Final Report (Revision 2)
21.03.2025

Summary of safety data on KSM-66® Ashwagandha

including comments on DTU National Food Institute's risk assessment report of ashwagandha/*Withania somnifera*

[redacted] Quality Manual, Data Integrity Policy

[redacted]

This report, recommendations and conclusions in it are presented based on the laws and regulations valid and applied at the time this report is dated and may not be valid once or if the laws and regulations are updated. Conclusions and views in this report are based on [redacted] evaluation and prior expert knowledge. If [redacted] verified partner opinion is used, [redacted] agrees to this opinion.

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Summary

1. Ashwagandha root extract KSM-66®

Ashwagandha root extract KSM-66® supplied by Ixoreal Biomed, is a standardized aqueous root-only extract. KSM-66® has been on the market for over 14 years as a food supplement, and it is currently used in over 3000 products in 52 countries around the world.

KSM-66® is produced under GMP conditions, and its composition and purity are accurately monitored and ensured.

2. Safety evaluation studies with ashwagandha root extract KSM-66

The data demonstrates that Ixoreal Biomed has been highly proactive in ensuring the safety of KSM-66®. Even though KSM-66® is not a novel food in the EU, Ixoreal has ensured its safety by carrying out all toxicological studies as generally required for novel foods. In addition, the extract has undergone numerous clinical trials that also focused on the assessment of safety related parameters.

The nonclinical safety evaluation studies with KSM-66® following the OECD test guidelines have been carried out and in accordance with the OECD Good Laboratory Practice (GLP) regulations. They include a comprehensive battery of genotoxicity tests *in vitro* and *in vivo*, an acute oral toxicity study in rats, a 14-day dose-range-finding study in rats, a subchronic (90-day) oral toxicity study in rats, and a reproduction/developmental toxicity screening test in rats. In addition, a 28-day oral toxicity study has been carried out following the OECD test guidelines.

Altogether, 29 clinical trials on KSM-66® have been published in scientific journals or preprinted, of which 27 were placebo-controlled and double-blinded. In addition, a few more studies have been completed and are under peer review for publication, and some more studies on KSM-66® are ongoing.

In clinical studies, the most common KSM-66® dose has been 600 mg/day, but in a high dose safety study the dose of 2000 mg/kg/day was given for 12 weeks. The treatment time has varied from 8 weeks to 12 months. These studies included assessment of safety parameters, such as assessment of vital signs, analysis of clinical chemistry and/or hematology parameters, including liver and kidney function, analysis of thyroid hormones and sex hormones in some studies and monitoring of adverse events. The total number of participants in placebo-controlled studies was about 3000 of which about half received KSM-66®.

Mild adverse and transient events were reported at about similar frequency for both KSM-66® and placebo, and the adverse events were not related to sexual health, sex hormones, thyroid hormones, liver function or hepatotoxicity. The overall outcome of these studies was that KSM-66® is very well tolerated, the measured parameters were within normal reference ranges or adjusted toward normal levels in study subjects with baseline values outside the standard limits, and no serious adverse effects were reported.

3. Conclusions

The safety evaluation studies (both nonclinical and clinical) indicate the safety of KSM-66® as it is currently used, and no safety concerns arose in the studies. Overall, the safety evaluation of ashwagandha extract KSM-66® is based on (1) the experience on the long history of use of traditional aqueous extracts of ashwagandha roots and KSM-66®, (2) reportedly quality control of production of KSM-66®, (3) nonclinical safety evaluation studies on KSM-66®, and (4) assessment of safety parameters in clinical trials with KSM-66®.

1 Introduction

1.1 Background

At the request of Shri Kartikeya Pharma (Ixoreal Biomed inc), [redacted] has summarized their scientific data on the safety of KSM-66® Ashwagandha, an aqueous ashwagandha root extract used in food supplements worldwide (later "KSM-66®", or "KSM-66® ashwagandha root extract" or "KSM-66® ashwagandha").

In 2020, the Danish National Food Institute (DTU) published a risk assessment for *Withania somnifera*. After that a few other risks assessments have been published (e.g. RIVM letter report 2024-0029) at least partially based on the DTU's conclusions. It is very important to note that the DTU Food Institute's report does not directly apply to KSM-66®. The report has been prepared in response to safety concerns raised by national authorities in the EU including a reference to the report "Risk assessment of the root from *Withania somnifera*" prepared by DTU Food Institute (Fødevareinstituttet) (Ref.: DTU DOCX. No. 19/1030299, dated May 15, 2020).

