteria*

The profile is valid if the bands representing Withaferin A, Withanolide A, various flavonol glycosides and Kaempferol 3-O-robinoside-7-O-glucoside are clearly distinguishable in the chromatogram of the reference standard and test materials, for both aqueous extracts and hydroethanolic extracts.

3.4 *Results*

The bands for Withaferin A and Withanolide A are clearly distinguishable in the chromatogram (Images 1, 2). For aqueous extracts in Method 1 (root), Withaferin A is present at R_f = 0.08 and Withanolide A is present at R_f = 0.25. For aqueous extracts in Method 2 (aerial), Kaempferol 3-O-robinoside-7-O-glucoside is present at R_f = 0.13 (Images 3, 4).

For hydroethanolic extracts in Method 1 (root), Withaferin A is present at R_f = 0.08 and Withanolide A is present at R_f = 0.26 (Images 1, 2). For hydroethanolic extracts in Method 2 (aerial), Kaempferol 3-O-robinoside-7-O-glucoside is present at R_f = 0.13 (Images 3, 4).

The summary of these values can be found in Table 3, below.

Table 3: System Suitability/Robustness, Rf Values

Compound	Method 1 (Root)		Method 2 (Aerial)	
	Aqueous	Hydroethanolic	Aqueous	Hydroethanolic
Withanolide A	0.25	0.26	n/a	n/a
Withaferin A	0.08	0.08	n/a	n/a
Kaempferol 3-O-robinoside-7-O-glucoside	n/a	n/a	0.13	0.13

4. Sensitivity and Specificity

Sensitivity (True Positive Result): Identification of Ashwagandha (*Withania somnifera*) by comparison of root material and aerial part material. **Specificity (True Negative Result):** Identification of Ashwagandha (*Withania somnifera*) root by differentiation of root material from aerial part material, including identifying the presence of aerial parts in root material to 5~10% depending on the extraction solvent, of aerial part material in root material.

4.1 *Description of experiment*

The method is executed according to Table 1. Refer to the Appendix section for the experimental design and HPTLC images described in the results section below.

4.2 *Acceptance Criteria*

Method 1 is sensitive if the phytochemical constituent (fingerprint) profiles obtained with the test material solutions for *Withania somnifera* root has the presence of Withanolide A and its fingerprint is consistent with respect to number and position of bands matching the chromatograms of root reference materials (Images 1, 2). The color and intensity of the bands may or may not vary due to natural processes. Samples of other identity yield different profiles.

Method 2 is specific for *Withania somnifera* root if the phytochemical constituent (fingerprint) profiles show the absence of the leaf marker compounds, shown in the chromatograms (Images 3, 4) and are differentiable from that of *Withania somnifera* aerial part reference material.

Method 2 is specific for *Withania somnifera* aerial parts if the fingerprints show the presence of leaf marker compounds and are differentiable from that of *Withania somnifera* root reference material (Images 3, 4). The color and intensity of the bands may or may not vary due to natural processes. Samples of other identity yield different profiles.

4.3 *Results*

The profiles of the *Withania somnifera* root reference materials show a similar phytochemical constituent profile with one another (Images 1, 2, Lanes 3,4) and can be distinguished from *Withania somnifera* aerial part reference materials (Images 3, 4, Lanes 9-11) based on a different phytochemical constituent profile, for both aqueous extracts and hydroethanolic extracts in Method 1 (root) and Method 2 (aerial).

The profile of the USP Ashwagandha root extract demonstrates some traces of flavonoid compounds in Method 2 (aerial) (Images 3, 4, Lane 2), possibly indicative of adulteration with leaf.

5. Robustness: Extraction Solvent

5.1 *Description of Experiment*

The method is executed according to Table 1. Test materials are analyzed as aqueous extracts as well as hydroethanolic extracts. Refer to the Appendix section for the experimental design and HPTLC images described in the results section below.

5.2 *Acceptance Criteria*

The profiles obtained with both extractions are similar with respect to number, position, color, and intensity of bands. In Method 1 (Root), the R_f value of Withaferin A and Withanolide A are not significantly different if $\Delta R_f \leq 0.05$. In Method 2 (Aerial), the R_f value of Kaempferol 3-O-robinoside-7-O-glucoside is not significantly different if $\Delta R_f \leq 0.05$.

5.3 *Results*

The difference in R_f compared to the average values of the aqueous extracts and the hydroethanolic extracts is ≤ 0.05 (see Table 3, above). The profiles obtained with different extractions are similar with respect to color and intensity of bands.

6. HPTLC Limit of Detection of Leaf in Root

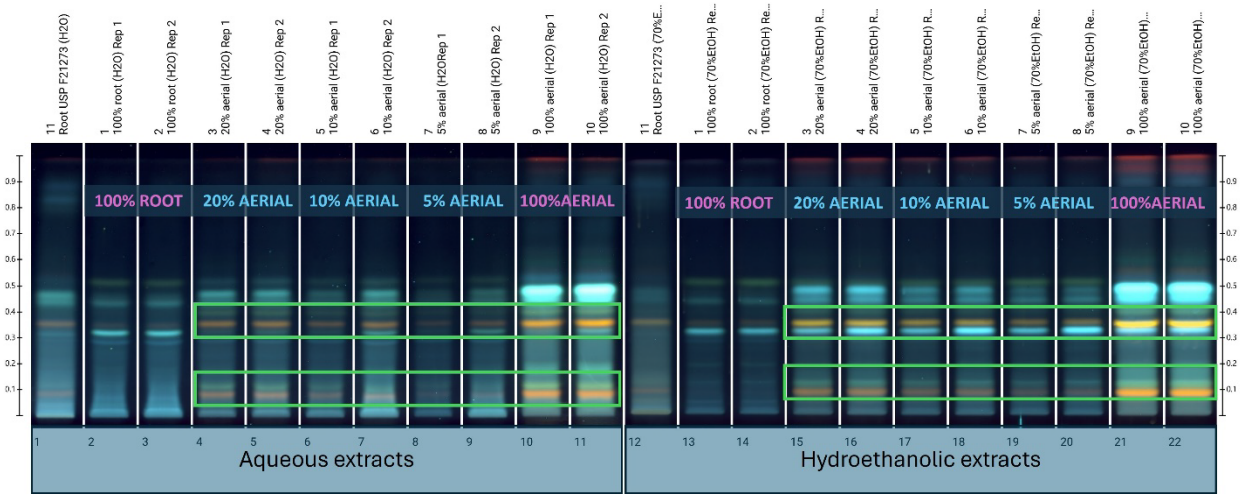
In Image 5, flavonoid compounds are detectable as low as 5% for both aqueous extracts (Lanes 8, 9) and hydroethanolic extracts (Lanes 19, 20). Flavonoid compounds are visible in the ranges of Rf 0.1-0.2 and Rf 0.3-0.45, as indicated by the green boxes.

Table 4. HPTLC Lane Assignment for LOD study

	Aqueous extracts		Hydroethanolic extracts	
Description	**Lane**	**Application Volume (µl)**	**Lane**	**Application Volume (µl)**
Root USP F21273	1	2	12	5
100% root Rep 1	2	2	13	5
100% root Rep 2	3	2	14	5
20% aerial Rep 1	4	2	15	5
20% aerial Rep 2	5	2	16	5
10% aerial Rep 1	6	2	17	5
10% aerial Rep 2	7	2	18	5
5% aerial Rep 1	8	2	19	5
5% aerial Rep 2	9	2	20	5
100% aerial Rep 1	10	2	21	5
100% aerial Rep 2	11	2	22	5

Image 5:

Method 2 (aerial); LOD of aerial part in root at 5 levels: 0%, 5%, 10%, 20%, and 100% of aerial part in root for both aqueous and hydroethanolic extracts.



7. HPLC Limit of Detection of Leaf in Root

7.1 *Description of Experiment*

To identify and quantify key ashwagandha chemical markers (Withanolides & Flavonol Glycosides) in root and leaf using the USP Ashwagandha Root Powder Monograph (Appendix 4 for reference). Additionally, to determine presence of leaf specific phytochemical markers in mixtures of root & leaf Authentic Reference material. Test materials are analyzed as USP methanol extracts, aqueous extracts and hydroethanolic extracts.

7.2 *Acceptance Criteria*

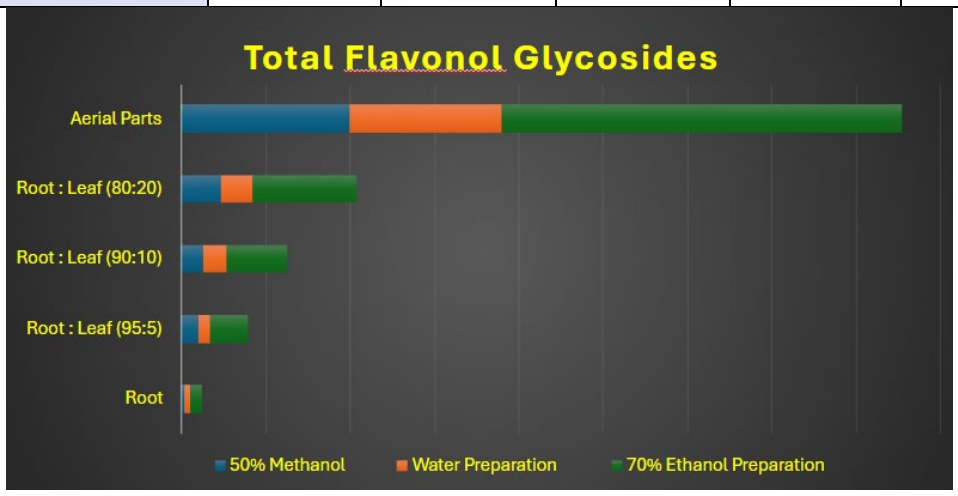
The content of withanolides and limit of flavonol glycosides derived from aerial parts were analyzed according to the HPLC conditions described in the USP Ashwagandha Root Powder Monograph (Appendix 4 for reference). Total Flavonol Gylcosides, Withaferin A, and Withanolide A were determined using three (3) different extraction methods. The extraction methods were the USP monograph methanol extraction, water extraction and hydroethanolic extraction.

7.3 *Results*

Total Flavonol Glycosides content for the three (3) different extraction methods are summarized in Table 5 and the chart below. Flavonol glycosides are compounds found in higher concentrations in the leaves of the ashwagandha plant (*Withania somnifera*). These compounds are markers used to detect adulteration in ashwagandha root products and to support and verify the HPTLC data.

Table 5: HPLC Flavonol Glycosides Results

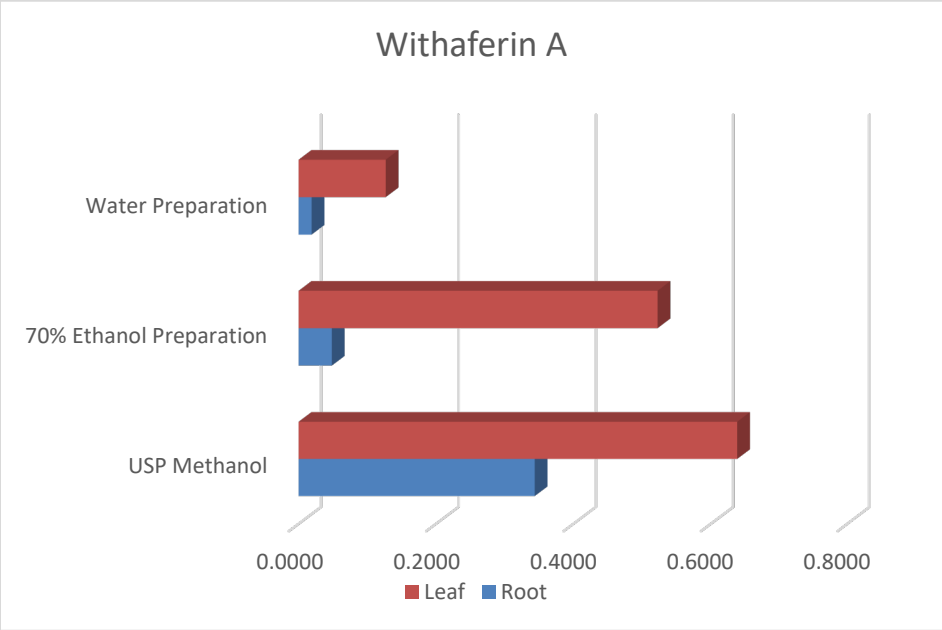
Total Flavonol Glycosides	100% Root	Root : Leaf (95:5)	Root : Leaf (90:10)	Root : Leaf (80:20)	100% Leaf
50% Methanol	0.0018	0.0107	0.0133	0.0238	0.1002
Water Preparation	0.0036	0.0064	0.0140	0.0191	0.0897
70% Ethanol Preparation	0.0074	0.0230	0.0357	0.0616	0.2376



Withaferin A content for the three (3) different extraction methods are summarized in Table 6 and the chart below. Withaferin A is a prominent bioactive compound found in the leaves of the ashwagandha plant (*Withania somnifera*). The leaves of ashwagandha contain higher levels of withaferin A compared to the roots, as seen below.

Table 6: HPLC Withaferin A Results

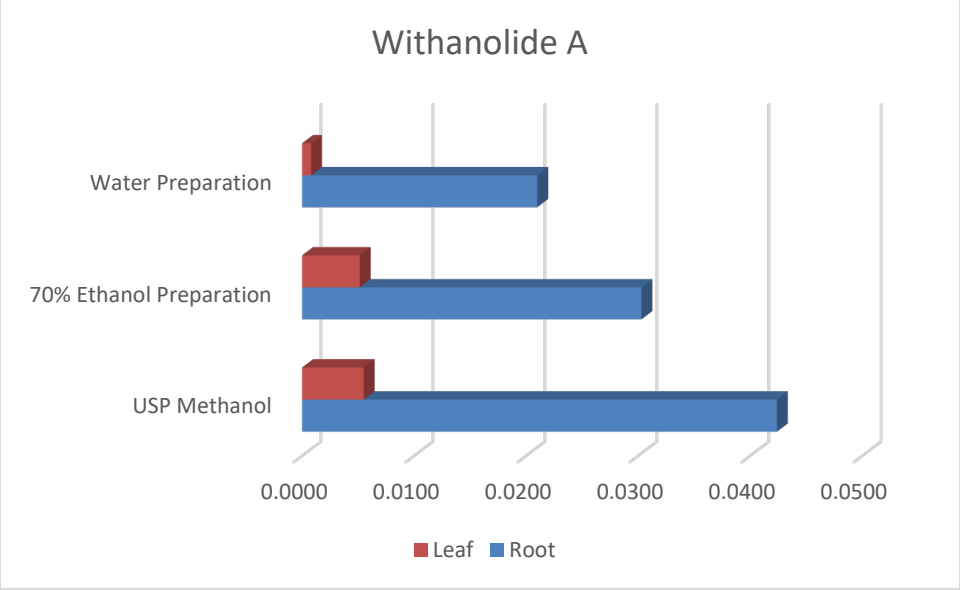
Withaferin A	Root	Leaf
USP Methanol	0.3437	0.6384
70% Ethanol Preparation	0.0484	0.5226
Water Preparation	0.0190	0.1268



Withanolide A content for the three (3) different extraction methods are summarized in Table 7 and the chart below. The root of ashwagandha contains higher levels of Withanolide A compared to the leaves, as seen below.

Table 7: HPLC Withanolide A Results

Withanolide A	Root	Leaf
USP Methanol	0.0424	0.0055
70% Ethanol Preparation	0.0303	0.0052
Water Preparation	0.0210	0.0008



8. Conclusion

This study has clearly demonstrated the ability of HPTLC to identify and characterize the Ashwagandha (*Withania somnifera*), root and leaf, as well as, differentiate the root and leaf plant parts from one another in three different extracts, Methanol, Hydroethanolic and Water extracts. We also clearly demonstrated the ability of these methods to detect the presence of Ashwagandha leaf adulteration of root, to a level of ~ 5%.

For HPTLC Method 1 (root), the intensity of the band for Withanolide A is more visible for hydroethanolic extracts than it is for aqueous extracts (See Images 1, 2). This is supported by the HPLC analysis of the increasing Withanolide A content in aqueous, hydroethanolic, and methanolic extraction solvents, seen in Table 7.

For HPTLC Method 2 (aerial), the presence of the various flavonol glycosides, not limited to the Kaempferol 3-O-robinoside-7-O-glucoside, have a greater solubility in the hydroethanolic extracts than water or methanol, which is supported with the HPLC analysis, seen in Table 5.

Images 1, 2 (Appendix 2) demonstrate the ability of the HPTLC method to identify Ashwagandha root by virtue of the presence of the Withanolide A at the R_f ~ 0.26, in Lanes 2 – 6, enclosed in the green colored box. This was confirmed by the HPLC quantitative analysis as shown in Table 7 and its associated chart for Withanolide A. By contrast, Lanes 7 – 11 (Images 1, 2) reveal the absence of the Withanolide A in the aerial parts, enclosed by the red box, and hence the identity of Ashwagandha aerial parts, also confirmed in Table 7.

Images 3, 4 (Appendix 2) demonstrate the ability of the HPTLC method to identify Ashwagandha leaf by virtue of the presence of the flavonol glycosides at the R_f values ~ 0.13, 0.95 and 0.38, in Lanes 7 – 11, enclosed in the green colored boxes. This was also confirmed by HPLC quantitative analysis, found in Table 5 and its associated chart for the flavonol glycosides. Again, by contrast, Lanes 2 – 6, (Images 3, 4) reveal the absence of the flavonol glycosides in the Ashwagandha root, enclosed by the red boxes, again confirmed in Table 5.

The results described above allow a clear determination of Adulteration of Ashwagandha root with Ashwagandha aerial parts and was demonstrated in Table 4 and Image 5 above, with the HPTLC and the HPLC analyses, revealing a Level of Detection to ~ 5% leaf in root, for all extracts.

While there is a slight presence of each of the marker compounds for aerial parts (flavonol glycosides) and root (Withanolide A) in both plant parts, the HPLC results clearly demonstrate the significant concentration differences found in each of the plant parts, thus allowing differentiation of one plant part from the other, as revealed in Tables 5, 6 and 7.

Appendix 1

HPTLC Experimental Design for Plate Lane Assignments

The same track assignments were applied to all plates. The application volumes employed throughout this validation were optimized to obtain a profile of the samples under study. The application volume of subsequent testing after validation (routine release) may or may not need adjustment; this is to ensure optimal profile is obtained for evaluation because of the natural variation of the botanical method. Testing of subsequent samples will consider the application volume employed during the validation. If an optimal profile is not obtained, the volume of application will be adjusted and documented accordingly as part of the release documentation.

Lane Assignment for Images 1 to 4

Lane	Description
1	Withanolide A
2	Root USP F21273
3	Root BRM (Botanical Reference Material) Mixture J22813NRPL1 + J15916NRPL1
4	Root BRM J12903PI
5	Root TS 1 25006YRK
6	Root TS 1 25006YRK
7	Aerial TS 2 25006UGM
8	Whole Plant TS 3 25006NQI
9	Aerial BRM J02218NRPL2
10	Aerial BRM J36110SC2
11	Aerial USP F054JO
12	Withaferin A
13	Quercetin
14	Kaempferol 3-O-robinoside-7-O-glucoside
15	Rutin

Appendix 2 – HPTLC Images

Image 1A:

Method 1 (Root) Aqueous Extracts, Derivatized in 10% Sulfuric Acid Reagent, visible light

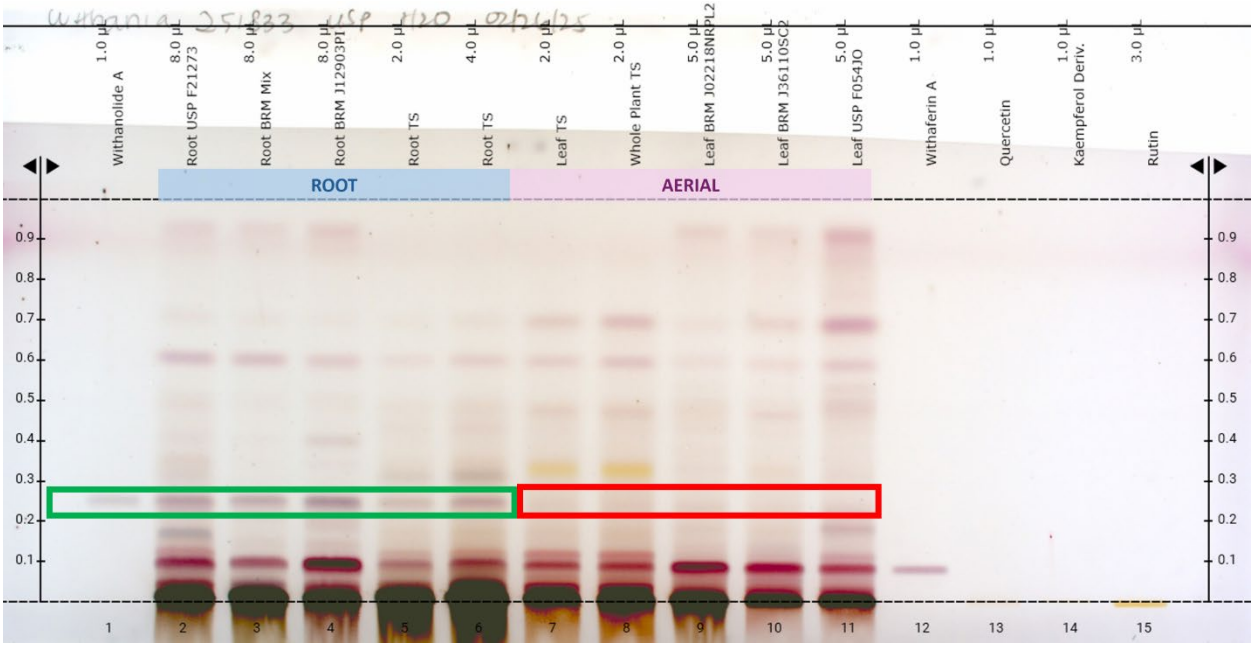


Image 1B:

Method 1 (Root) Aqueous Extracts, Derivatized in 10% Sulfuric Acid Reagent, UV 366nm

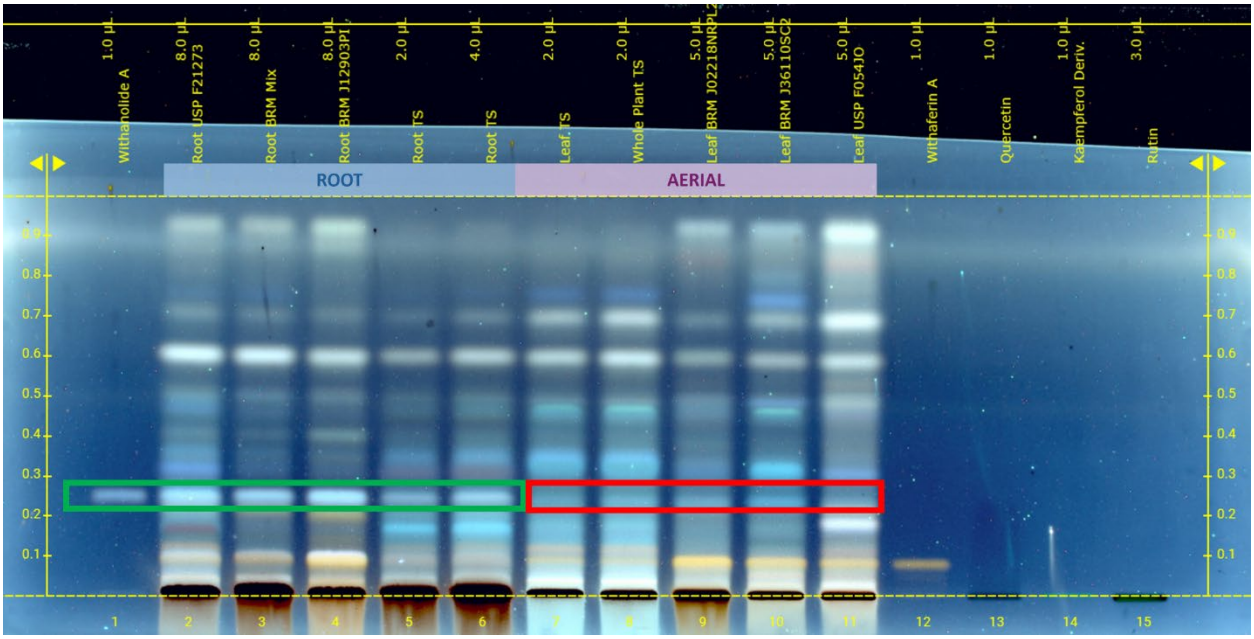


Image 2A:

Method 1 (Root) Hydroethanolic Extracts, Derivatized in 10% Sulfuric Acid Reagent, visible light

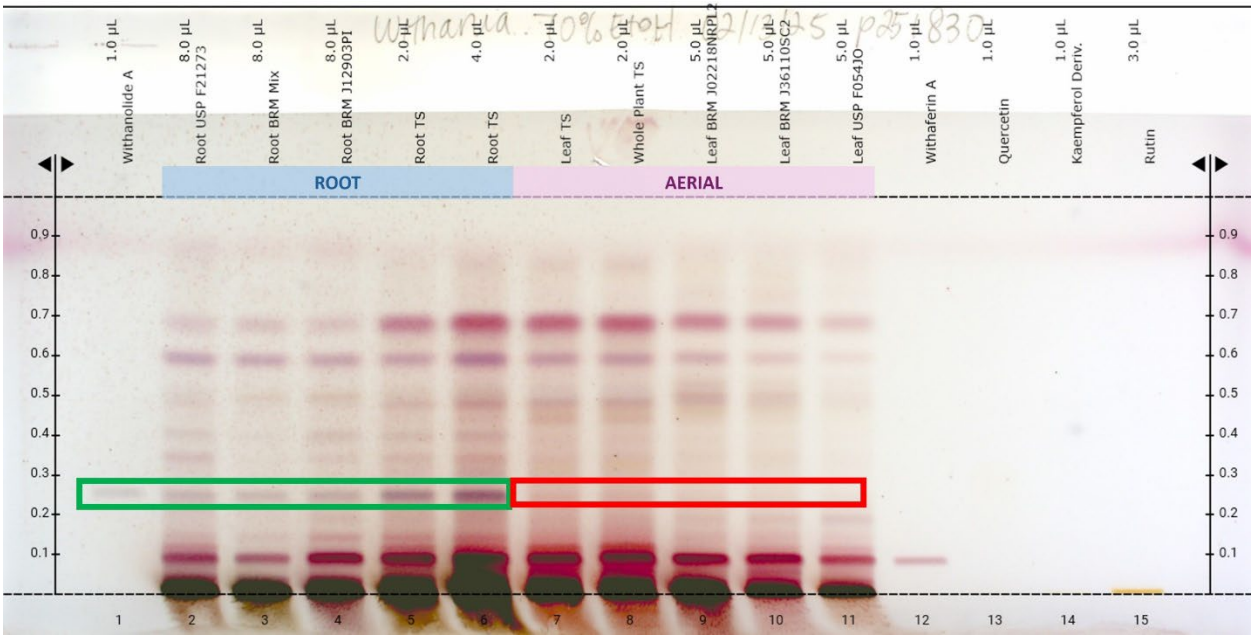


Image 2B:

Method 1 (Root) Hydroethanolic Extracts, Derivatized in 10% Sulfuric Acid Reagent, UV 366nm

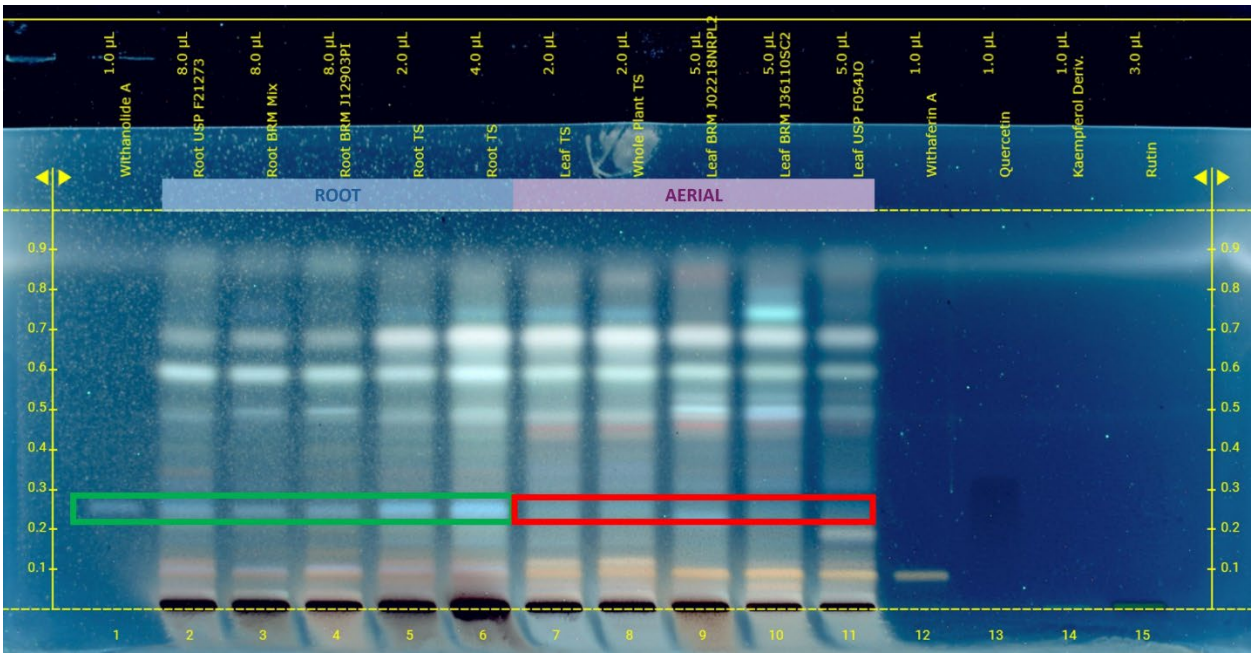


Image 3:

Method 2 (Aerial) Aqueous Extracts, Derivatized in Natural Products & PEG Reagent, UV 366nm

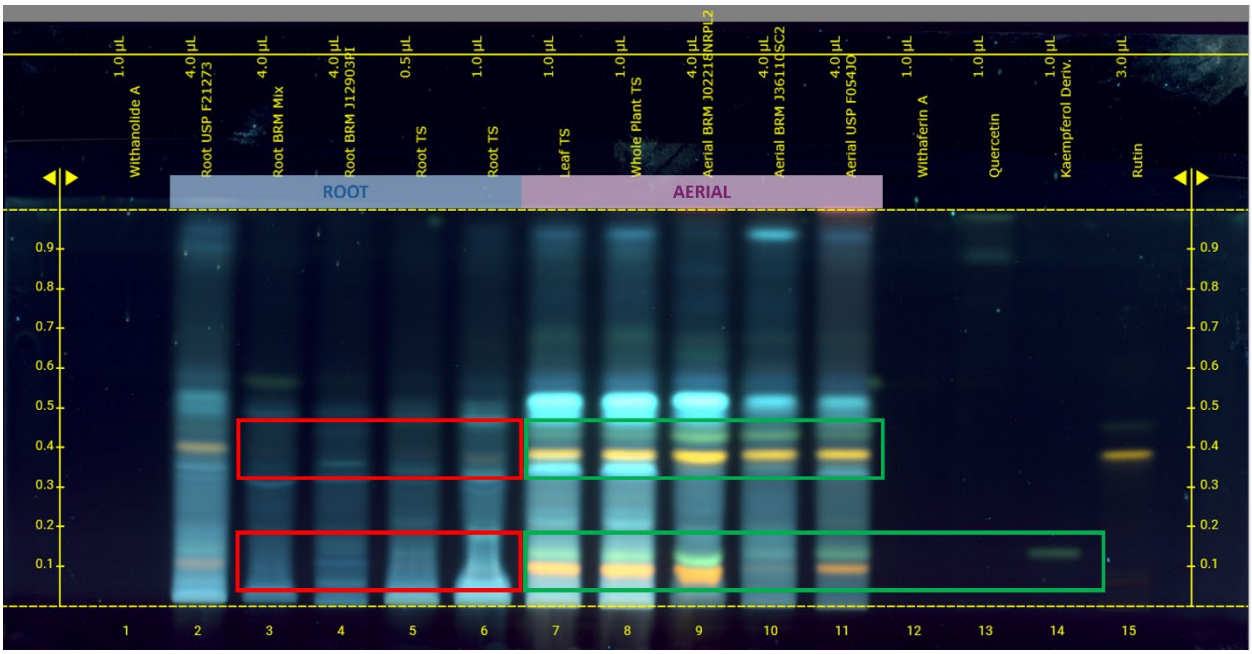
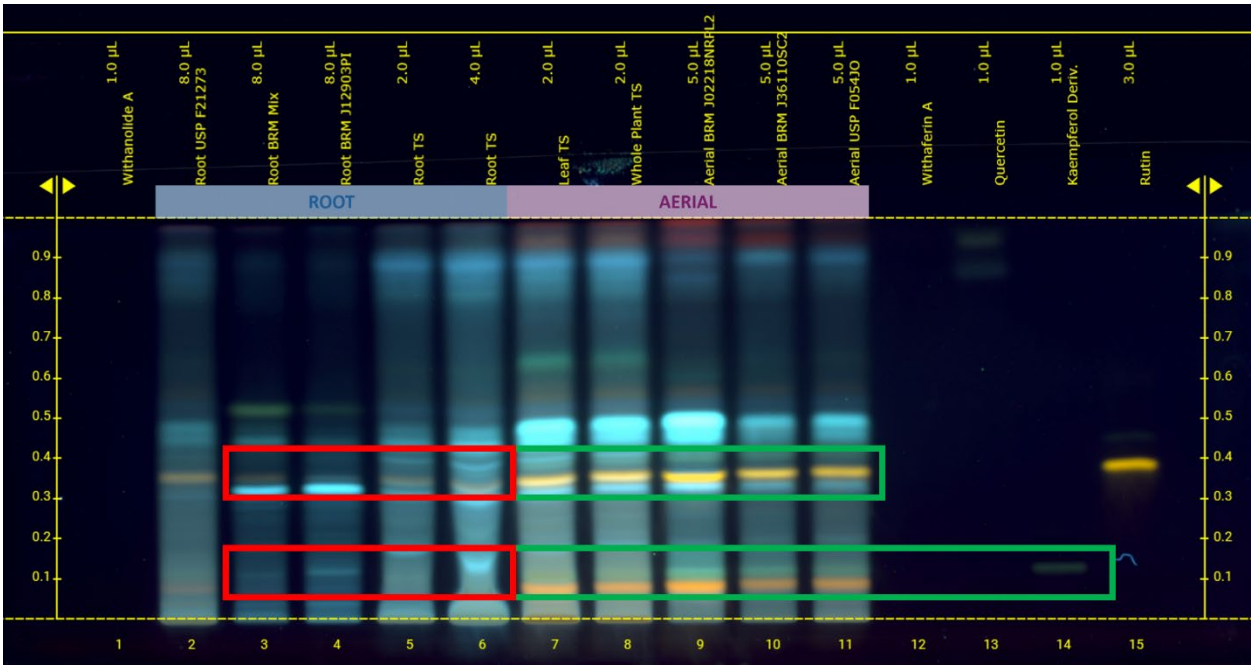


Image 4:

Method 2 (Aerial) Hydroethanolic Extracts, Derivatized in Natural Products & PEG Reagent, UV 366nm



Appendix 3

Experimental Parameters:

Test Materials

Name	Source	Sample #	Plant Part
Test Sample (TS) 1: *Withania somnifera*	Ixoreal	25006YRK	Root
TS 2: *Withania somnifera*	Ixoreal	25006UGM	Leaf
TS 3: *Withania somnifera*	Ixoreal	25006NQI	Whole Plant
Withania somnifera	Natural Remedies Private Limited	J22813NRPL1	Root
Withania somnifera	Natural Remedies Private Limited	J15916NRPL1	Root
Withania somnifera	Pathway International	J12903PI	Root
Withania somnifera	Natural Remedies Private Limited	J02218NRPL2	Aerial Parts
Withania somnifera	Sabinsa Corp	J36110SC2	Aerial Parts

Chemical Reference Standards

Name	CAS#	Manufacturer	Part #	Lot #	Grade	Expiration
Powdered Ashwagandha Root Extract	1043309	USP	1043309	F2I273	USP	10/31/26
Powdered Ashwagandha Aerial Dry Extract	1354965	USP	1354965	F054J0	USP	05/31/26
Withanolide A	32911-62-9	USP	1719500	R143A0	USP	11/30/27
Withaferin A	5119-48-2	Covance dba ChromaDex Analytics	ASB-00023250-010	00023250-004	P	05/31/29
Quercetin	117-39-5	Covance dba ChromaDex Analytics	ASB-00017030-100	00017030-336	P	12/31/25
Kaempferol 3-O-robinoside-7-O-glucoside	114924-89-9	USP	1354954	R176B0	USP	07/31/27
Rutin	470-82-6	Covance dba ChromaDex Analytics	ASB-00005375	00005375-M01	P	07/31/28

Stationary Phase

Plate	Size	Manufacturer	Part No.	Lot No.
Glass Plates HPTLC Silica gel 60 F_{254}	20 x 10 cm	Merck	1.05642.0001	HX33978942

Instruments and Software

Technique	Instrument	Manufacturer
HPTLC	Linomat 5	CAMAG
	Automatic TLC Sampler 4	
	Automatic Developing Chamber 2	
	Automatic Developing Chamber 2	
	TLC Visualizer 3	
	Immersion Device III	
	TLC Plate Heater III	
	visionCATS software	
HPLC	Alliance 2695	Waters
	PDA Detector	
	Empower software	

Appendix 4

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DOI: https://doi.org/10.31003/USPNF_M2788_02_01
Doc ID: GUID-43057A36-34DF-4B91-9458-BF4CA58205D4_2_en-US

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DOI: https://doi.org/10.31003/USPNF_M2789_08_01
Doc ID: GUID-1655E3C0-6B65-4F49-9990-72050ACFA6F3_8_en-US

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Comprehensive metabolic fingerprinting of *Withania somnifera* leaf and root extracts

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ABSTRACT

Profiling of metabolites is a rapidly expanding area of research for resolving metabolic pathways. Metabolic fingerprinting in medicinally important plants is critical to establishing the quality of herbal medicines. In the present study, metabolic profiling of crude extracts of leaf and root of *Withania somnifera* (Ashwagandha), an important medicinal plant of Indian system of medicine (ISM) was carried out using NMR and chromatographic (HPLC and GC–MS) techniques. A total of 62 major and minor primary and secondary metabolites from leaves and 48 from roots were unambiguously identified. Twenty-nine of these were common to the two tissues. These included fatty acids, organic acids, amino acids, sugars and sterol based compounds. Eleven bioactive sterol–lactone molecules were also identified. Twenty-seven of the identified metabolites were quantified. Highly significant qualitative and quantitative differences were noticed between the leaf and root tissues, particularly with respect to the secondary metabolites.

1. Introduction

Withania somnifera (L.) Dunal (Solanaceae) commonly known as Ashwagandha/Indian ginseng/winter cherry, is one of the most esteemed medicinal plants used in Indian Ayurveda for over 3000 years (Gupta and Rana, 2007; Singh et al., 2001). It is used as herbal medicine in various forms (decoctions, infusions, ointments, powder and syrup) in different parts of the world (Archana and Namasivayam, 1999; Davis and Kuttan, 2001; Kumar et al., 2007), for all age groups of patients without any side effects even during pregnancy (Gupta and Rana, 2007; Sharma et al., 1985). The extracts as well as different isolated bioactive constituents of *W. somnifera* have been reported to possess adaptogenic, anticancer, anti-convulsant, immunomodulatory, antioxidative and neurological effects. The plant is also considered efficacious in the treatment of arthritis, geriatric, behavioural and stress related problems (Dhuley, 2001; Gupta and Rana, 2007; Kaur et al., 2001; Mishra et al., 2000; Ray and Gupta, 1994; Schliebs et al., 1973; Sethi et al., 1970). Several bioactive alkaloids and sterol-lactone based phytochemicals, e.g. ashwagandhine, cuscohygrine, isopelletierine, anaferine, anhygrine, tropine, sitoindosides (sapo-nins), the diversely functionalized withanolides, withanamides, and glycowithanolides have been isolated from different parts of this plant (Matsuda et al., 2001; Mishra et al., 2005, 2008; Rahman et al., 1993, 1999, 2003). Its increasing therapeutic benefits continuously attract the attention of pharmacologists for biomedical investigations on plant extracts and isolated phytochemicals (Bani et al., 2006; Chang et al., 2007; Chen et al., 2008; Kaieh et al., 2007; Malik et al., 2007; Mulabagal et al., 2009; Nair and Jayaprakasam, 2007a,b; Pan et al., 2009).

The metabolic constituents, particularly secondary metabolites differ with the variety of *W. somnifera*, tissue type and sometimes with growth conditions (Abraham et al., 1968). Such variations often lead to inconsistent therapeutic and health promoting properties of various commercial *Withania* preparations (Dhar et al., 2006; Sangwan et al., 2004). This causes difficulties in the compositional standardization of herbal formulations and the commercial exploitation of this plant since in a multi-component therapeutic system, different constituents could influence the health effects through complex multi-target interactions. A recent review (Deocaris et al., 2008) narrates cases where multi-component *W. somnifera* extracts showed better medicinal efficiency than the purified compounds. Hence, instead of tracking a few marker compounds Chaurasiya et al. (2008), comprehensive phytochemical fingerprinting needs to be carried out on the plant material to be used for health benefits (Mohn et al., 2009; Shyur and Yang, 2008; Wang et al., 2005).

The analysis of total metabolome of a plant is important to extend our understanding of complex biochemical processes within a

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plant. Significant technological advances in analytical systems like NMR, GC–MS and HPLC have opened up new avenues for plant metabolomics research aimed at rapidly identifying a large number of metabolites quantitatively and qualitatively. This has become an important area of investigations in pharmacology and functional genomics of medicinal plants. Comprehensive chemical analysis is required not only to establish correlation between complex chemical mixtures and molecular pharmacology but also to understand complex cellular processes and biochemical pathways via metabolite-to-gene network (Nakabayashi et al., 2009). We report the application of various approaches for broad based chemical analysis to identify targeted and non-targeted metabolites in roots and leaves of *W. somnifera* and quantify some of those.

2. Results and discussion

Leaves and root tissues of *W. somnifera* were extracted with *n*-hexane followed by warm (~35 °C) methanol–water (90–70%, MeOH, step wise successively). After liquid–liquid partition of methanolic water portion with $CHCl_3$ followed by *n*-BuOH, metabolites repertoire of the plant was distributed into four fractions of different polarities (*n*-hexane, aqueous-methanol, chloroform and *n*-butanol). Each fraction was then subjected to NMR, GC–MS and sometimes to HPLC–PDA analysis. Metabolic content of leaf and root tissues extracted by solvents of different polarities are presented in Table 1. Quantity of metabolite in leaves was much higher than that in roots, particularly in the aqueous-methanolic fraction.

Table 1
Metabolite content of different fractions from leaf and root of *W. somnifera*.

Extract partition	Total metabolite content mg/gm of DW	
	Leaf	Root
Hexane	34.29 ± 2.0	4.44 ± 0.8
$CHCl_3$	35.71 ± 1.5	10.00 ± 1.0
n-BuOH	28.57 ± 1.6	11.11 ± 1.2
Methanolic water	228.57 ± 5.2	15.00 ± 1.6

2.1. Metabolic analysis of n-hexane extract

Metabolomic analysis of *n*-hexane extract of *W. somnifera* leaves and roots was performed by NMR spectroscopy and GC–MS. ^{1}H NMR spectra of both leaf (Fig. 1) and root (Supplementary Fig. 1) extracts predominantly contained different saturated and unsaturated fatty acids. Signals at δ 1.6 and at δ 2.3 represented the β-CH_2 and α-CH_2 of the fatty acids (Knothe and Kenar, 2004). The signals of all other protons of hydrocarbon chain of fatty acids appeared at δ 1.3. The appearance of olefinic protons at δ 5.35 indicated the presence of unsaturated fatty acids whereas signals at δ 2.07 indicated the allylic protons of unsaturated fatty acid. In addition, the characteristic bis-allylic signals (triplet) of di and tri unsaturated fatty acids appeared at δ 2.8. Homo decoupling experiment of the olefinic protons (δ 5.35) altered the triplet signals of bis-allylic protons into two distinguished singlets at δ 2.77 and δ 2.83, indicating the presence of di and tri unsaturated fatty acids. The 18:3 fatty acids were identified by their characteristic triplet methyl signals at δ 0.99 particularly in the spectrum of leaf *n*-hexane extract. The downfield shift of methyl signals for 18:3 (linole-

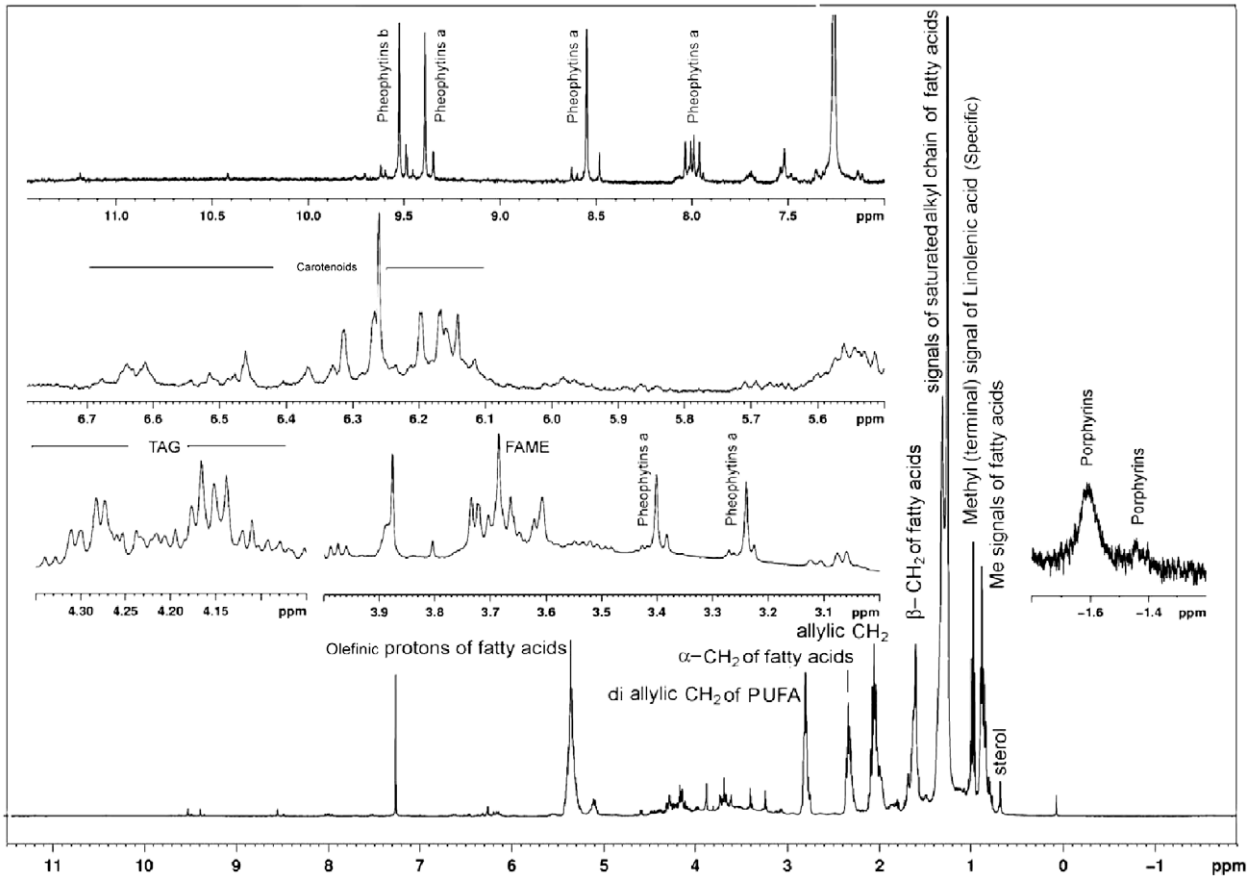

Fig. 1. ^{1}H NMR spectra of the hexane extract of *W. somnifera* leaves.

nic acid) fatty acids occurred due to proximity of the unsaturated double bond (Knothe and Kenar, 2004). Terminal methyl signals of other fatty acids appeared collectively at δ 0.90. In case of root samples, the signals of the terminal methyl were not very distinct due to overlap.

The ^{1}H NMR spectrum of *n*-hexane extract showed characteristic double doublet (dd) signals (δ 4.1–4.3) of sn1 and sn3 protons of triacylglycerol (TAG) (Annarao et al., 2008). Minor amounts of methyl esters of fatty acids were indicated by singlet (for O–Me group) signals at δ 3.6. The characteristic signal of 18-CH_3 group of sterol appeared distinctly at δ 0.7. Several signals at δ 6.0–6.5 might be attributed to the carotenoids (Sobolev et al., 2005). Integration ratio with respect to TSP signals indicated that the carotenoid content of leaf extract was higher than that of root. This is expected, as major role of carotenoids in leaves is to protect leaf from excessive light stress. It was further observed that percentage of TAG was much higher in root extract than in leaves. The presence of two signals in the up field region (δ −1.43 and δ −1.6) of the spectrum of leaves was characteristic for N–H group of the porphyrins (Sobolev et al., 2005). Pheophytins are the degraded products of chlorophylls. During metabolite extraction, the chlorophylls lose their magnesium ions and become pheophytins. The signals of part of phytal fragments (–O–CH_2–CH=C(CH_3)–) of chlorophylls and other part of pheophytins also appeared at δ 9.52, 9.35, 8.5, 8.0, 3.4 and 3.24. The ratio of chlorophylls *a* and *b* was determined as 3:1 by integration of singlet signals at δ 9.37 and δ 9.55. Other minor signals in the range of δ 11–7 may have appeared due to oxidised products of chlorophylls. The observed signals of all the protons and the corresponding ^{13}C signals (identified by ^{1}H–^{13}C HSQC mapping) are presented in Table 2.

To determine the composition of individual fatty acids and sterols, the *n*-hexane extract was subjected to GC–MS analysis after esterification (methyl ester). Palmitic acid (16:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) were major fatty acids present in the leaf and root samples (Supplementary Fig. 2). These fatty acids belong to membrane lipids of plant tissues. Percentage peak area of the GC chromatograms revealed that palmitic acid and linolenic acid were the predominant fatty acids present in the leaves whereas roots were richer in palmitic acid and linoleic acid. Quantitative data of all major fatty acids in the leaves and roots are documented in Table 3. GC–MS analysis further suggests the presence of many others minor but very long chain fatty acids (Fig. 2) particularly in the root samples, indicating higher activity in the stearoyl-CoA elongation activity in the root tissue as compared to the leaf tissue (Schreiber et al., 2005). Presence of each fatty acid was indicated by respective molecular ions peak in the mass spectrum together with the characteristic peak at *m*/*z* 74 (base peak) that appeared due to McLafferty rearrangement and the peak at *m*/*z* 87 that appeared due to loss of $(CH_2)_2CO_2CH_3^+$. Other respective logically defined mass fragments also appeared in the spectrum. Presence of campesterol and stigmasterol at t_R 53.09 and 53.35 were indicated in the GC–MS analysis, particularly in the *n*-hexane extract of root (Supplementary Fig. 2, Supplementary Table 1).

Table 3
GC–MS identified major metabolites of hexane extract of *W. somnifera*.

Metabolites	t_R (min)	MS data (*m*/*z*)	Peak area (%)	Amount mg/g of DW
Palmitic acid (16:0) (L & R)	30.6	270 (M^+), 87 (58%), 74 (100%), 55 (12%)	10.35 (L) 26.64 (R)	3.55 ± 0.5 (L) 1.18 ± 0.2 (R)
Oleic acid (18:1) (L & R)	34.3	298 (M^+), 87 (60%), 74 (100%), 55 (18%)	2.08 (L) 8.84 (R)	0.71 ± 0.1 (L) 0.39 ± 0.1 (R)
Linoleic acid (18:2) (L & R)	34.6	294 (M^+), 109 (22), 95 (38%), 81 (100%), 67 (72%), 55 (34%)	4.43 (L) 29.52 (R)	1.52 ± 0.2 (L) 1.31 ± 0.2 (R)
Linolenic acid (18:3) (L & R)	35.1	292 (M^+), 121 (20%), 108 (22%), 74 (100%), 55 (24%)	12.74 (L) 3.30 (R)	4.38 ± 0.5 (L) 0.15 ± 0.1 (R)

Leaves (L), roots (R).

2.2. Metabolic analysis of $CHCl_3$ and n-BuOH partition

^{1}H NMR spectrum of *W. somnifera* leaf extract is presented in Fig. 3. The presence of double doublet at δ 6.8 and δ 6.5 together with distinguished doublet signals at δ 6.3 and δ 5.8 indicated the presence of withanolide skeleton. Characteristic singlet signals of methyl series of withanolide frame work were observed in the range of δ 0.6–2.2. Comparison of the spectrum with the purified withanolide standards (Supplementary Figs. 3–5) established the presence of withaferin-A and withanone in the mixture. The ^{13}C signals of each metabolite were recognized by ^{1}H–^{13}C HSQC spectrum (Supplementary Fig. 6a and b) and compared with the literature data of the pure compounds (Tuli and Sangwan, 2009). Both ^{1}H and ^{13}C signals clearly indicated the presence of withaferin-A and withanone as the major metabolite in the $CHCl_3$ partition of leaves. All the related NMR data are presented in Table 4. Branch signals at δ 0.9 of the ^{1}H spectrum appeared due to aliphatic chain of β-sitosterol. GC–MS analysis of this partition indicated the presence of β-sitosterol in this partition. There were some unassigned

Table 2
NMR identified metabolites from hexane extract of *W. somnifera*.

Metabolites	1H Chemical shift δ ppm	13C Chemical shift δ ppm
Porphyrines (L)[a]	−1.43 (bs), −1.60 (bs)	
Carotenoids (L & R)[a]	6.0–6.6	126.9, 127.5, 130.0, 132.5, 132.6
Saturated (L & R)[a]	0.90 (t), 1.27–1.33, 1.60, 2.33	39.8, 34.5, 32.0, 31.5, 29–30, 27.5, 25.5, 24.5, 22.5, 20.8, 14.5179.0
Mono unsaturated (L & R)[a]	0.90 (t), 1.27–1.33, 1.60, 2.07, 2.33, 5.35	39.8, 34.5, 32.0, 31.5, 29–30, 27.5, 25.5, 24.5, 22.5, 20.8, 14.5128.0, 130.0, 179.0
Di-unsaturated (L & R)[a]	0.90 (t), 1.27–1.33, 1.60, 2.07, 2.33, 2.77 (t), 5.35	39.8, 34.5, 32.0, 31.5, 29–30, 27.5, 25.5, 24.5, 22.5, 20.8, 14.5128.0, 130.0, 179.0
Tri & poly-unsaturated (L & R)[a]	0.99 (t), 1.27–1.33, 1.60, 2.07, 2.33, 2.83 (t), 5.35	39.8, 34.5, 32.0, 31.5, 29–30, 27.5, 25.5, 24.5, 22.5, 20.8, 14.5, 128.0, 130.0, 179.0
Fatty acid methyl ester (S) (L & R)[a]	3.5–3.7 (s)	Not detected
Pheophytin a (L)[a]	9.37 (s), 8.55 (s), 8.0 (m), 3.40 (s), 3.24 (s)	Not detected
Pheophytin b (L)[a]	9.55 (s)	Not detected
Sterol (L & R)[a]	0.65 (s)	Not detected
TAG (L & R)[a]	4.1–4.35, 5.05–5.15 (bs)	Not detected

Leaves (L), roots (R).
[a] Identified by NMR.

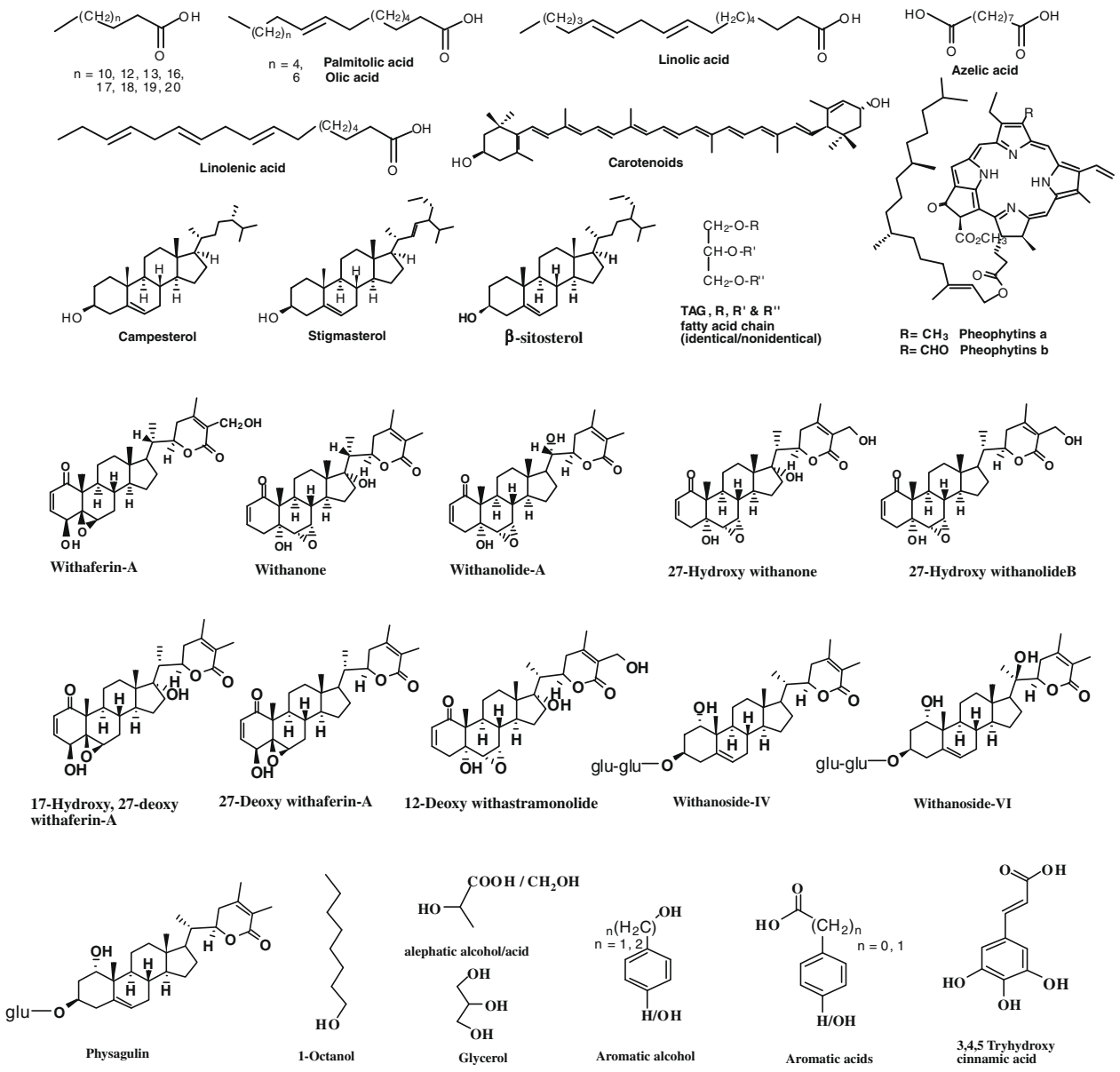

Fig. 2. Some of the phytochemicals identified in *W. sominfera*.

signals appearing in the range of δ 5.2–5.7 of the ^{1}H NMR spectrum which may be due to the presence of minor amounts of withanolides in the mixture. The corresponding ^{1}H NMR spectrum of the root (Supplementary Fig. 7) was not distinct like leaf but it was clear enough to indicate the presence of withanolide skeleton. The ^{1}H signals at δ 6.6 (m), 5.85 (d), 4.2 (m), 1.95 (s), l.85 (s), 1.2 (s), 0.95 (s) and corresponding ^{13}C signals at δ 12.0, 13.0, 14.0, 22.0, 57.0, 129.5140.0 were identified by HSQC spectrum (Supplementary Fig. 8) provided sufficient evidence for the presence of withanolide A. Broadness of the spectrum indicated the presence of significant amounts of similar type of compounds. HPLC analysis of the $CHCl_3$ partition of both leaf and root samples (Supplementary Fig. 9) were performed for further identification of other NMR-non identifiable withanolides. Assignments of resultant chromatograms were performed by matching with the chromatogram of purified withanolides and subsequently by co-chromatography. The analysis suggested that withaferin-A and withanone were the major metabolites present in the leaf as shown by NMR and withanolide A and withanone are major metabolites in the root. Other metabolites detected are 27-hydroxy withanone, 17-hydroxy 27-deoxy withaferin-A, 27-hydroxy withanolide B, 27-deoxy withaferin-A, 12-deoxy withastramonolide. Each of the withanolides was quantified by HPLC using the calibration curve of the standard samples. The qualitative and quantitative data on the metabolites established by NMR and HPLC are presented in the Table 4 and the structures of the identified metabolites are presented in Fig. 2. However, NMR analysis was not enough to provide any useful information about chemical constituents of the *n*-BuOH fraction. The HPLC–PDA analysis (Supplementary Fig. 10) of the *n*-BuOH fraction of leaf is indicating the presence of physagulin, withanoside IV and withanoside VI. However, chromatogram of same fraction of root is cumbersome but reasonable enough to indicate the presence of withanoside IV and withanoside VI. Presence of these compounds was further confirmed by co-chromatography with standard. To explore further the metabolic composition of $CHCl_3$ and *n*-BuOH fractions, GC–MS analysis was carried out. It indicated the presence of 1-octanol, different aromatic alcohols and aromatic acids. Presences of these compounds are logically

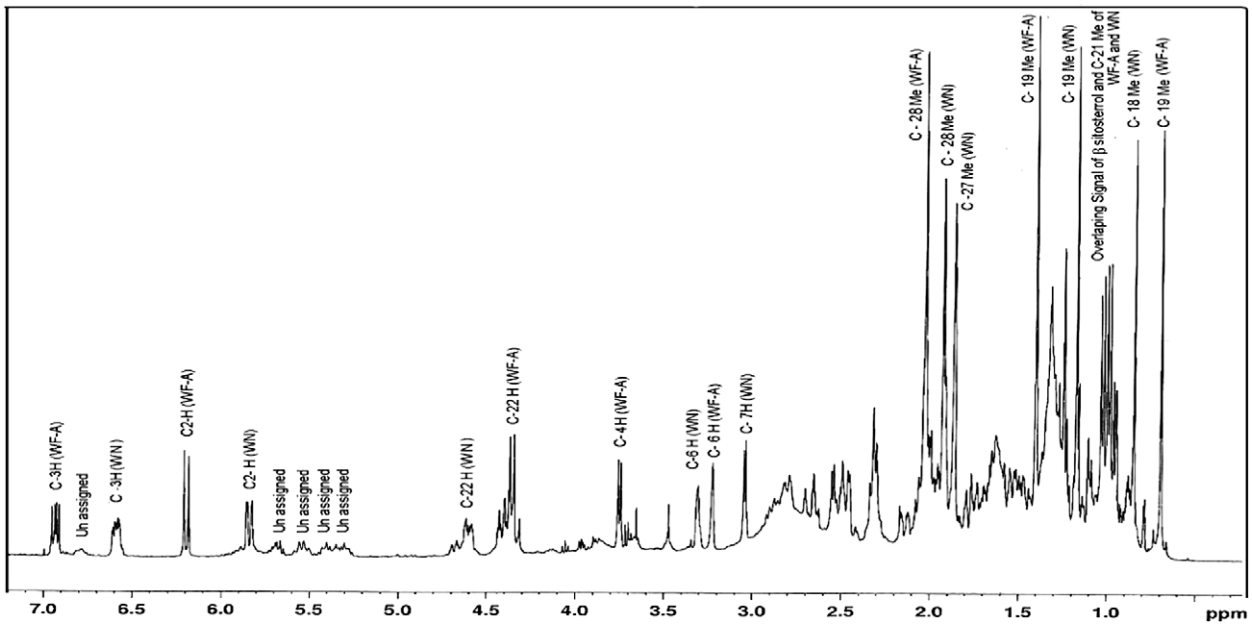

Fig. 3. ^{1}H NMR spectrum of the $CHCl_3$ partition of the *W. somnifera* leaves. WF-A represents withferin-A and WN represent withanone.

Table 4
Identified metabolites in $CHCl_3$ and *n*-BuOH partition of *W. somnifera*.

Metabolites	NMR (δ ppm) and MS/MS (*m*/*z*) data		t_R (min)	*k*′	α	Amount mg/g of DW
	^{1}H Chemical shift δ ppm	^{13}C Chemical shift δ ppm				
$CHCl_3$ fraction						3.45 ± 0.4 (L)[c]
27-Hydroxy withanone (L & R)[b]			16.9 ± 0.5	7.45	1.81	0.50 ± 0.1 (R)[c]
17-Hydroxy, 27-deoxy withaferin-A (L & R)[b]			28.9 ± 0.5	13.45	1.17	3.61 ± 0.5 (L)[c] 0.66 ± 0.2 (R)[c]
Withaferin-A[a,b]						
493 [M+Na$^+$], 471.2, 299.0 (L & R)	6.9 (dd, *J* = 10), 6.21 (d, *J* = 10), 4.3–4.45 (m), 3.75 (d), 3.2 (bs), 2.0 (s), 1.40 (s), 1.20 (d), 0.7 (s)	12.0, 17.5, 20.0, 63.0, 70.0, 74.5, 132.5, 143, 202	33.5 ± 0.5	15.75	1.07	22.31 ± 1.0 (L)[c] 0.92 ± 0.4 (R)[c]
27-Hydroxy withanolide B (L & R)[b]			35.8 ± 0.5	16.9	1.04	2.78 ± 0.5 (L)[c] 0.55 ± 0.2 (R)[c]
Withanolides A 493[M+Na$^+$] (L & R)[a,b]	6.6 (dd, *J* = 10), 5.85 (d, *J* = 10), 4.2 (m), 1.95 (s), l.85 (s), 1.2 (s), 0.95 (s)	12.0, 13.0, 14.0, 22.0, 57.0, 129.5, 140, 202	37.2 ± 0.5	17.6	1.03	2.11 ± 0.5 (L)[c] 3.88 ± 0.7 (R)[c]
Withanone 493 [M+Na$^+$], 431.1263.1 (L & R)[a,b]	6.6 (dd, *J* = 10), 5.85 (d, *J* = 10), 4.6 (m), 3.05 (d), 1.90 (s), 1.85 (s), 1.60 (bm), 1.20 (d), 0.8 (s)	13.0, 14.7, 15.0, 20.5, 56.5, 57.0, 78.0, 129, 140, 203	38.3 ± 0.5	18.15	1.15	18.42 ± 0.8 (L)[c] 5.54 ± 0.4 (R)[c]
12-Deoxy withastromonolide (L & R)[b]			43.8 ± 0.5	20.9	1.14	2.15 ± 0.5 (L)[c] 1.90 ± 0.5 (R)[c]
27-Deoxy withaferin-A (L & R)[b]			49.7 ± 0.5	23.85	–	1.63 ± 0.2 (L)[c] 3.94 ± 0.4 (R)[c]
β-Sitosterol (L)[a]	0.90–1.10 (m)				Not quantified	
n-BuOH fraction						
Withanoside IV (L & R)			26.9 ± 0.5	25.9	1.07	1.60 ± 0.2 (L)[c] 0.44 ± 0.1 (R)[c]
Physagulin (L & R)			28.6 ± 0.5	27.6	1.14	3.46 ± 0.4 (L)[c] Not detected (R)[c]
Withanoside VI (L & R)			32.4 ± 0.5	31.4	–	1.90 ± 0.2 (L)[c] 3.74 ± 0.2 (R)[c]

Leaves (L), roots (R). *k*′ is the capacity factor and α is the separation factor; *k* and α indicates the quality of the HPLC analysis (Sidhu et al., 2003).
[a] Identified by NMR.
[b] HPLC–PDA.
[c] Quantified by HPLC.

supported by their respective mass fragmentation pattern obtained from GC–MS analysis (Table 5).

2.3. Analysis of methanolic water partition

Metabolic profiling of water extract was analyzed mainly by NMR, though in case of root samples, GC–MS was also applied. ^{1}H spectrum of the water extract is presented in Fig. 4. Assignment of the compounds was thoroughly done comparing the ^{1}H spectra of reference compounds together with Biological Magnetic Resonance Data Bank (http://www.bmrb.wisc.edu/metabolomics/) and wherever necessary, by spiking with appropriate internal standards. 2D COSY and HSQC spectra were also extensively used to resolve the complexity of the overlapping/interfering spectral regions to identify the exact molecule in the extract.

Table 5
Identified metabolites by GC–MS in $CHCl_3$ and *n*-BuOH partition.

Metabolites	t_R (min)	Molecular formula of the derivative	MS data (*m*/*z*)
2-Hydroxy propanol (L)	3.80 ± 0.3	$C_9H_{24}O_2Si_2$	220 (M^+), 205 (M^+-CH_3, 40%), 147, 117, 73 (Me_3Si, 100%)
2-Hydroxy propanoic acid (L)	6.80 ± 0.3	$C_9H_{22}O_2Si_2$	219 (M^+), 147, 117, 73 (Me_3Si, 100%)
1-Octanol (L & R)	7.11 ± 0.5	$C_{11}H_{26}OSi$	187 (M^+-CH_3, 24%), 147 (20%), 103 (32%), 73 (Me_3Si, 100%)
Glycerol (L)	11.98 ± 0.2	$C_{12}H_{32}O_3Si_3$	293 (M^+-CH_3, 24%), 218, 205, 147, 73 (Me_3Si, 100%)
Benzyl alcohol (R)	12.14 ± 0.1	$C_{10}H_{16}OSi$	180 (M^+, 12%), 165 (M^+-CH_3, 100%), 135 (M^+-3CH_3, 42%), 91($PhCH_2$, 64%), 73 (Me_3Si, 60%)
2-Phenyl ethanol (R)	14.21 ± 0.3	$C_{11}H_{18}OSi$	180 (M^+, 12%), 179 ($M^+-CH_3^{\pm}$, 54%), 105 (Ph-$CH_2CH_2^{\pm}$, 20%), 73 (Me_3Si, 100%)
Benzoic acid (L & R)	15.81 ± 0.5	$C_{10}H_{14}O_2Si$	194 (M^+), 179 (M^+-CH_3, 100%), 135 (34%), 105 ($PhCO^+$, 40%), 77 (Ph^+, 46%), 75 (18%)
Butandioic acid (L)	16.14 ± 0.3	$C_{10}H_{22}O_4Si_2$	247 (M^+-CH_3, 12%), 147, 73 (Me_3Si, 100%)
Phenyl acetic acid (L & R)	17.63 ± 0.5	$C_{11}H_{16}O_2Si$	208 (M^+), 193 ($M-CH_3$, 12%), 164 ($M-2CH_3$, 22%), 91 ($PhCH_2^+$, 10%), 73 (Me_3Si, 100%)
p-Hydroxy, phenyl ethanol (L)	22.44 ± 0.5	$C_{14}H_{26}O_2Si_2$	282 (M^+), 223, 179 (100%), 73 (Me_3Si, 45%)
p-Hydroxy benzoic acid (L)	24.24 ± 0.5	$C_{13}H_{22}O_3Si_2$	282 (M^+, 26%), 267 (M^+-2CH_3, 88%), 223 (M^+-Me_3Si, 84%), 193 (80%), 126 (26%), 73 (Me_3Si, 100%)
p-Hydroxy, phenyl acetic acid (R)	24.57 ± 0.0	$C_{12}H_{18}O_3Si_2$	238 (M^+, 36%), 179 ($M^+-Me_3Si-CH_3$, 100%), 163 ($M^+-Me_3SiO-3Me_3$, 34%), 73 (Me_3Si, 42%)
3,4,5-Trihydroxy cinnamic acid (R)	37.17 ± 0.5	$C_{15}H_{22}O_5Si$	310 (M^+, 68%), 280 (M^+-2CH_3, 100%), 249 (12%), 73 (Me_3Si, 68%)
β-Sitosterol (L)	51.91 ± 0.5	$C_{32}H_{58}OSi$	488 (MH^+), 396, 357, 129, 75, 44

Leaves (L), roots (R): some of the molecules may appear in either of the fractions depending upon the ratio of the solvent used during partitioning.

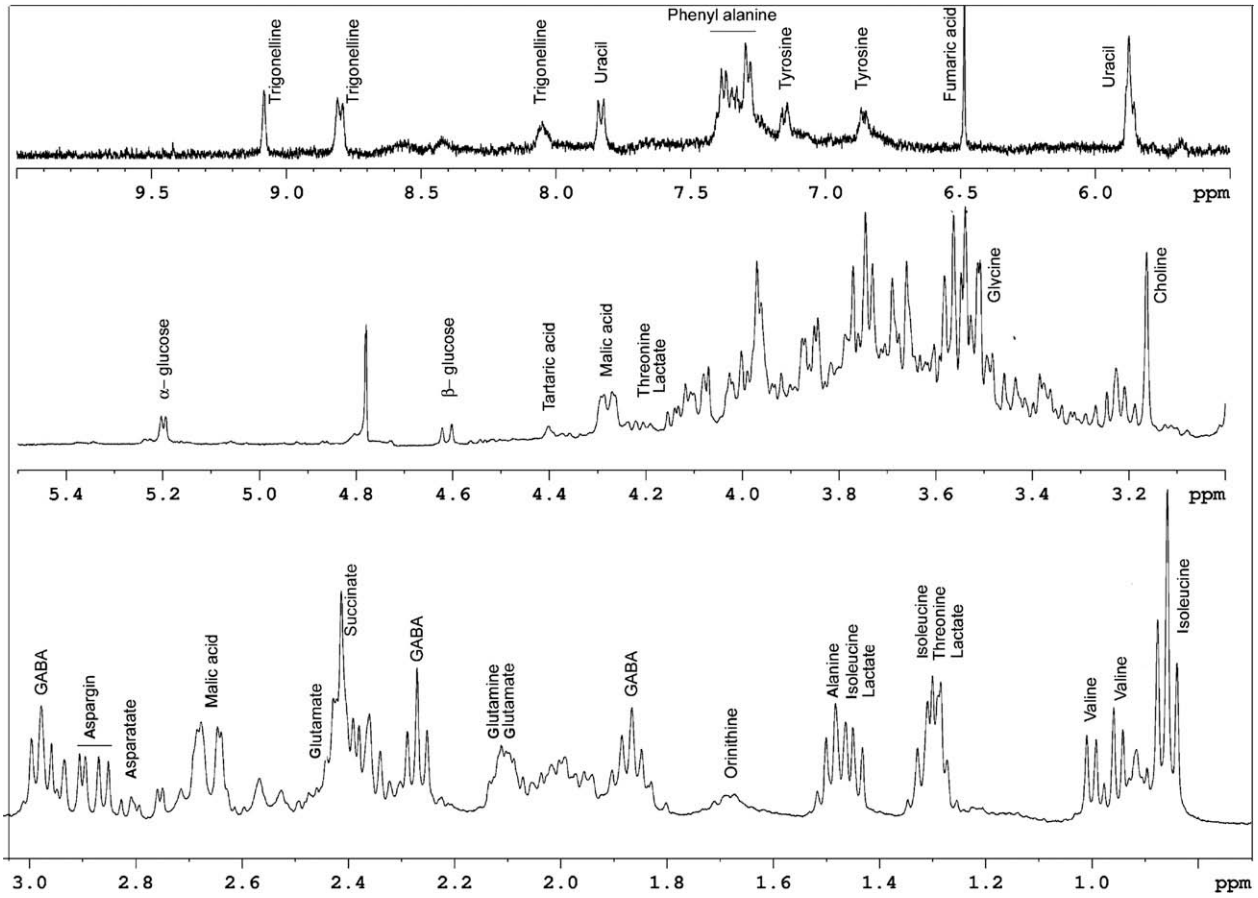

Fig. 4. ^{1}H NMR spectrum of the aqueous fraction of *W. somnifera* leaves.

The entire ^{1}H spectrum of aqueous fraction may be divided into three major regions. δ 0.0–3.5 region is rich with amino acids. δ 3.5–5.5 contains sugars and rest of the spectrum is dominated by aromatic compounds. In the ^{1}H NMR spectrum of the leaves, the region of amino acids started with distinct triplet signals of isoleucine, followed by two sharp doublets of valine. The doublet signals of two δ-CH_3 (0.95, 0.96) leucine were detected by enlarging the spectral segment using Bruker X-win NMR software. Multiplet signals of γ-CH_2 of isoleucine appeared at δ 1.35 and it also shows distinct COSY interactions with δ-CH_3 of isoleucine. Complex multiplet signal at δ 1.48 was observed due to overlapping signals of β-CH_3 of alanine (doublet) and multiplet signals of γ-CH_2 of isoleucine. Branch broad multiplet signals at δ 1.6–1.8 may be due to the presence of ornithine in the extract (Lee et al., 2009). The presence of GABA in the mixture was indicated by characteristics signals at δ 1.90 (m, β-CH_2), 2.29 (t, α-CH_2), 3.01 (t, γ-CH_2) and then distinct correlation in COSY spectrum. Characteristic dd signals of malic acid appeared at δ 2.47–2.68 (geminal protons) and it showed the expected correlation with dd signals of neighbouring protons at δ 4.34 (H attached with C–OH). Respective carbon signal appeared at δ 43.5 and δ 71.5. The dd signal at δ 2.68–2.79 is indicative of β-CH_2 of aspartate whereas similar signals at δ 2.85–2.90 suggested the presence of asparagine. Strong singlet signal at δ 2.40 was the signature of succinic acid (Sobolev et al., 2005). The presence of glutamine and glutamate in the water part was identified mainly by COSY cross peak. Overlapping signals of associated protons appeared in regions at δ 2.0–2.44. Strong singlet signal at δ 3.2 is indicative of $N(Me_3)_3$ of choline in the extract. Related carbon signals appeared at δ 55.1 and δ 75.3. The 1D spectral regions at δ 3.2–4.2 of the carbohydrate region is highly congested. It was very difficult to identify any particular signals but all the carbon signals related to carbohydrate skeleton were relatively distinct (δ 63.0, 73.0, 74.2, 76.6, 78.2 and 96.9) in the HSQC spectrum. Overall nature of the spectrum suggested the presence of high amount of sugars in the

Table 6
Identified metabolites from aqueous fraction of *W. somnifera*.

Metabolites	NMR and MS data		t_R (min)	Amount mg/g of DW
	^{1}H δ ppm	^{13}C δ ppm		
Alanine (L)[a]	1.48, 3.77 (q)	17.9, 53.0		Detected
Aspartate (L)[a]	2.68–2.79 (dd), 3.88	36.3		Detected
Asparagine (L & R)[a]	2.85–2.90 (dd), 3.90	36.5		Detected
Choline (L & R)[a]	3.19 (s), 3.52	55.1, 75.3		3.52 ± 0.5
Citric acid (L)[a]	2.58 (m)	46.2, 76.6, 180.6, 182.9		Detected
Fructose-5 TMS (R)[b]	*m/z* 437 (38%), 217 (30%), 204 (68%), 147 (30%), 103, 73 (Me_3Si, 100%)		23.73	Detected
Fructose-5 TMS, $MeOX_1$ I (R)[b]	*m/z* 307 (72%), 217 (62%), 147, 103, 73 (Me_3Si, 100%))		25.68	Detected
Fructose-5 TMS, $MeOX_1$ II (R)[b]	*m/z* 307 (72%), 277, 217 (62%), 147, 103, 73 (Me_3Si, 100%)		26.03	Detected
Fumaric acid (L)[a]	6.52 (s)	135.9		0.6 ± 0.2
GABA (L & R)[a]	1.91(m), 2.29(t), 3.01	41.0		16.74 ± 0.8 (L)
GABA-N,N-TMS, O-TMS $C_{13}H_{33}NO_2Si_3$[b]	*m/z* 319 (M^+, 100%), 304 (M^+-CH_3), 174, 147, 73 (Me_3Si, 88%)		19.79	Detected (R)
Galactose (L & R) $C_2H_{55}NO_6Si_5$[b]	*m/z* 554 (M^+-CH_3), 319 (100%), 217, 205 (28%), 147, 73 (Me_3Si, 88%)		26.24	Detected
Glycerol (R) $C_{12}H_{32}O_3Si_3$[b]	*m/z* 293 ($M-CH_3$), 218 (20%), 205 (40%), 147 (46%), 117 (34%), 73 (Me_3Si, 100%)		12.00	Detected
Glutamate (L & R)[a]	2.06, 2.11, 2.36			Detected
Glutamic acid N-TMS, 2 O-TMS[b] $C_{14}H_{33}NO_4Si_3$	*m/z* 363 (M^+), 348 (M^+-CH_3,12%), 246 (100%), 147, 128 (22%), 73 (Me_3Si, 49%)		22.79	
Glutamine (L & R)[a]	2.14, 2.44, 3.79			Detected
α-Glucose (L & R)[a,b]	4.64 (d, *J* = 3.7)	63.3, 73.0, 74.2, 76.6, 78.2, 93.1		6.11 ± 0.5 (L)
β-Glucose (L & R)[a,b]	5.20 (d, *J* = 7.8)	63.3, 73.0, 74.2, 76.6, 78.2, 96.9		10.22 ± 0.9 (L)
Glycine (L)[a]	3.57 (s)			Detected
Myo-inositol (L)[b]	612 (M^+), 320 (M^+-4TMS), 305($M^+-4TMS-Me$), 217 $M^+-5TMS-2Me$), 147, 73 (Me_3Si, 49%)		28.84	
Isoleucine (L)[a]	0.88 (t, *J* = 7.6), 1.96 (m)			19.83 ± 0.8
Lactic acid (L)[a]	1.33, 4.11			Detected
Lysine (L)[a]	1.47, 1.72, 1.88, 3.02, 3.76	22.5		Detected
Leucine (L)[a]	0.96 (d), 1.69, 3.72			Detected
Succinate (L & R)[a]	2.68–2.79 (dd), 4.31 (dd)	71.5, 43.5		
Malic acid 3 TMS $C_{13}H_{30}O_5Si_3$[b]	*m/z* 335 (M^+-CH_3), 245, 233, 147, 73 (Me_3Si, 100%)		19.64	Detected
N-Acetyl Glucosamine (L) $C_{21}H_{50}N_2O_6Si_4$[b]	*m/z* 538 (M^+), 450, 348, 147, 73 (Me_3Si, 100%)		27.02	
Orinithine (L)[a]	1.6–1.8 (bm)			21.5 ± 0.8
Phenyl alanine (L)[a]	7.3–7.45 (bm)			Detected
Succinate (L)[a]	2.40 (s)			12.75 ± 0.5
Tartaric acid (L)[a]	4.38 (s)			4.10 ± 0.4
Tyrosine (L)[a]	3.08, 3.17, 3.94, 6.88 (d), 7.2 (d)			Detected
Threonine (L)[a]	1.32 (d)	21.92		Detected
Trigonelline (L)[a]	9.1 (s), 8.8, 8.1			1.33 ± 0.3
Uracil (L)[a]	5.95 (s), 7.75 (m),			3.90 ± 0.2
Valine (L)[a]	0.98 (d, *J* = 7.0), 1.04 (d, *J* = 7.0), 2.25 (m)	18, 19		5.60 ± 0.5

Leaves (L), roots (R).
[a] Identified by NMR.
[b] Identified by GC–MS.

extract. However, the presence of α and β anomers of glucose were clearly identified by their respective doublet signals (β; δ 4.61, J = 7.9 Hz; α; δ 5.2, J = 3.8 Hz). GC–MS analysis further indicated presence of other sugars, *i.e.* galactose, N-acetyl glucosamine and myo-inositiol in the extract. The singlet signals at δ 4.4 appeared due to presence of tartaric acid (Sobolev et al., 2005). ^{1}H signals of threonine and lactate generally appeared side by side at δ 1.33 and δ 4.2 due to their low abundance in the extract. The characteristic signals were not clearly observed in the 1D spectrum but in the 2D COSY spectrum they appeared distinctly. The respective carbon peak appeared at δ 22.5. Strong singlet signals at δ 5.8 indicated one of the olefinic protons of uracil and showed clear cross peak at δ 7.85 corresponding to olefinic protons signals. Sharp singlet around δ 6.5 represented fumaric acid in the extract. Signals at δ 6.88 and δ 7.18 were assigned to tyrosine, which was supported by COSY experiments. The branch spectral band from δ 7.3 to δ 7.45 regions and corresponding COSY analysis suggested the occurrence of phenyl alanine. The signatures of trigonelline were observed at δ 8.1, 8.8 and 9.1 ppm. Detailed COSY relations of the individual metabolite are presented as supplementary information (Supplementary Fig. 11a and 11b, Table 2).

^{1}H spectral complexity (overlapping signals) did not allow quantification of all the metabolites. However, a number of them were quantified by integrating the distinct characteristic signals of each metabolite with respect to signal intensity of quantified amount of TSP. NMR spectroscopy of the aqueous aliquots of root samples was not distinctly informative as very high concentration of sugar in this fraction masked other minor signals. However, GC–MS analysis of the extract of root samples indicated the presence of higher amounts of fructose, galactose, glucose and glycerol besides some minor amino acids. Summary of the quantitative and qualitative outcome is presented in Table 6.

3. Concluding remarks

Metabolomic fingerprinting of herbal extracts is desirable to standardise drugs and to establish the scientific basis of their pharmacological action. This study recruited 1D and 2D NMR, HPLC–PDA, GC–MS techniques for rapid metabolome analysis of *Withania* leaf and root extracts. Such analysis is desirable for developing herbal drugs and establishing association with their action through functional genomics and molecular pharmacology. Such knowledge will evolve directions for genetic improvement of medicinal plants for the enhancement of pathways leading to the biosynthesis of bioactive molecules. A total of 62 major and minor primary and secondary metabolites from leaves and 48 from roots were unambiguously identified in *W. somnifera* in this investigation. Twenty-seven of the identified metabolites were quantified. Significant qualitative as well as quantitative differences between the leaf and root tissue, particularly with respect to secondary metabolites were noticed. High resolution NMR like 800/1000 MHz or high resolution instruments like Fourier Transform Ion Cyclotron Resonance Mass Spectrometer can resolve even higher number of molecules and establish those quantitatively in different plant parts. Such a wide metabolomic analyses not only can help set discrete parameters for better defining and quality control of herbal extract but could also serve as diagnostics for their true identity and adulteration with other plants or non-usual parts of the same plant.

4. Experimental

4.1. Plant samples and chemicals

Fresh leaves and roots of *W. somnifera* genotype NMITLI-101 were collected at early flowering stage from 50 different plants of the same age grown in similar conditions at NBRI experimental farm. Collected leaves were dried in shade on blotting papers until constant weight. Dried leaves and roots were ground to powder using grinder mill. All the solvents used for the extraction of phytochemicals from plant tissues were purchased from Qualigen (ExcealR). All deuterated solvents for NMR were purchased from Sigma Chemical Company (USA). All the solvents used for HPLC were of HPLC grade from E. Merck.

4.2. Extraction of metabolites

The dried plant material was extracted with ten times weight of warm (~35 °C) hexane. The solvent portion was collected by filtration and this procedure was repeated five more times until the hexane layer became almost colourless. Separated solvent layer was concentrated under reduced pressure. The resulting sticky mass was stored at −20 °C until analyzed. The remaining solid plant material was further extracted thrice with fivefold excess (w/w) of 90% and then with 70% warm methanol–water. Volume of the extract was reduced to 1/3rd using rotavapour and defatted with equal volume of hexane. Defatted water–methanol layer was partitioned (liquid–liquid) with equal volume of $CHCl_3$ (five times) followed by *n*-BuOH (five times). Each of the layers was dried separately over sodium sulphate, concentrated to semisolid mass and stored at −20 °C till further analysis. Remaining methanolic water layer was lyophilized to dryness and the resulting solid was again saved for analysis.

4.3. NMR analysis

^{1}H NMR spectra of the hexane and aqueous-methanolic extracts were obtained on Bruker Biospin Avance 400 MHz NMR spectrometer using a 5 mm broad band inverse probe head, equipped with shielded z-gradient accessories. 1D ^{1}H NMR spectral analyses of hexane extracts were carried out using one-pulse sequence by dissolving samples in 500 µl deuterated chloroform taken in 5-mm NMR tubes. A reusable sealed capillary tube containing 30 µl of 0.375% of TSP in deuterium oxide was inserted into the NMR tube before recording the spectra. TSP served as chemical shift reference as well as internal standard for quantitative estimation. ^{1}H NMR experiments were also performed with homonuclear decoupling to olefinic CH=CH protons. Typical parameters for both the extractions were: spectral width: 6000 Hz; time domain data points: 32 K; For quantification purpose the effective flip angle of 45° was used, optimized and standardized instead of 90° using total relaxation delay of 7.73 s for complete recovery of the magnetization by taking consideration of our earlier studies on amino acids (Bharti et al., 2008) so that the quantified results are precise; spectrum size: 32 K points; and line broadening for exponential window function: 0.3 Hz. To confirm the assignments, two-dimensional (2D) correlation spectroscopy (COSY), ^{1}H–^{13}C heteronuclear single quantum correlation (HSQC) were carried out using the Bruker's standard pulse program library. The spectral widths of COSY were 6000 Hz in both dimensions, and 512 t_1 increments for each t_1. Sixteen transients using 2.5 s relaxation delays were added with 2048 complex data. The phase-sensitive data were obtained by the time proportional phase incrementation (TPPI) method. The resulting data were zero-filled up to 1024 in t_1 dimension and were weighted with 90° squared sine window functions in both dimensions prior to double Fourier transformation. Heteronuclear 2D ^{1}H–^{13}C chemical shift correlations were measured using gradient HSQC with a gradient ratio of GPZ1:GPZ2 as 80:20. The experiments were performed with a spectral width of 6000 Hz in F_2 dimension and 24,000 Hz in F_1

dimension, 400 t_1 increments. For each t_1, 96 transients using 1.5 s relaxation delay was added with 2048 complex data points.

4.4. GC–MS analysis

GC–MS analysis was performed using Thermo Trace GC Ultra coupled with Thermo fisher DSQ II mass spectrometers with electron impact ionisation at 70 eV to generate mass spectra. 30 m × 0.25 mm Thermo TR50 column (polysiloxane column coated with 50% methyl and 50% phenyl groups) was used for chromatographic separation of metabolites. To prepare the sample for GC–MS analysis of non-polar hexane extract, 10 mg portion was heated at 60 °C for 6 h with 5 ml of methanolic sulphuric acid (5%, v/v). After cooling, the reaction mixture was diluted and vigorously shaken with 25 ml hexane and 20 ml water. Separated hexane layer was washed with 20 ml water containing 5% (w/v) sodium bicarbonate followed by equal volume of 5% (w/v) sodium chloride solution. Hexane layer was collected and concentrated using rota vapour after drying over anhydrous sodium sulphate. Resulting oily mass was dissolved in 1 ml of GC-grade *n*-hexane and 0.4 μl of the solution subjected to analysis on GC. With an initial 5-min solvent delay time at 70 °C, the oven temperature was increased to 330 °C at 5 °C/min, 5 min isocratic and cooled down to 70 °C followed by an additional 5-min delay. Helium flow was maintained at 1 ml/min and split ratio was maintained 1/60. The resulting GC–MS profile was analyzed using WILLY and NIST mass spectral library and by matching the chromatogram with supelco FAME (fatty acid methyl ester) mixture and whenever is possible, with appropriated standards. For the GC–MS analysis of other than hexane extracts, the TMS derivative of the sample was prepared. Approximately 5 mg of the sample was suspended in 40 μl of the solution of methoxylamine hydrochloride in pyridine (20 mg/ml). The mixture was shaken for 4 h at 37 °C before adding 70 μl of the 2,2,2-trifluoro-N-methyl-N-trimethylsilyl-acetamide (MSTFA). Shaking was continued for another 30 min. Subsequently, 40 μl of the derivatized solution was sampled with 20 μl of *n*-hexane. The GC–MS running conditions were same as mentioned earlier. Quantification of metabolite was done using its percentage peak area appeared at the total ion chromatogram in GC–MS analysis.

4.5. HPLC analysis of the samples

HPLC–PDA analysis of $CHCl_3$ fraction was performed on the system from waters (Milford, MA, USA). The separation was carried out using waters reverse phase column (3.9 × 150 mm, 5 μm) and binary gradient elution. The two solvents used for the analysis consisted of water containing 0.1% acetic acid (solvent A) and methanol containing 0.1% acetic acid (solvent B). Gradient programming of the solvent system was carried out at 27 °C, initially at 60% A changed to 40% A at 30 min, maintained for the next 2.0 min, changed to 25% A at 45 min and then to 5% A at 54 min at flow rate of 0.6 ml/min and then at a flow rate of 1.0 ml/min. The mobile phase was changed to 0% A at 55 min. The solvent composition was maintained until the run time reached 60 min. All the gradient segments were linear (curve type 6). The wavelength scan range of the PDA was set to 190–350 nm. The chromatograms were recorded at 227 nm. HPLC–PDA analysis of *n*-BuOH fraction was carried out using the protocol-I (acetonitrile: water gradient) of Malik et al. (2007) for glycosylated withanolides using waters reverse phase column (3.9 × 250 mm, 5 μm). Quantification of withanolides was carried out as by using the peak area of the sample chromatogram in the regression equation of the calibration curve for each withnaolides and glyco-withanolides standard.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2010.04.001.

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Review Article

Chemical Composition, Biological Activity, and Health-Promoting Effects of *Withania somnifera* for Pharma-Food Industry Applications

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The *Withania* genus comes from the Solanaceae family and includes around 23 species, spread over some areas of the Mediterranean, Asia, and East Africa. Widely used in traditional medicine for thousands of years, these plants are rich in secondary metabolites, with special emphasis on steroidal lactones, named withanolides which are used as ingredients in numerous formulations for a plethora of diseases, such as asthma, diabetes, arthritis, impotence, amnesia, hypertension, anxiety, stress, cancer, neurodegenerative, and cardiovascular diseases, and many others. Among them, *Withania somnifera* (L.) Dunal is the most widely addressed species from a pharmacological and agroindustrial point of view. In this sense, this review provides an overview of the folk uses, phytochemical composition, and biological activity, such as antioxidant, antimicrobial, anti-inflammatory, and cytotoxic activity of *W. somnifera*, although more recently other species have also been increasingly investigated. In addition, their health-promoting effects, i.e., antistress, anxiolytic, adaptogenic, antirheumatoid arthritis, chemoprotective, and cardiorespiratory-enhancing abilities, along with safety and adverse effects are also discussed.

1. Introduction

The genus *Withania* (Solanaceae) includes 23 species [1], mostly occurring in North Africa, Canary Islands, Southern Europe, and Asia (Figure 1) [2–7]. Of the known species, there are two of huge economic importance that are also mostly grown due to their wide applicability in natural medicine [8], namely, *Withania somnifera* (L.) Dunal and *Withania coagulans* (Stocks) Dunal. Both species are grown mainly in subtropical regions of India. However, *W. somnifera* even presents a greater economic significance [9, 10]. In Morocco and Algeria, *Withania adpressa* Cors. is also found as an endemic species [11], although both the morphological form and phytochemical composition of such plants undergo polymorphisms, conditioned by its occurrence in a given geographical area [5].

Although various *Withania* spp. have been used in traditional medicine for the management of different pathologies [12], *W. somnifera* and *W. coagulans* are the most widely recognized species not only for their economic value but also for their therapeutic potential, and they are largely commercialized and cultivated in Afghanistan, Iran, India, and Pakistan [13–20]. In this sense, this review aims to provide an overview of the botanical features, traditional uses, phytochemical composition, biological activities, and health-promoting effects observed in preclinical and clinical studies of *W. somnifera*, along with updated data on its safety and adverse effects.

2. Botanical Features

Plants under the *Withania* genus are evergreen with heights ranging from 0.5 to 2.0 m, present grasses, bush suburbs, branched or unbranched [21, 22]. The flowers are green or yellow, little pedicelled or pentameric umbels, sessile to subsessile, and hermaphrodites. They have simple leaves, petiolate, ovate, alternate, or in unequal pairs with a sharp apex. Fruits are berry of 6 mm in diameter, with orange-red color when mature, globous, and enclosed in the green calyx. Seeds are compressed, small, flat, yellow, reniform, reticulate to smooth, and very light [2, 23–28].

3. Traditional Uses

From a folk medicinal point of view, *W. somnifera,* known as "winter cherry," is the most important species belonging to the *Withania* genus, and that evidences the most renowned therapeutic abilities. This plant has been used in Indian medicine for a long time, and its roots are used in more than 200 formulations [2, 29, 30].

W. somnifera (called Ashwagandha, Indian ginseng) is the best-known species, widely used in natural medicine as it helps in many different ailments, namely, in boosting the immune and hematopoietic system, has an anti-inflammatory activity that helps in skin diseases and osteoarthritis, and also has antiaging effects. In addition, it is also used in hypothyroidism, cardiovascular diseases, diabetes, depression, and chronic stress [31, 32]. More recently, several clinical trials have also confirmed their therapeutic uses, namely, in the treatment of anxiety, insomnia, and Parkinson's disease [33]. In Ayurveda, *W. somnifera* is used for over 3000 years [9] and is considered to have excellent rejuvenating abilities, while it prolongs life and has strong aphrodisiac effects. Indeed, this plant is traditionally used in India to promote youthful vigor, strength, endurance, and health [20, 33], so that such restorative properties have led to *W. somnifera* roots being called Indian ginseng. *W. somnifera* may also be useful to treat various central nervous system (CNS) disorders, such as epilepsy, stress, and neurodegenerative conditions, like Parkinson's disease (PD), Alzheimer's disease (AD), and even cerebral ischemia. Ethnobotanically, it can be used as a hallucinogenic agent [34].

With the rising number of literature available, it has also been indicated that such species may also exert cytotoxic effects, opening the possibility of its use in oncological therapies. According to Verma and Kumar [33], the chemopreventive properties of *W. somnifera* make it a potentially useful adjunct for patients undergoing radiation and chemotherapy. *W. somnifera* stimulates the immune system by stimulating the production of T lymphocytes and macrophages [35, 36], while Ziauddin et al. [37] stated a general increase in the number of white blood cells after administration of a root extract. *W. somnifera* application has also

Figure 1: Red spots indicate the geographical distribution of *Withania* spp.

been shown to be able to reduce the number of skin lesions relative to the control group and showed inhibition of cancer cell growth in breast, lung, and colon cancer, which, apart from its cytotoxic abilities, is linked to their excellent antioxidant effects [38, 39]. Other authors, namely, Panda and Kar [40] and Andallu and Radhika [41], also stated an increase in T4 thyroid hormone concentration following *W. somnifera* root powder application, so that its use may be helpful in controlling the levels of hormones in diseases linked to hypothyroidism. Some authors have also indicated that *W. somnifera* root may be used for preventing cardiovascular disease, such as atherosclerosis [40–42]. For instance, in a human trial, a significant decrease in blood glucose and cholesterol levels to the extent of 10% and 12%, respectively, was observed when compared to the group that received the conventional oral drug for type 2 diabetes (Daonil). These therapeutic effects could be due to one or more active principles in the roots of the plant. The hypoglycemic effect of *W. somnifera* root could be specifically attributed to its ability to enhance serum insulin levels and/or the antioxidant activities of catalase, superoxide dismutase, and glutathione peroxidase [40–42].

4. Phytoconstituents

Chemical analysis of different plant parts of *W. somnifera* has afforded numerous compounds belonging to various chemical classes. The biologically active chemical constituents of *W. somnifera* are alkaloids (isopelletierine, anaferine), steroidal lactones (withanolides, withaferins), saponins containing an additional acyl group (sitoindoside VII and VIII), and withanolides with glucose at carbon 27 (sitoindoside XI and X). Among them, withanolides (steroidal lactones) have been used in an increasing number of drug formulations, given their promissory therapeutic abilities [43].

Despite being widely reported by a plethora of studies, Table 1 and Figure 2 present some of the most important withanolides isolated from *Withania* spp., considering its abundance and bioactive effects and representative structures, respectively. Misra et al. [44] reported withanolide A, withanolide B, 27-hydroxy withanolide B, withanolide D, withaferin A, 16β-acetoxy-6α, 7α-epoxy-5α–hydroxy-1-oxowitha-2, 17 (20), 24-trienolide, 5, 7α-epoxy-6α, 20α–dihydroxy-1-oxowitha-2, 24- dienolide along with common steroids, β-sitosterol and sitosterol, and their glucosides in *W. somnifera*. Matsuda et al. [45] isolated 7 new withanolide glycosides from *W. somnifera* roots, named withanoside I to VII, among which class VI is more abundant. Similarly, Bessalle and Lavie [46] isolated two chlorinated withanolides, namely, withanolide C and 4-deoxyphysalolactone from dried leaves of *W. somnifera* (Table 1).

There have been also reports on other constituents from plants of the *Withania* genus, namely, fatty acids and volatile compounds. Misra et al. [57] have reported new ergosterol and 1, 4-dioxane derivatives along with various fatty acids

Table 1: List of selected withanolides and other compounds identified from *Withania somnifera* (L.) Dunal.

Plant parts	Compounds	References
Roots	Withanolide A, withanolide B, 27-hydroxy withanolide B, withanolide D, withaferin A, 16β-acetoxy-6α, 7α-epoxy-5α–hydroxy-1-oxowitha-2, 17 (20), 24-trienolide, 5, 7α-epoxy-6α, 20α–dihydroxy-1-oxowitha-2, 24-dienolide	[44]
	Withanoside I, withanoside II, withanoside III, withanoside IV, withanoside V, withanoside VI, withanoside VII, withaferin A, physagulin D, coagulin Q	[45]
	Withasilolide A, withasilolide B, withasilolide C, withasilolide D, withasilolide E, withasilolide F	[47]
	Withanolide E, withanolide F, withanolide G, withanolide H, withanolide I, withanolide J, withanolide K, withanolide L, withanolide M	[48]
	Withanolide Q, withanolide R	[49]
	Withanolide E, withanolide F, withanolide S, withanolide P	[48]
	Withanolide T, withanolide U	[50]
	Glucosomniferanolide	[51]
Stem bark	Withasomnilide, withasomniferanolide, somniferanolide, somniferawithanolide, somniwithanolide	[52]
Leaves	Withanolide C, 4-deoxyphysalolactone	[46]
	(20R, 22R)-14α, 2OαF-dihydroxy-1-oxowitha-2, 5, 16, 24-tetraenolide	[53]
	Withaferin A	[54]
	24,25-Dihydrowithanolide A, withanolide A, withanone, withaferin A, 27-hydroxy withanone, and 17-hydroxy withaferin A, 27-deoxy-16-en-withaferin A, 2, 3-dihydro-3β-hydroxywithanone, 2,3-dihydro withanone-3β-O-sulfate	[55]
Fruits	24,25-Dihydrowithanolide VI, withanoside IV, withanoside V, withanoside VI, withanamide A, withanamide B, withanamide C, withanamide D, withanamide E, withanamide F, withanamide G, withanamide H, withanamide I	[56]

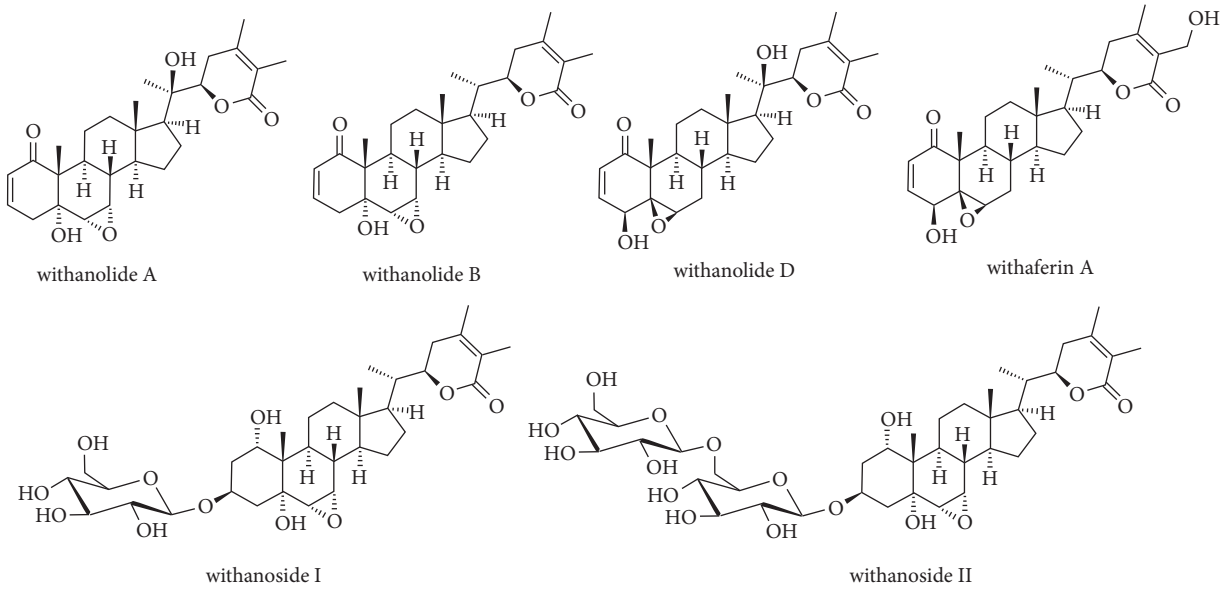

Figure 2: Chemical structures of some withanolide derivatives isolated from *Withania somnifera*.

(octacosane, oleic and stearic fatty acids), steroids, and oleanolic acid from *W. somnifera* roots. For example, Rautela et al. [58] studied the constituents of both ethanol and methanol extracts of *W. somnifera* leaves and roots and analyzed components by gas chromatography-mass spectrometry (GC-MS). Various compounds, including withanolide B, rosifoliol, and phytol, were reported [58]. Gulati et al. [59] studied the chemical composition of various extracts from *W. somnifera* roots of different genotypes and stated several metals in its composition, along with different concentrations of total sugars, alkaloids, and tannins. Bhatia et al. [60], studying the effect of chemotype variations in the chemical composition of *W. somnifera* fruits using GC-MS and nuclear magnetic resonance (NMR) spectroscopy, stated clear variations in metabolites contents in different chemotypes.

5. Biological Activities

Given the wide range of *Withania* species applications in Ayurvedic medicine for multiple aims, an increasing number of studies have progressively addressed their biological effects (Figure 3). Furthermore, with the popularization, the use of this plant as a food supplement in the market is also increasing. Indeed, both extracts and compounds isolated from the *Withania* species exhibit excellent

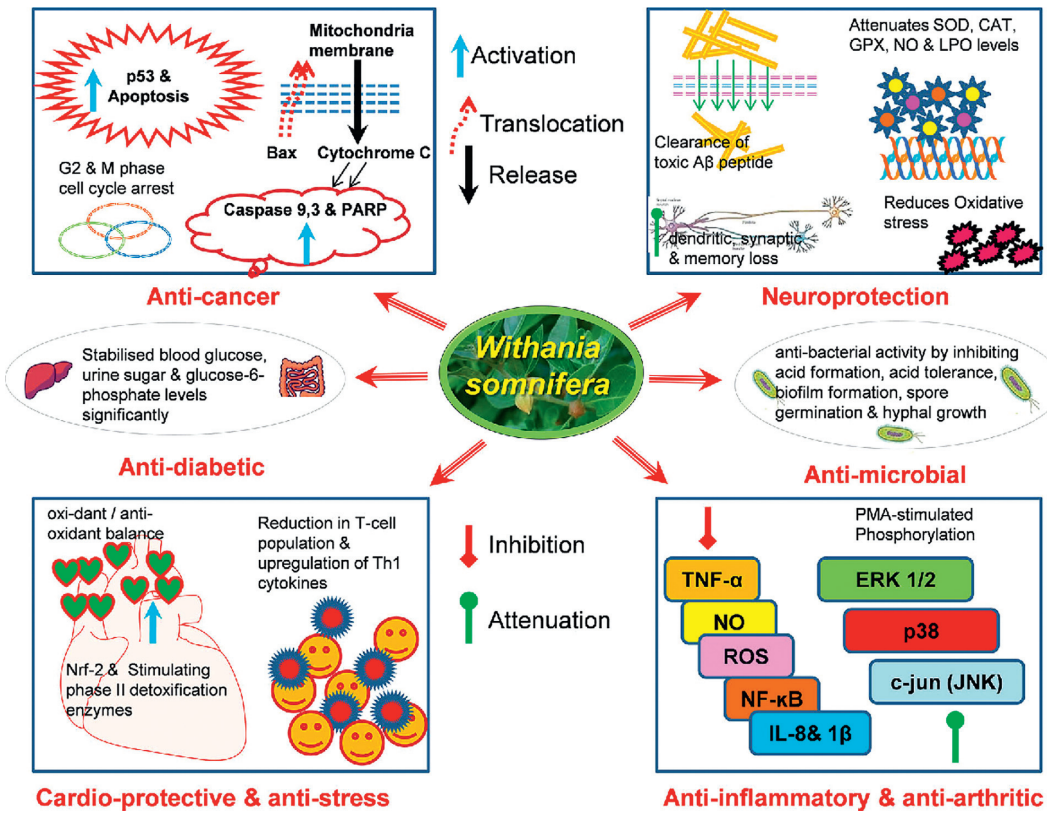

FIGURE 3: Major biological activities of *Withania somnifera*. Anticancer effects: *W. somnifera* exerts anticancer effects via multiple pathways, including nuclear factor (NFK-beta) and signal transducer and activator of transcription 3 (STAT3) signaling, PI3K (phosphoinositide 3-kinase)/AKT (a serine-threonine protein kinase) and mitogen-activated protein kinase (MAPK) signaling, angiogenesis inhibition, oxidative stress induction, and p53 signaling. Melanoma cells were destroyed by withaferin A via ROS-mediated apoptosis. This process activated the mitochondrial pathway, resulting in the downregulation of Bcl-2, translocation of Bax to the mitochondrial membrane, release of cytochrome c into the cytosol, abolition of transmembrane potential, and concomitant activation of caspases 9 and 3, resulting in the downregulation of proapoptotic protein, poly (ADP-Ribose) polymerase-1 (Parp-1) and DNA fragmentation. Neuroprotection: *Withania somnifera* reduced blood glucose, tissue lipid peroxidation (LPO), and glutathione (GSH) levels while increasing the activities of antioxidant enzymes such as glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), superoxide dismutase (SOD), and catalase (CAT). This demonstrates *W. somnifera's* significant free radical scavenging activity, as well as its ability to improve non-enzymatic and enzymatic antioxidants. *W. somnifera* root extract and withanolide A protected isolated hippocampus cells against hypobaric hypoxia-induced memory loss and neurodegeneration in vitro by stimulating the glutathione production pathway and decreasing glutathione (GSH) concentration. Furthermore, in cortical neurons treated with amyloid beta peptide, Withanolide A promoted both axonal and dendritic change as well as synaptic repair. Antidiabetic effects: *W. somnifera* leaf and root extracts showed antidiabetic activity by normalizing glucose uptake in skeletal myotubes and adipocytes in a dose-dependent manner. Furthermore, it considerably attenuated levels of urine and blood glucose, glucose 6-phosphatase, and tissue glycogen levels through nonenzymatic and enzymatic antioxidant mechanisms. Antimicrobial effects: the antimicrobial effect of *Withania somnifera* is attributed by inhibiting acid formation, acid tolerance, biofilm formation, spore germination, and hyphae growth, which in turn is mediated through gene silencing, immunopotentiation and cytotoxicity. Cardioprotective and antistress effects: the cardioprotective and cardiotropic properties of *W. somnifera* are demonstrated via nuclear factor erythroid 2-related transcription factor (Nrf)-2 and by activating phase II detoxification enzymes and abrogating apoptosis. Moreover, it is capable of alleviating chronic stress induced reduction of T-cell population and upregulated Th1 cytokines, thereby ensuring better stress endurance in animals as well as humans. Anti-inflammatory and antiarthritic effects: *Withania somnifera* alleviated inflammation by suppressing cytokines such as interleukin- (IL-) 8 and 1, tumor necrosis factor- (TNF-) α, nitric oxide (NO), and reactive oxygen species (ROS). Furthermore, withaferin A, one of the active ingredients of *W. somnifera,* inhibited the expression of cell adhesion molecules, leukocyte adhesion and migration, IL-6 and TNF-a production, and NF-k activation (nuclear factor kappa-light-chain-enhancer of activated B cells). Furthermore, it inhibited the phosphorylation of p38, extracellular regulated kinases (ERK 12), and c-Jun N-terminal kinase by phorbol-12-myristate-13-acetate (PMA) (JNK).

biological activities, including antioxidant, antimicrobial, anti-inflammatory, and chemopreventive abilities, as assessed by both in vitro and in vivo studies. Concerning its in vitro biological effects, studies performed so far generally focused on their antioxidant activity and total phenolic content (spectrophotometric and/or chromatographic analyses) [61–68] and antimicrobial effects (disc diffusion assay and/or minimum inhibitory concentration (MIC)) [65, 69–81]. In addition to in vitro studies, there has been a significant number of *in vivo* studies addressing the anti-proliferative, cytotoxic, and anti-inflammatory effects of *W. somnifera* extracts in animal models [62].

5.1. Antioxidant Activity. The biological effects, and particularly the antioxidant potential and phytochemical constituents of *W. somnifera*, along with the other plants of the *Withania* genus, vary depending on the extraction method [61]. Methanol-chloroform-water (1 : 1 : 1) extract of *W. somnifera* roots, with the highest content of all phytochemical constituents except tannins, had higher antioxidant and reducing activities when compared to water, acetone, and aqueous methanol (1 : 1) extracts (i,e. total antioxidant capacity of methanol-chloroform-water (1 : 1 : 1) was 83.354 ± 1.828, aqueous methanol (1 : 1) was 76.978 ± 2.210, and water was 68.439 ± 1.000) [62]. Alkaloid content was found to be a leading contributor to the overall antioxidant and reducing activities of the extracts, closely followed by flavonoids and withanolides. Moreover, different parts of the plant may have different levels of antioxidant capacity [62]. For instance, Sumathi and Padma [82] reported that the leaves and fresh and dry tubers of *W. somnifera* had high contents in antioxidant compounds, while those present in tender roots and stems were not so high. Similar findings were also stated in other studies [63–65], with Alam et al. [66] also reporting that *W. somnifera* presents a good antioxidant activity, with catechin being the major polyphenol present in the highest concentration (13.01 ± 8.93 to 30.61 ± 11.41 mg/g). High concentrations of polyphenols (gallic, syringic, benzoic, p-coumaric, and vanillic acids as well as catechin, kaempferol, and naringenin), flavonoids, and DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging activities were detected in 80% methanolic extracts of *W. somnifera* fruits, roots, and leaves, ranging from 17.80 ± 5.80 to 32.58 ± 3.16 mg/g (dry weight), 15.49 ± 1.02 to 31.58 ± 5.07 mg/g, and 59.16 ± 1.20 to 91.84 ± 0.38 mg/g, respectively [66]. Other authors also reported that *W. somnifera* root extract (0.7 and 1.4 mg/kg daily by gastric intubation method for 20 days) improves oxidative damage due to lead intoxication in mice by significantly decreasing lipid peroxidation and significantly increasing superoxide dismutase and catalase enzyme activities [67]. Free radical scavenging activity (FRSA) and metabolic profile of in vitro cultivated and field-grown *Withania somnifera* roots were examined by Samir et al. [68]. In vitro produced roots had significantly higher levels of FRSA, total phenolic content (TPC), and total flavonoid content (TFC) than field-grown samples. Furthermore, as compared to 45-day-cultured samples, 30 day-cultured in vitro root samples had considerably greater FRSA, TPC, and TFC. Gas chromatography-mass spectrometry study detected a total of 29 compounds in in vitro cultivated and field-grown roots. Alcohols, organic acids, purine, pyrimidine, sugars, and putrescine were among the metabolites identified. Vanillic acid was found only in in vitro cultured root samples, and it was found in higher concentrations in 30 day-cultured in vitro root samples than in 45 day-cultured samples. As a result, 30 day-cultured in vitro root samples are recommended as a substitute for field-grown roots in the development of medicinal and functional food products.

5.2. Anticancer, Anti-Inflammatory, and Cytotoxic Activity. Regarding the anticancer and cytotoxic effects of *Withania* species, Samir et al. [68] reported that ethanol extracts of aerial parts of *W. somnifera* demonstrated cytotoxic activity against human liver (HEPG-2) and breast (MCF-7) cell lines with half-maximal inhibitory concentration (IC_{50}) of 8.5 μg/mL and 9.4 μg/mL for HEPG-2 and MCF-7, respectively. Cytotoxic activity of *W. somnifera* extracts was found to be at the stage of the G2/M phase and sub-G0 by arresting the cell cycle. Similarly, Naidoo et al. [83] reported that *W. somnifera* root extract effectively regulates the levels of the inflammatory cytokines while inhibiting the cancer cells' growth. Closely linked to the antioxidant activity, the cytotoxic activity of *W. somnifera* leaf extract against hepatocellular carcinoma cell line was also reported by Ahmed et al. [84]. In another study, it was observed that hydroalcoholic extract of *W. somnifera* root exhibited chemopreventive activity in mice with skin cancer [39] and fibrosarcoma [85]. Similarly, Padmavathi et al. [86] reported that *W. somnifera* root exerts chemopreventive effects against forestomach and skin carcinogenesis in mice.

On the other hand, closely linked to both antioxidant and anti-inflammatory effects, Khadrawy et al. [87] reported that *W. somnifera* demonstrated excellent effects against aluminum chloride ($AlCl_3$)-induced neurotoxicity in rats. Aluminum increased lipid peroxidation and nitric oxide levels in the cortex, hippocampus, and striatum while lowering glutathione levels in the hippocampus and striatum. Lipid peroxidation, nitric oxide, and reduced glutathione levels were not significantly different in rats protected with *W. somnifera* extract. Furthermore, it inhibited the increased activity of acetylcholinesterase and Na^+, K^+, ATPase in the cortex, hippocampus, and striatum caused by $AlCl_3$, apart from preventing a significant increase in tumor necrosis factor-α induced by $AlCl_3$ in the cortex and hippocampus. These findings imply that *W. somnifera* extract can protect against aluminum neurotoxicity by acting as an antioxidant and anti-inflammatory agent. Furthermore, it helps to prevent the decline in cholinergic activity by maintaining normal acetylcholinesterase activity. The latter effect may support the use of *W. somnifera* as a memory booster. Also, Pingali et al. [88] reported that withaferin A of *W. somnifera* can cause type II collagen expression and increase reactive oxygen species and cyclooxygenase-2 expression in rabbit articular chondrocytes depending on dose and time.

5.3. Cardioprotective Activity. Udayakumar et al. [89] suggested that the flavonoids and phenolics present in both root and leaf extracts of *W. somnifera* can be effective in reducing the blood glucose levels in diabetic rats. It was also reported that *W. somnifera* was effective in decreasing hyperlipidemia and oxidative stress in type 2 diabetic rats. When *W. somnifera* was given orally to type 2 diabetic rats at dosages 200 mg/kg and 400 mg/kg, it led to significantly reduced serum levels of total cholesterol, triglyceride, low-density lipoprotein-cholesterol, and very-low-density lipoprotein-cholesterol while high-density lipoprotein-cholesterol levels increased significantly when compared to the diabetic control group [90]. Moreover, Udayakumar et al. [89] claimed that phenolic contents of the extracts of *W. somnifera* leaf and root were helpful in decreasing blood

glucose levels in diabetic rats. Elkady and Mohamed [91] also reported that *W. somnifera* can be effective in protecting the occurrence of cardiotoxic effects induced by γ-rays in rats. A similar finding was also reported by Hosny Mansour and Farouk Hafez [92] that *W. somnifera* reduced hepatotoxicity in rats exposed to γ-radiation by significantly lowering serum hepatic enzymes, hepatic nitrate/nitrite, and malondialdehyde levels, significantly increasing antioxidant activity, and significant heme oxygenase (HO-1) induction. HO-1 enzymes protect the cell from injury due to oxidative and pathological stress, having a central role in cardiovascular protection [93].

5.4. Antimicrobial Activity. The antimicrobial activity of the *Withania* species is also remarkable. For example, methanol extracts of *W. somnifera* roots, fruits, and leaves have been revealed to be highly effective against gram-negative bacteria, including *Klebsiella pneumoniae, Citrobacter freundii, Salmonella typhi, Pseudomonas aeruginosa, and Escherichia coli*, as shown by Alam et al. [65]. Modulation of physiological functions of gut microbiota is involved in the mode of action of Withania somnifera root extracts. Similarly, the dichloromethane and ethyl acetate extracts of aerial parts of *W. somnifera* also evidenced excellent effects against *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* by disc diffusion assay, as shown by Mwitari et al. [69] and Hussain et al. [94].

The antimicrobial activity depends on the extraction method where ethanolic and methanolic extracts of *W. somnifera* root did not exhibit antibacterial activity against *K. pneumoniae* and methicillin-resistant *S. aureus*, whereas these microorganisms were inhibited by chloroform extracts of stem and leaves [70]. Moreover, the antimicrobial activities of different extracts of *W. somnifera* against different bacteria were reported by AbdEislam et al. [71]. The antibacterial activity of aqueous extract of *W. somnifera* against *E. coli* was higher compared to that of the alcoholic extract [72]. The extracts of *W. somnifera* root were also effective against multidrug-resistant *S. aureus* [73], with methanol extract of *W. somnifera* being also effective in inhibiting oral bacteria, like *Streptococcus mutans* and *Streptococcus sobrinus* [74]. Halamova et al. [75] investigated the antimicrobial activity of *W. somnifera* against human pathogenic bacteria and observed that those pathogens were more susceptible to extracts compared to beneficial *Bifidobacteria*. Interestingly, Zahran et al. [95] also reported that the dietary supplementation with *W. somnifera* root powder exhibited immunotherapeutic activity against *Aeromonas hydrophila* in Nile tilapia.

When looking at the effect of *W. somnifera* isolated constituents, flavonoids have shown excellent antimicrobial effects against *C. albicans, S. aureus, Proteus mirabilis, E. coli*, and *P. aeruginosa*, although no effects were noted against *Aspergillus flavus* or *Aspergillus niger* [76]. Interestingly, the minimum inhibitory concentration (MIC) of *W. somnifera* methanol extract against *C. albicans* and *Neisseria gonorrhoeae*was reported as 20 mg/mL and 0.5 mg/mL, while that of water extract against *N. gonorrhoeae*was 10 mg/mL [77]. In addition, *W. somnifera* glycoprotein revealed antibacterial effects against *Clavibacter michiganensis* subsp. *michiganensis* and antifungal activity against *A. flavus, Fusarium oxysporum,* and *Fusarium verticillioides* [78]. Also, it was reported that *W. somnifera* can be utilized in the synthesis of silver nanoparticles with excellent antioxidant, antimicrobial, and anticancer potential [79–81].

6. Health-Promoting Effects

As previously mentioned, *Withania* has been used since a long time ago for different clinical purposes. In the traditional system of medicine, *Withania somnifera* has been used for anti-inflammatory, anticancer, antioxidant, adaptogenic, and antistress purposes, along with as an immunomodulator. Moreover, it also exerts a positive influence on endocrine, cardiorespiratory, and central nervous system (CNS) levels. For instance, it was stated that *W. somnifera* is a powerful help in cancer management, with good tolerance [96]. Recently, upon evaluating the clinical evidence base and investigating the potential role of *W. somnifera* in managing cognitive dysfunction, Ng et al. [97] found that *W. somnifera* extract improved performance on cognitive tasks, executive function, attention, and reaction time. It also appears to be well tolerated, with good adherence and minimal side effects. Using standardized *W. somnifera* extracts or its bioactive ingredients, new and more effective medications to treat cognitive impairment could be produced [97]. Notwithstanding, despite the broad spectrum of preclinical data available, the number of clinical trials performed using *W. somnifera* is markedly scarcer (Table 2).

7. Food-Pharma Industry: Safety and Adverse Effects

W. somnifera has traditionally been available in the form of capsules and powder, being most often sold as a supplement. However, it can now be found in a variety of food products, including ghee, honey, and kombucha. More recently, *W. somnifera* has also been incorporated in baked goods, juices, and beverages, respectively, sweets (candies/snacks), and dairy products marketed as "Functional Foods" or "Nutraceuticals." The worth of note is that the amount of *W. somnifera* in food can vary widely, where the addition of powder can range from 1 to 10% depending on the product (baked good vs. beverage). Also, levels of *W. somnifera* up to 5% have also been found to have acceptable sensorial features [114].

Herbal cookies designed as functional foods have also been developed with *W. somnifera* leaf powder, with the final product presenting with an acceptable color, taste, and texture while maintaining an acceptable shelf-life [115]. Incorporating *W. somnifera* into foods can serve several functions; for example, it can provide excellent antioxidant and human health benefits. Moreover, the addition of *W. somnifera* to ghee (clarified butter fat) was found to be an effective natural antioxidant to prevent oxidative degradation (less than synthetic antioxidant BHA, butylated hydroxyanisole) apart from providing health-promoting benefits. The antioxidant activities evaluated were

Table 2: Health-promoting effect of *Withania somnifera*.

Biological activity	Dose/duration	Study design/subjects	Effect	References
Antistress and antianxiety	500 mg dried aqueous extract of roots and leaves/twice a day for 14 days	Double-blind, placebo-controlled, randomized, crossover study ($n = 20$ healthy men)	Decrease aortic pressure	[88]
	300 mg roots extract/day, 45 days	Prospective double-blind, randomized, placebo-controlled trial ($n = 64$ subjects with a history of chronic stress)	Reduce cortisol levels and the scores of stress-assessment scales	[98]
	500 mg powder capsule/ twice a day, twice a day, 30 days	Single-trial group ($n = 30$ subjects with generalized weakness)	Reduce fatigue symptoms, improve workability and quality-of-life dimension scores	[99]
	120 mg root extract/day, six weeks	Double-blind placebo-controlled trial ($n = 30$ individuals with the obsessive-compulsive disorder)	Improve effect in Yale-Brown obsessive-compulsive scale (symptoms severity)	[100]
	300 mg root extract/day 12 weeks	Clinical control-placebo trial ($n = 55$ type II diabetics, under oral hypoglycemics)	Improvement in stress and complaints	[101]
	250 mg root ethanol extract/twice a day, 6 weeks	Double-blind, placebo-controlled study ($n = 39$ subjects with generalized anxiety disorder, mixed anxiety and depression, panic disorder, and adjustment disorder with anxiety)	Improvement in anxiety score across time	[102]
	1000 mg standardized root extract/day, 12 weeks	Randomized, placebo-controlled, double-blind ($n = 66$ patients with depression and anxiety symptoms)	Improvement in depression single-item and anxiety-depression cluster scores and anxiety symptoms	[103]
Cognitive	500 mg standardized root extract/day 8 weeks	Randomized placebo-controlled ($n = 53$ patients with bipolar disorder)	Improvement in auditory-verbal working memory (digit span backward)	[104]
	250 mg dried aqueous extract of roots and leaves/twice daily, 14 days	Prospective, double-blind, placebo-controlled, crossover ($n = 20$ healthy men)	Improvement in the cognitive and psychomotor performance	[105]
	300 mg root extract/twice daily, 8 weeks	Prospective, randomized, double-blind, placebo-controlled ($n = 50$ healthy man and female adults)	Improvement in general memory and executive function	[106]
Cardiorespiratory	300 mg roots extract/ twice daily, 12 weeks	Randomized, double-blind, and placebo-controlled ($n = 50$ healthy athletic male and/or female adult)	Enhances the cardiorespiratory endurance, improvement in the self-reported quality-of-life questionnaire	[107]
	250 mg standardized root extract/twice daily, 14 days	Prospective, double-blind, randomized, and placebo-controlled ($n = 50$ healthy men)	Increased velocity, power, and maximum oxygen consumption	[108]
	500 mg standardized root extract/day Sensoril®, 12 weeks	Randomized, double-blind, placebo-controlled ($n = 40$ healthy, recreationally active men)	Improves upper- and lower-body strength in active men	[109]
Analgesic/anti-inflammatory	1000 mg standardized root extract/day, 10–14 days	Randomized placebo-controlled ($n = 26$ healthy men)	Increased mean pain threshold time	[110]
	250–125 mg standardized root extract/twice daily, 12 weeks	Randomized, double-blind placebo-controlled ($n = 16$ patients with knee joint pain and discomfort)	Reduced pain and disability scores (both doses), and promoted a better response (at a higher dose)	[111]
	450 mg root extract/day, 15 days	Double-blind, placebo-controlled, crossover ($n = 42$ patients with osteoarthritis)	Reduced severity pain and a disability score	[112]
Chemoprotective	2000 mg root extract/day every 8 h during chemotherapy cycles	Open-label prospective nonrandomized comparative trial ($n = 100$ patients with breast cancer in all stages)	Reduce score Piper's fatigue score Reduced Schwartz's cancer fatigue score and improved quality-of-life questionnaire scores	[113]

β-carotene bleaching assay, DPPH assay, and Rancimat method, and the doses evaluated were 1.0% and 0.5% (w/w) for aqueous and ethanolic *W. somnifera* extract, respectively. Perhaps not surprisingly, much food product development research has focused on incorporating *W. somnifera* into foods commonly consumed in India. Nonetheless, as foods containing *W. somnifera* are becoming widely available, increasing attention and consideration must be given to the potential occurrence of adverse effect(s) as a result of overingestion [116].

7.1. From Therapeutic to Safety Profile. Animal and human studies have been conducted to determine the potential impact in the treatment of a wide range of diseases, including but not limited to cancer, immunosuppressive diseases, anxiety and depression, Parkinson's disease (PD), and fertility [117]. Studies performed so far suggest that the consumption of up to 100 mg per kg of body weight in a single dosage or approximately 21 g per day is safe. Typically, a therapeutic dose is ≤10 g/day, so that a total intake can be more closely controlled when consumed in a capsule form. In an animal model, *W. somnifera* extract was given for 28 days at oral doses of 0, 500, 1000, and 2000 mg/kg body weight, and data obtained suggest that the administration of *W. somnifera* extract up to 2000 mg/kg/day did not trigger adverse effect [118].

Several review articles broadly cover various human clinical trials suggesting that *W. somnifera* has no adverse health effects during long-term (≥one-year) administration [119]. For example, a group of 64 subjects aged from 18 to 54 received a 300 mg capsule of *W. somnifera* root extract for a period of 60 days [98]. Any incidences of adverse events were comparable in the placebo-control group and *W. somnifera* group, with the difference being not statistically significant. Another study investigated the use of *W. somnifera* in reproductive issues; for that, a group of 41 men received a dose of 4 tablets (500 mg each) 3 times/day (i.e., 6 g/day) containing *W. somnifera* root powder through oral route after intake of food for 60 days [120]. The placebo (wheat powder) received a tablet form, consisting of 4 tablets (500 mg each) 3 times/day (i.e., 6 g/day) ($n = 45$). No adverse health effects were stated using the *W. somnifera* root powder.

The impact of *W. somnifera* root extract supplementation in muscle strength and recovery of 57 male subjects (18 to 50 years old) was also evaluated [121]. Subjects in the treatment group received 300 mg of *W. somnifera* root extract twice daily for 8 weeks, and no adverse health events were reported. Taken together, data obtained so far appears to support that *W. somnifera* has no toxic effects; however, such studies were not specifically designed to address safety and adverse effects. Also, most studies were of short duration and, as such, may not be indicative of the long-term impact of *W. somnifera* intake in human health.

7.2. Pregnancy and Teratogenicity. To what concerns, the safe use of *W. somnifera* during pregnancy, whether as a supplement or in food, remains uncertain. Reports suggest that *W. somnifera* might have abortifacient properties during pregnancy, indicating classification under toxic plants that cause abortion and sterility [122, 123]. In this way, some researchers addressed the concern by orally administering *W. somnifera* root extract to pregnant rats during a period of major organogenesis and histogenesis (days 5 to 19 of gestation). Briefly, pregnant rats received a dose of 500, 1000, and 2000 mg/kg/day and were monitored for a range of clinical symptoms, although no evidence of maternal or fetal toxicity was stated. The root extract provoked no changes in body weight of parental females, the number of corpora lutea, implantations, viable fetuses, and external, skeletal, and visceral malformations. Thus, the authors proposed evidence of safety related to *W. somnifera* root extract at least at 2000 mg/kg/day [124]. Regardless, caution must be exercised concerning the use of *W. somnifera* during pregnancy given the limited number of published studies addressing the issue [122, 123]. According to the National Institutes of Health [125], *W. somnifera* contains several compounds that may cause miscarriage, premature birth, or uterine contractions [124]. *W. somnifera* is commonly safely used by adults in doses up to 1000 mg per day, for up to 12 weeks, but pregnant and breastfeeding women should not consume [125].

Collectively, the wealth of research suggests that oral intake of *W. somnifera* is safe with a possible exception during pregnancy. In addition, given that *W. somnifera* is being formulated into a wide range of commercially available food and beverages, the total day consumption by consumers of such products may need to be more closely considered. In this sense, future research may focus on differences in bioavailability of the various forms (leaf and root powder, extracts, and essential oils) related to safety and adverse effects.

8. Conclusion

The *Withania* genus has been traditionally used for its therapeutic potential in numerous diseases, of which insomnia, depression, and immunostimulant effects stand out. However, remarkable anti-inflammatory and rejuvenating activities have also been stated, with in vitro and in vivo studies highlighting excellent antioxidant, antiproliferative, cytotoxic, anti-inflammatory, and antimicrobial activity. However, not all species present the same activity, with the most studied and economically important one being the roots of *W. somnifera*. More importantly, the clinical studies performed so far have progressively affirmed the *W. somnifera* therapeutic effects, namely, its excellent ability to increase vitality, physical performance, and hematopoietic capacity and to treat insomnia. Moreover, *W. somnifera* is being valued for its ability to promote longevity and strengthen the immune system without stimulating the body's reserves. Nonetheless, despite the advances stated so far, further clinical trials and more precise and deeper studies, namely, addressing the bioavailability and effect of pure compounds and the occurrence of synergistic effects when used in combination, along with the development of methods to standardize the percentage composition of active compound(s) in marketed products, are the fields that most

need to be intensively explored. Actually, although it is possible to find various products containing *W. somnifera* at variable amounts and safety studies do not report adverse effects, it is of utmost importance to have deeper knowledge on synergistic effects that may possibly occur with other food components and to know what are the effects when high doses are used and even what are the effects in pregnancy.

Data Availability

The data supporting this review are from previously reported studies and datasets, which have been cited. The processed data are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

All authors contributed equally to the manuscript. Conceptualization was done by Javad Sharifi-Rad, Hari Prasad Devkota, Beraat Özçelik, Miquel Martorell, William C. Cho, and Natália Cruz-Martins; Cristina Quispe, Seyed Abdulmajid Ayatollahi, Farzad Kobarfard, Mariola Staniak, Anna Stępień, Katarzyna Czopek, Surjit Sen, Krishnendu Acharya, Karl R. Matthews, Bilge Sener, Celale Kırkın, Montserrat Victoriano, Deepak Chandran, Manoj Kumar, and Hafiz Ansar Rasul Suleria contributed to validation, investigation, data curation, and writing the draft of the manuscript; review and editing of the manuscript were performed by Javad Sharifi-Rad, Hari Prasad Devkota, Beraat Özçelik, Miquel Martorell, William C. Cho, and Natália Cruz-Martins. All authors read and approved the final manuscript.

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Estimating the production of withaferin A and withanolide A in *Withania somnifera* (L.) dunal using aquaponics for sustainable development in hill agriculture

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Introduction: Humanity is suffering from huge and severe difficulties, including changes in climate, soil degradation, scarcity of water and the security of food and medicines, among others. The aquaponics system acts as a closed loop consisting of aquaculture elements and hydroponics, which may contribute to addressing these problems. The aquaponics method is quickly expanding as the requirement to increase the production of sustainable herbal products, including medicinal compounds and foods, in freshwater systems and replenish phosphorous reserves shrinks.

Methods: The current work is designed to increase the production of the antioxidants withaferin A and withanolide A in two varieties (Jawahar-20 and Poshita) of *W. somnifera* using the aquaponics technique. Total 100 seedlings (one month old) grown in soil initially were taken to be grown in aquaponics for a time period of 6 months.And 100 seedlings were placed in pots containing soil as control for study after six months.

Results: It was observed that the higher content of withaferin A was analyzed in the root and stem samples of Jawahar-20 and Poshita from the six-month-old plant of *W. somnifera*. The maximum content of withanolide A was examined in the root samples of the six month-old plants of Poshita (1.879 mg/g) and Jawahar-20 (1.221 mg/g). While the 6 month old Poshita seedling grown in soil recorded less withaferin A (0.115 ± 0.009^{b}) and withanolide A (0.138 ± 0.008^{d}).

Discussion: It is concluded that Poshita was found to be more promising for the enhanced production of withaferin A and withanolide A in the aquaponics

system. Moreover, the root was observed as the best source for the production of withaferin A and withanolide A and the best age of the plant is 2 years for the production compounds in medicinal plants with futuristic perspective to hill agriculture integrated farming. compounds. Thus aquaponics can be an effective approach with enhanced yield of bioactive compounds in medicinal plants with futuristic perspective to hill agriculture and integrated farming.

KEYWORDS

aquaponics, withania somnifera, metabolites, withaferin A, withanolide

Introduction

Farmers in the hilly regions face a major constraint due to undulating topography, leading to soil erosion gradually causing loss of its fertility. Thus most of the farming is affected and is performed on fragile land set-ups, then lack of infrastructure, lack of transport, irrigational problems, lack of capital are another huge challenges faced by farmers of hilly regions. This affects the growth and production of quality crops inspite of hard efforts and funds of the farmer. Moreover the exposure of crops to different types of biotic and abiotic stress make the crops more vulnerable affecting its yield. Thus in order to sustain such harsh conditions integrated farming approach offers a great advantage to the farmers by utilization of available resources in a precise manner to get the best results. Aquaculture offers a promising approach to cater to the needs of farmer and also enable him to raise his income. Aquaculture comprises three entities: fish, plants, and nitrifying bacteria. Where by the nitrifying bacteria convert the unfed food and faecal waste of the fish into utilisable simpler forms, i.e., converting ammonia into nitrates that can be taken up by the hydroponic plants. Thus, aquaponics serves as a source of nutrition for the hydroponic plants under controlled conditions. Aquaponics is resilient integrated farming with less water, labour, and land, providing better nutrition and wellness to the growing plants and crops. Different plants grow successfully in aquaponic systems. Fish rearing and hydroponic vegetable components must be operated continuously in order to have enhanced production (Rakocy et al., 2007). Hydroponically grown plants require a lower pH, from 5.5 to 6.5 (Rakocy et al., 2007). The maintenance of nutrient solution pH is a significant challenge in aquaponics systems, as not only it promotes plant growth but also influences the bioactive metabolite production, root rhizosphere and apoplastic pH. In a study on Taraxacum officinale and Reichardia picroides, low pH levels (pH = 4.0) seemed to be beneficial to nutritional and dietary value in both species highlighting the potential of commercial cultivation under adverse conditions, especially in sustainable farming systems (Alexopoulos et al., 2021). Aquaponics is a symbiotic combination of growing fish and hydroponics, where nutrient-enriched water is used for growing plants in a soil-less culture. Thus, aquafarming is an integrated farming method for growing plants under controlled environmental conditions. It can be a powerful method for the development of elite germplasm with enhanced pharmaceutically active ingredients from medicinal plants in hilly and desert areas where land and water are scarce. Traditional practices of uprooting the whole plant for the extraction of plant metabolites can lead to the extinction of many important plant varieties of pharmaceutical significance. Thus, conservation of such endangered medicinal plant species is the prime need for adopting sustainable agricultural and integrated farming methods.

A hydroponic-centered system for the production of crops provided the necessary nutrients to produce cherry tomatoes (Schmautz et al., 2016) and reported microbial niche distinctions within the aquaponics system (Schmautz et al., 2017). The aquaponics system is also regulated by the type of fish being used for the maintenance of the aquaponics system. Generally, the species used for aquaponics production include Arctic char, trout, perch (Diver and Rinehart, 2010), bluegill, largemouth bass, channel catfish, barramundi, Murray cod, jade perch (Nelson and Pade, 2008), koicarp, goldfish, pacu, and common carp (Rakocy et al., 2007).

Medicinal plants have a very significant role in curing different ailments naturally (Bhasin et al., 2019). The pharmacologically significant medicinal plants are in huge commercial demand owing to the presence of bioactive ingredients (Singh et al., 2017; Gupta et al., 2019). The bioactive metabolites of *Withania somnifera* possess adaptogenic, anticancer, anti-convulsant, immunomodulatory, antioxidative and neurological effects. The plant has also been found to be efficient in the treatment of osteo-arthritis, geriatric, behavioural and anxiety (Singh et al., 2022). The root extract of Ashwagandha containing withanolide A is reported to have health promoting effects such as anti-stress, anti-arthiritic, anti-inflammatory, analgesic, anti-pyretic, anti-oxidant and immunomodulatory properties (Singh et al., 2022). Withanolide B, D, F and withanosides (glycosylated steroids) secreted by WS having neuroprotective, anticancer, hepatoprotective, anti-aging, diuretic, antipogenic, hemopoietic, immunomodulatory functions and antioxidant activities (Singh et al., 2017). It is also reported to improve the overall health so works as a rejuvenating drug to the aged persons. However roots of the plant is used in preparing tonic which promote anti-aging properties and also helpful in the treatment of infectious diseases.

The major bottleneck is the availability of raw materials to meet commercial demands (Singh et al., 2018). It has been reported that the contents of bioactive compounds vary from tissue to tissue and variety to variety, so the selection of elite plant varieties is crucial (Singh et al., 2019). The bioactive compounds can be enhanced by the application of different elicitors, in different environmental regimes, and through tissue culture techniques (Singh et al., 2020). It has been reported that 15 mg/L ammonium nitrate increased the withanolide contents (1.74 mg/g DW withanolide A, 0.92 mg/g DW withanolide B, 0.52 mg/g DW withaferin A, and 1.54 mg/g DW withanone) in a culture of roots formed from shoot regeneration (Sivanandhan et al., 2015).

In aquaponics systems, ammonia is an important nitrogen source for plants, and fish species that accumulate ammonia and urea in higher quantities are mostly in demand. As a result of the stability of the nitrogen metabolism processes, tolerant temperature species are preferred for higher urea and ammonia excretion ratios. The main obstacle to the aquaculture technique is represented by its first drawback: high initial capital investments to maintain a constant water quality that will respond to the physiological requirements of cultured species. People have also reported that excess solid wastes increase the BOD, causing a lowering of oxygen levels in the rhizosphere and the accumulation of ammonia and nitrate, which are toxic for plant growth (Rakocy et al., 2012; Danaher et al., 2013). Pharmaceutically active metabolites of *Withania somnifera* Withanolide A and Withaferin A are of immense health benefits to humans.The studies have shown that withanolide A possess neuroprotective, anticancer, hepatoprotective, anti-aging, diuretic, antipogenic, hemopoietic, immunomodulatory functions and antioxidant activities, possess strong neuropharmacological efficacy (Singh et al., 2022). Withaferin A serves as a potent anti-cancerous compound. It also has diverse pharmacological activities, including antitumor, antiangiogenic, cardioprotective, anti-inflammatory, and immunomodulatory effects. Recently it was observed that Withaferin A having therapeutic potential, protects from COVID-19 infection to mitigate the virus made cardiovascular disease (Singh et al., 2022).The two predominant phytochemicals of the plant contribute in drugs as well as assists with getting the physiological property for the treatment of various illnesses.

Keeping in mind the significance of aquaponics, the present study focuses on an aquaponics system that has been employed for the enhancement of withanolide contents under the controlled micro and macro environments of rhizogenesis.

Materials and methods

Material prepared or purchased

The seeds of commercially available varieties of *Withania somnifera* (L.), viz. Jawahar-20 and Poshita, were purchased from CIMAP, Lucknow. The fish and their meals were purchased from the certified center of the G. B. Pant University of Agriculture and Technology, Pantnagar.

Aquaponic culture for Poshita and Jawahar-20 varieties of *Withania somnifera*

The study was performed to analyze the production of antioxidants withaferin A and withanolide A in two elite varieties, Jawahar-20 and Poshita, of *W. somnifera* Dunal using modern hydro-chemical aquaponics culture techniques. Total 100 seedlings which one month old grown in soil initially were taken to be later grown in aquaponics for 6 months and 100 seedlings were placed in pots containing soil as control for study after six months. The aquaponics culture condition was maintained between 65-85°F, and the pH between 5.5 to 6.5 was maintained and monitored at every hour interval. The fishes used in aquaculture were Rohu (*Labeo rohita*), Glass Catfish (*Kryptopterus bicirrhis*), Basa (*Pangasius bocourti*), and Singhi (*Heteropneustes fossilis*) fishes were used in the aquaponics system. The feed for the fish was wheat flour (Atta), wheat bran (Choker), azolla (*Azolla caroliniana*), linseed meal, and rice husk powder. The element composition of water used was rich in NH_3 and other important minerals (Table 1). The cultivation time of studied plant of aquaponics was 1 month (control) and 6 (month) seedlings inorder to assess the withanolides production.

Comparison of *Withania somnifera* seedlings grow in soil and aquaponics

The seedlings of the Jawahar-20 and Poshita varieties of *W. somnifera* were also grown in soil. The comparative studies were performed on seedling growth in soil and aquaponics techniques.

Estimate the content of withaferin A and withanolide A in the leaves, roots and stem samples of Poshita and Jawahar-20 varieties of *Withania somnifera*

The samples of fresh leaves, stems, and roots were taken from the one- and six-month-old seedlings of Jawahar-20 and Poshita varieties of *W. somnifera*. The tissue samples were subjected to drying in a hot air oven at 40°C for 3–4 days until a constant dry weight was obtained. Then the plant tissue was ground with the help of a clean and dry mortar and pestle. Dry powder (1g) of plant tissue was taken and percolated in 50 ml of 80% methanol, then sonicated for 20 minutes and placed on a rotatory shaker at 30°C at 100 rpm overnight. The procedure was repeated three times, and the methanolic extracts thus obtained after percolation were pooled together and filtered through Whatman filter paper (pore size 11µm). Then the methanolic extract was subjected to drying using a rotatory vacuum

TABLE 1 Element analysis of the water used in aquaponic system.

S. No.	Parameters	Tap water	Aquaponic water
1.	pH	7.7	6.5
2.	EC	251 µS/cm	205 µS/cm
3.	Temperature	25°C	28.66°C
4.	Dissolved O_2	4.1 mg/l	6.81 mg/l
5.	TS	281 mg/l	298 mg/l
6.	TDS	161 mg/l	150 mg/l
7.	BOD	5.95 mg/l	2.95 mg/l
8.	COD	6.38 mg/l	11.71 mg/l
9.	Salinity	12	21
10.	Nitrite(NO_2)	0.009 mg/l	0.018 mg/l
11.	NH_3	0.253 mg/l	0.590 mg/l
12.	PO_4	0.95 mg/l	0.06 mg/l
13.	Cd	0.112 µg/ml	0.034 µg/ml
14.	Zn	0.086 µg/ml	0.035 µg/ml
15.	Cu	0.037 µg/ml	0.308 µg/ml
16.	Cr	0.00	0.00
17.	Pb	0.00	0.297 µg/ml
18.	Na	4.68 ppm	15 ppm
19.	K	0.95 ppm	08 ppm
20.	TDS	161 mg/l	309 mg/l
21.	Mn	0.097 mg/l	0.84 mg/l
22.	Ca	3.205 µg/ml	1.19 mg/l
23.	Mg	14.76 µg/ml	11.02 µg/ml
24.	Hg	7.376 µg/ml	6.02 µg/ml
25.	Fe	2.373 µg/ml	3.56 µg/ml

evaporator maintained at 60°C until a completely dried residue was obtained. The dried residue was dissolved in HPLC-grade methanol (4 ml). A pinch of charcoal was added to the extract in order to decolorize the sample, which was centrifuged at 8000 rpm for 15 min. The supernatant was then filtered through nylon filter membranes (0.22). The samples were used for the estimation of the content of withaferin A and withanolide A. The extra samples were kept in the vials at 4°C for future uses (Singh et al., 2018; Singh et al., 2020). The quantification of antioxidants was done through HPLC in one-month-old seedlings and six-month-old seedlings of Jawahar-20 and Poshita varieties of *W. somnifera* (Singh et al., 2018).

Essential elements and other parameters analysis in the water sample of aquaponics culture

The analysis of elements existing in the aquaponics water sample used for the elicitation experiment was done through the protocol of *Standard Methods for the Examination of Water and Wastewater* by the *American Public Health Association* (APHA) with a few modifications (Lipps et al., 2023).

Atomic absorption spectrophotometer

The presence of cadmium, zinc, copper, chromium, and lead was determined by AAS (Atomic Absorption Spectrophotometer (AAS), Benchtop Thermo Fisher Ice 3500 Atomic Absorption Spectrometer. Digestion of the sample (50 ml) with HNO_3 (20 ml) then the volume of the sample was reduced on a hot plate to 15 ml at 100°C. The final volume was made up to 100 ml with double-distilled water. The sample was then filtered with Whatman filter paper, and the reading of the sample was taken through AAS.

Spectrophotometric analysis

The presence of Nitrite (NO_2^-), Ammonia (NH_3^+), and Phosphate (PO_4^-) was done through a spectrophotometer, Evolution 201 UV-Vis spectrophotometer (Thermo Scientific, USA),. The wavelength used was 190 nm for Nitrite, Ammonia and Phosphate estimation.

Flame photometer

Five ml of the sample was taken and filtered with Whatman filter paper for the analysis of the presence of sodium (Na) and potassium (K). The equipment (Microprocessor Flame Photometer, LABTRONICS Model Name/Number: LT-6710) was calibrated with the standard solution of Na and K with a capillary tube (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 ppm). The sample was put into the equipment to take readings after calibration.

Biochemical oxygen demand

BOD is the measurement of the amount of dissolved oxygen needed by aerobic biological organisms to break down the organic material present in a given water sample at a certain temperature over a certain time period.

Procedure to determine the biochemical oxygen demand of water

To determine BOD, two bottles (300 ml) were filled with 10 ml of sample, and the remaining volume was made up with water. The other two BOD bottles were only filled with water, which served as a control. The bottles were closed immediately to avoid any air bubbles in the bottles. Then the bottles were incubated at 20°C for 5 days. After 5 days, the BOD of the sample was analyzed.

Neutralization of sample

A sample of 50 ml was taken in a 100-ml beaker. The pH of the solution was kept at 7.0 by using 1N H_2SO_4 or 1N NaOH. The volume of H_2SO_4 or NaOH used to adjust the pH of the 50-ml sample to 7.00 was recorded. Then the volume of sulfuric acid or sodium hydroxide required to neutralise the 1000-ml sample was calculated.

Removal of chlorine content

The removal of chlorine from the water sample was done by adding sodium sulfite to the sample. A 50-ml water sample was taken, to which 2.5 ml of acetic acid (50%) was added, followed by 2.5 ml of a 10% w/v solution of potassium iodide. After some time, 1 ml of starch indicator was added and titrated with a 0.025 N sodium sulfite solution.

Preparation of alkali-Iodide-azide reagent

An amount of 500 g of sodium hydroxide (NaOH) and 135 g of sodium iodide (NaI) were dissolved in distilled water, and the final volume was made up of 1000 ml of distilled water. To which 10 g of sodium azide were added.

Preparation of aqueous solvent

Five liters of double-distilled water were taken in a glass container and aerated with clean compressed air for 12 hours. It was then allowed to get stable for at least 6 hours at 20°C. After that, 5 ml of a 27.5% (w/v) solution of calcium carbonate, 5 ml of a 22.5% (w/v) solution of magnesium sulphate, 5 ml of a 0.15% (w/v) solution of ferric chloride, and 5 ml of a phosphate buffer solution were added. The solution was mixed well and allowed to stand for 2 hours.

Chemical oxygen demand

Standards were prepared using KHP (potassium hydrogen phthalate). Water sample (2 ml) was added to each vial. In the case of the "blank," 2 ml of double distilled water was added. Then 2 ml of the standard was added to the corresponding vials. Each vial was mixed well and placed into the COD reactor block for two hours. After two hours, the vials were removed from the block to a cooling rack for about 15 minutes. The readings were taken with the help of a colorimeter.

Analysis of essential elements and other parameters in the soil sample

Various essential elements and other parameters were analyzed in the soil samples of the mango garden, the transgenic laboratory, and the control soil sample. The soil (vermin-compost added and autoclaved) used for potting the plants of *W. somnifera* in the mango garden, Dept. of Plant Physiology, G.B. Pant University of Agriculture and Technology, and the other in the controlled environment of the Transgenic Laboratory, Dept. of Molecular Biology and Genetic Engineering, G.B. Pant University of Agriculture and Technology, was done through DTPA (diethylene triamine pentaacetic acid).

Estimation of micronutrients in soil by extraction method

Ten gram of air-dried soil was weighed in a 150-ml conical flask to which 20 ml of DTPA extraction buffer was added (1.967 g DTPA, 14.9 g TEA (triethanolamine), and 1.47 g $CaCl_2.2H_2O$ were dissolved in 200 ml DW). The pH of the solution was adjusted to 7.0, and the final volume was made up to 1000 ml. The flask was tightly capped with a polyethylene stopper and then kept on a horizontal shaker (120 cycles/min) for 2 h. After shaking, the filtrate was filtered through Whatman filter paper No. 42, and the filtrate was used for the estimation of micronutrients. The calculation of the extractable micronutrient content was done with the help of the following equation:

$$\text{DTPA extractable micro} - \text{nutrient content}(\text{mg/kg soil}) = \text{filtrate concentration}(\mu\text{g/ml}) \times 20/10$$

Results and discussion

Aquaponic culture for Poshita and Jawahar-20 varieties of *Withania somnifera*

The seedlings of the two promising varieties, Jawahar-20 and Poshita of *W. somnifera*, were grown in controlled conditions using the hydro-chemical technique of aquaponics for the estimation of biomass and content of withaferin A and withanolide A (Figure 1).

Comparison of seedlings of *Withania somnifera* grown in soil and aquaponics

The comparative studies showed more growth in the seedling of the one-month-old *W. somnifera* plant in aquaponics culture in comparison to soil culture. Similarly, the results demonstrated more length and biomass in aquaponics culture in six-month-old *W.*

FIGURE 1
Seedlings of *Withania somnifera* in the control condition of aquaponics.

somnifera plants (Figure 2). The growth of seedlings in the aquaponics system was also positively controlled by different species of fish (Knaus and Palm, 2017). The fish used in the present study of the aquaponics system were Rohu (*Labeo rohita*), glass catfish (*Kryptopterus bicirrhis*), Basa (*Pangasius bocourti)*, and Singhi (*Heteropneustes fossilis*). Previous studies have investigated the importance of African catfish (*Clarias gariepinus*) in aquaponics systems (Endut et al., 2009; Palm et al., 2014). The different types of feed given to the fish in our aquaponic system were also helpful for the regulation of the growth of the plant *W. somnifera*. One study has demonstrated that the fish feed rate is associated with the growth of plants, but the conversion of feed and nutrient assimilation vary with feed type and plant crop type (Rakocy et al., 2007).

Estimation of Withaferin A and Withanolide A in different plants tissues of Ashwagandha

The higher content of withaferin A was examined in the stem (0.446 mg/g), followed by the root (0.367 mg/g), and leaves (0.336 mg/g) of Jawahar-20 in one-month-old plants, while the higher content of withanolide A was examined in the root (0.149 mg/g), followed by stem (0.143 mg/g), and leaves. The one-month-old plants of Poshita showed a higher content of withaferin A in the stem (0.516 mg/g), followed by the leaves (0.400 mg/g), and the root (0.175 mg/g). The one-month-old plants of Poshita showed a higher content of withanolide A in the root (0.252 mg/g), followed by the leaves (0.168 mg/g), and the stem (0.066 mg/g) (Figure 3).

The higher content of withaferin A was examined in the stem (1.407 mg/g), followed by the leaves (1.166 mg/g), and the root (0.331 mg/g) of Jawahar-20 in six-month-old plants. While the higher content of withanolide A was examined in the root (1.221 mg/g), followed by stem (0.177 mg/g), and leaves (0.133 mg/g) of Jawahar-20 in one-month-old plants. The six-month-old plants of Poshita showed a higher content of withaferin A in the stem (1.977 mg/g), followed by the leaves (1.499 mg/g), and the root (0.543 mg/g). While the six-month-old plants of Poshita showed a higher content of withanolide A in the root (1.879 mg/g), followed by the leaves (0.495 mg/g), and the stem (0.196 mg/g) (Figure 4). The summary of results is demonstrated in the Supplementary Table. S1. Hence, the aquafarming technique is an integrated farming method of growing plants under controlled environmental conditions. It is concluded that the stem and root have excellent contents of withaferin A and withanolide A antioxidant in the Jawahar-20 and Poshita varieties. Previous research on cherry tomatoes showed that the hydroponic system is very helpful in increasing the yield of crops by providing the necessary nutrients (Schmautz et al., 2016). Another published work also suggested the positive role of the microbial niche in the production of cherry tomatoes in the hydroponic system (Schmautz et al., 2017).

Analysis of essential elements and other parameters in the water sample of aquaponic culture

Various essential elements and other parameters were analyzed in the water sample of the aquaponics system and tap water used to water potted plants as control (Table 1).

The pH value was 6.5 in the aquaponic water sample and 7.7 in the control sample. The plants require a lower pH (5.5 to 6.5) for excellent growth in a hydroponic system (Rakocy et al., 2007).

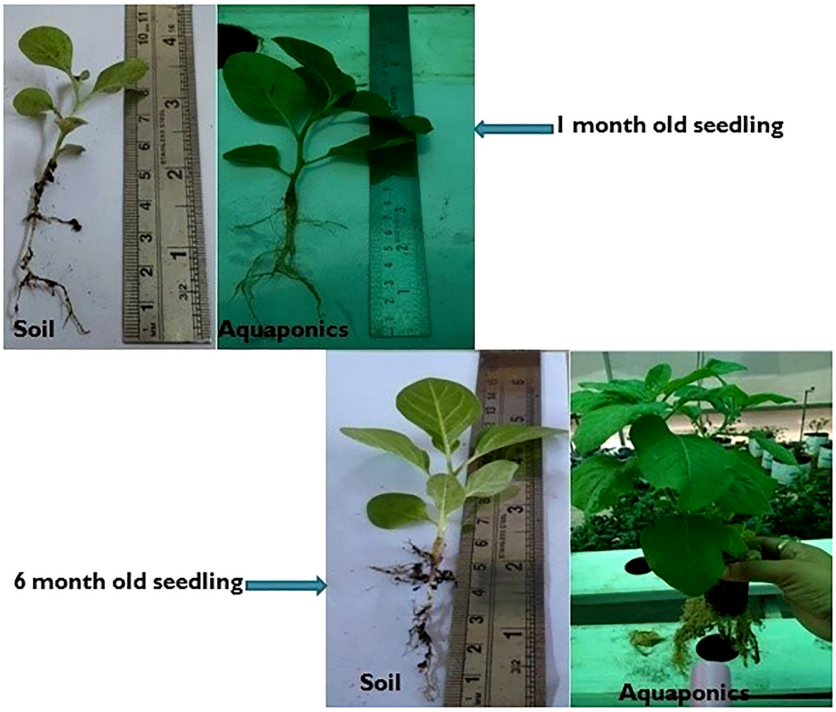

FIGURE 2
Comparison of seedlings of *Withania somnifera* grown in (i) Soil (ii) Aquaponics.

The results showed a high content of ammonia in the aquaponic water sample (i.e., 0.590 mg/l) with respect to the control sample (i.e., 0.253 mg/l). The content of Cu and Zn was found to be 0.308 µg/ml and 0.035 µg/ml in the water sample of the aquaponics system, respectively. The BOD was analysed at 2.95 mg/l in the water sample of the aquaponics system and 5.95 mg/l in the control sample, which provides a good condition for the aquaponics system (Table 2). The important role of BOD has been investigated in various studies, which illustrate that excess solid wastes are responsible for increasing BOD. The increase in BOD has promoted hypoxic conditions in the rhizosphere and may generate toxic concentrations of ammonia and nitrate (Rakocy et al., 2012; Danaher et al., 2013).

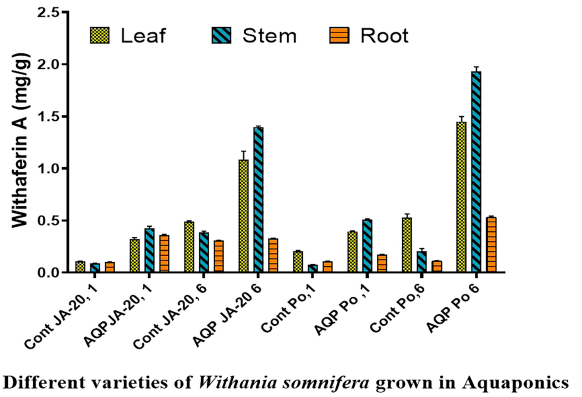

FIGURE 3
Withaferin A, content in the two varieties of *Withania somnifera* (Jawahar-20 and Poshita) grown in aquaponics (where Cont JA-20, 1 = 1 month Jawahar seedling grown in soil; AQP JA-20, 1 = 1 month Jawahar seedling grown in aquaponics; Cont JA-20,6 = 6 month Jawahar seedling grown in soil; AQP JA-20,6 = 6 month Jawahar seedling grown in aquaponics; Cont PO1 = 1 month Poshita seedling grown in soil; AQP PO1 = 1 month Poshita seedling grown in aquaponics; Cont PO,6 = 6 month Poshita seedling grown in soil; AQP PO,6 = 6 month Poshita seedling grown in aquaponics.

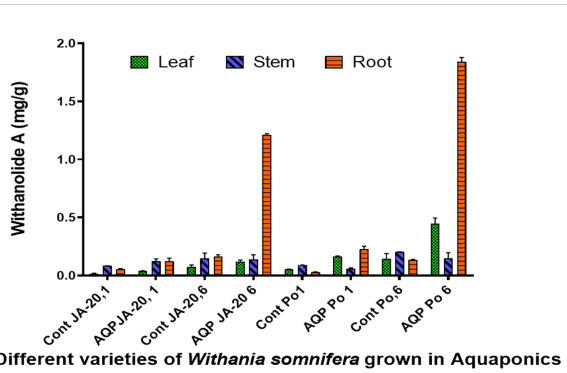

FIGURE 4
Withanolide A contents in Jawahar-20 and Poshita varieties of *Withania somnifera grown* in aquaponics (where Cont JA-20, 1 = 1 month Jawahar seedling grown in soil; AQP JA-20,1 = 1 month Jawahar seedling grown in aquaponics; Cont JA-20,6 = 6 month Jawahar seedling grown in soil; AQP JA-20,6 = 6 month Jawahar seedling grown in aquaponics; Cont PO1 = 1 month Poshita seedling grown in soil; AQP PO1 = 1 month Poshita seedling grown in aquaponics; Cont PO,6 = 6 month Poshita seedling grown in soil; AQP PO,6 = 6 month Poshita seedling grown in aquaponics).

TABLE 2 Effect of aquaponics on plant biomass and yield of withaferin A and withanolide A in WS.

Aquaponics					
Treatments	Plant Height (cm)	Plant Biomass FW (g)	Plant Biomass DW (g)	Yield (Withaferin A) mg/ Plant	Yield (Withanolide A) mg/ Plant
Cont JA-20, 1	7.5^{a}	1.2 ± 0.2^{ab}	0.05 ± 0.01^{ab}	0.23^{a}	0.03^{a}
AQP JA-20,1	18^{b}	3.2 ± 0.5^{bc}	0.1 ± 0.02^{cb}	0.21^{b}	0.01^{e}
Cont JA-20,6	20^{b}	2.9 ± 0.03^{ab}	0.18 ± 0.01^{bc}	0.2^{f}	0.02^{f}
AQP JA-20,6	25^{c}	3.3 ± 0.2^{bc}	0.2 ± 0.03^{cd}	0.28^{d}	0.03^{e}
Cont PO1	9^{a}	2.5 ± 0.28^{cd}	0.04 ± 0.01^{cd}	0.09^{e}	0.03^{a}
AQP PO1	20^{b}	3.1 ± 0.2^{ab}	0.0 ± 0.03^{ab}	0.05^{a}	0.01^{a}
Cont PO,6	21^{c}	3.2 ± 0.5^{cd}	0.25 ± 0.02^{ab}	0.28^{c}	0.08^{c}
AQP PO,6	32^{d}	3.5 ± 0.04^{ab}	0.3 ± 0.01^{cd}	0.3^{b}	0.09^{b}

(Cont JA-20, 1 = 1 month Jawahar seedling grown in soil; AQP JA-20,1 = 1 month Jawahar seedling grown in aquaponics; Cont JA-20,6 = 6 month Jawahar seedling grown in soil; AQP JA-20,6 = 6 month Jawahar seedling grown in aquaponics; Cont PO1 = 1 month Poshita seedling grown in soil; AQP PO1 = 1 month Poshita seedling grown in aquaponics; Cont PO,6 = 6 month Poshita seedling grown in soil; AQP PO,6 = 6 month Poshita seedling grown in aquaponics).
Data shown are mean _ SEm (n = 3). The genotypes with same superscript within each assay (parameter) are not significantly different at p _ 0.05, according to Duncan multiple comparison procedure (ANOVA).

The micro/macro elements and physio-chemical properties play important roles in the growth of plants and the content of bioactive components (photochemical, antioxidant metabolites, proteins, enzymes, etc.) in aquaponics systems. The published work showed that among the different nitrogen sources tested, 15 mg/l ammonium nitrate considerably improved the levels of withanolides (1.74 mg/g DW withanolide A, 0.92 mg/g DW withanolide B, 0.52 mg/g DW withaferin A, and 1.54 mg/g DW withanone) in the roots of regenerated shoots after 4 weeks of culture (Sivanandhan et al., 2015). It has also been reported that aquaponics systems that solely rely on fish excreta have lower concentrations of phosphorous, potassium, iron, manganese, and sulphur, resulting in poor plant growth (Graber and Junge, 2009). So from the above data, it may be concluded that Poshita has a higher content of withaferin A and withanolide A as compared to Jawahar-20. The content of withaferin A and withanolide A was found to be highest in 6-month-old seedlings as compared to one-month-olds, which confirms that the content of withaferin A and withanolide A increases with age.

Analysis of essential elements and other parameters in the soil sample

The results of various essential elements and other parameters were demonstrated in the soil samples of the mango garden, transgenic laboratory, and control sample using different techniques (Table 3).

Both the growth of the plant and the content of withaferin A and withanolides are enhanced by the use of vermicompost as a biofertilizer. Vermicompost is a good source of plant nutrients, which may be very sustainable for crop production (Wani, 2002). Previous studies have reported that the fresh weight and dry matter of cowpea (*Vigna unguiculata*) were high when the soil was amended with vermicompost (Karmegan et al., 1999; Karmegam and Daniel, 2000). A similar positive response was obtained in sorghum (*Sorghum bicolor*) (Patil and Sheelavantar, 2000) and sunflower (*Helianthus annus*) (Devi and Agrawal, 1998). Kaur et al. (2018) have reported organic cultivation of ashwagandha with increased biomass and higher quantities of bioactive withanolides by using vermicompost. As the usage of synthetic fertilizer for improving crop production increases the cultivation cost and causes long-term harm to the biological ecosystem (Savci, 2012), it also results in the acidification of soil, reducing nutrient uptake (Campbell et al., 2008; Coolon et al., 2013).

Conclusions

It is concluded that the Poshita variety has a higher content of withaferin A and withanolide A compared to Jawahar-20. The content of withaferin A and withanolide A was also found to increase with age. However, the major bottleneck is the availability of quality planting material/elite germplasm for enhanced active ingredients. The conventional propagation method cannot meet the increasing demand for this plant as a raw material for the preparation of pharmaceutical products or herbal formulations. Since environmental factors influence the secondary metabolite and antioxidant biosynthesis, it is therefore important to assess the withanolide A contents of elite germplasm and compare the withanolide A contents of field-grown and *in vitro*-grown promising varieties. The tissue culture technique can be an alternative for the continuous production of plantlet stocks for large-scale field cultivation and shoot multiplication. The most outstanding advantage offered by the aseptic mass propagation technique over conventional methods is that a large number of plants can be produced from a single plant. Unlike conventional methods of plant propagation, micropropagation of even temperature-specific species may be carried out throughout the year without any agro-climatic barriers. In order to enhance the contents of bioactive compounds, certain other strategies have been adopted by the researchers, like *Agrobacterium rhizogenes*-mediated transformation to increase the root biomass, the use of vermicompost, elicitors, suspension cultures, bioaugmented soil,

TABLE 3 Element analysis of the soil sample used in the elicitation study for potting the plants in the mango garden and transgenic polyhouse.

S. No.	Parameters	Control	Mango Garden	Transgenic Laboratory
1.	Salinity(PSU)	33	44	94
2.	Electrical conductivity(µS/cm)	65	56	187
3.	pH	7.23	7.40	7.86
4.	Temperature(°C)	29	28.5	25
5.	Organic Carbon	12	6.3	4.3
6.	Potassium(K)	18.3	22.6	69.9mg/g
7.	Phosphorous	0.04 mg/g	0.035 mg/g	0.032 mg/g
8.	Nitrogen(N_2)	3.3	3.8	4.2
9.	Vanadium	112.35 µg/g	117.96 µg/g	121.96 µg/g
10.	Chromium	79.06 µg/g	65.03 µg/g	69.50 µg/g
11.	Manganese	452.56 µg/g	402 µg/g	418.03 µg/g
12.	Iron	52063.91 µg/g	50569.36 µg/g	45235.25 µg/g
13.	Cobalt	62.36 µg/g	36.25 µg/g	30.24 µg/g
14.	Nickel	7.45 µg/g	6.98 µg/g	56.32 µg/g
15.	Copper	8.65 µg/g	75.25 µg/g	60.24 µg/g
16.	Zinc	39.57 µg/g	48.27 µg/g	52.45 µg/g

etc. Looking into the facts and importance of *W. somnifera* as a medicinal herb that is being continuously depleted from its natural habitat in India, the present research aims to develop elite germplasm with higher withaferin A and withanolide A contents derived from the promising genotypes. Moreover, the developed germplasm should be maintained. The hardening and acclimatization of this germplasm is an important effort to develop *in vitro* accession lines so that it can be made available to farmers in fulfilment of commercial demands. The present study demonstrates that aquaponics can work as an essential driver for the development of integrated crop and food production systems. The dry regions, such as Saudi Arabia and the desert regions of India, suffering from scarcity of water will specifically gain huge benefits from aquaponics methods being started in the commercial environment.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

MS, SB, ND: conducted the experiments, manuscript preparation. MS, KP, AA, MK, SCB: data analysis and editing. MS, AM, DCS: review, finalization of the manuscript and correspondence. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1215592/full#supplementary-material

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