The data in this report demonstrates that Ixoreal Biomed has been highly proactive in ensuring the safety of KSM-66®. In addition to numerous clinical studies, KSM-66® has been studied for several toxicological effects in various *in vitro* and animal models. The extract has undergone all the main toxicological preclinical studies required by the European Food Safety Authority (EFSA) for novel foods over the past decades.

Contrary to the comments in the DTU Food Institute's risk assessment report on ashwagandha extracts, KSM-66® has undergone a 90-day subchronic feeding study and therefore, a safe intake limit can be calculated and established for its daily dose. In addition, KSM-66® has undergone numerous clinical trials over the past several years, including in relation to safety. These studies were not available to the DTU Food Institute or other national experts at the time of the risk assessment.

Furthermore, this report shows that DTU Food Institute's assumption concerning the safety risks when consuming ashwagandha does not directly apply to a well-specified KSM-66® ashwagandha root extract due to the fact that the composition of KSM-66® is different compared to ashwagandha extracts derived from other parts of the plants and/or with alcoholic solvents. Commercialised ashwagandha products can have a very different chemical composition that is influenced, for example, by extraction method, the part of the plant used, growth conditions and analytical methodology. The variations in these active compounds, can lead to very different safety outcomes in studies. As such, the characterisation and specific part of the plant and its method of manufacture must be considered when drawing conclusions as to a product's safety.

2 KSM-66® ashwagandha root extract

2.1 Botanical identification

- Latin name: *Withania somnifera* (L.) Dunal
- Family name: *Solanaceae*
- Synonyms: *Physalis somnifera* L., *Withania kansuensis* Kuang & A.M. Lu, *Withania microphysalis* Suess, *Withania macrocalyx* (Chiov.) Chiov.
- Common name: Ashwagandha, Indian ginseng, Winter Cherry
- Other common names: Arabic (Bahman, ubad), Bengali (Ashvagandha, dhuppa), Gujaran (Ghodakun), Hindi (Asgandh), Punjabi (Asgand), Sanskrit (Ashvagandha).

The commercial (cultivated rather than wild) form of the plant consists of dried roots of *Withania sominifera*, which occur in small pieces, 10-17.5 cm long and 6-12 mm diameter. The pieces are dark brown to grey-yellow, with longitudinal wrinkles with a creamy interior. The roots are straight, unbranched, conical and bear fibre-like secondary roots following breaks that are short and uneven fractures. The outer surface of the stems is green, variously thickened, cylindrical and longitudinally wrinkled.

The odour is described as horse-like with a taste that is sweetish, bitter, astringent and slightly mucilaginous. A microscopic identification shows that the transverse section has a narrowband of yellowish cork, a narrow cortex packed with starch grains, a wide central woody region radiating many long-distance medullary rays, and a few xylem parenchyma.

2.2 Production process of KSM-66®

This section provides the specifics related to KSM-66®, a root extract that is prepared using a standardised method of an aqueous-based extraction process. The manufacturing procedure assures a consistent and high-quality product.

Collection
Commencing the cultivation process, the fields located in Kota, Rajasthan (India) will be ploughed in April, left fallow in May and June, and the sowing of seeds will commence after two to three rains in July or August, utilising the broadcasting method. Subsequently, as the seeds sprout, manual labour will be employed to remove weeds 3 to 4 times as necessary. The roots are poised for harvest in February. Good Agricultural and Collection Practices (GACP) are adhered to during root gathering.

Selection
Post-harvesting, the stems and roots are segregated. The stem part, comprising leaves and seeds, is left for natural drying. Once dried, a harvester machine aids in collecting the seeds, while the roots undergo thorough drying. The leaves are, according to tradition, left on the field as natural fertilizers. Following the drying process, the roots are subjected to sorting and grading. These roots are then quarantined and the quality roots (solid and bright, approximately 7 centimetres in length and with a diameter of 1–1.5 centimetres) are selected. The selected roots are washed with water to remove sand, dust and unwanted material in a specially designed washer then blow-dried by hot air.

Extraction
KSM-66® Ashwagandha Root Extract (*Withania somnifera* (L.) Dun) is derived from a water-based extraction process.

The dried roots are milled into a multi-miller, and subsequently the milled roots are mixed with an equal amount of hot water in the extraction tank. The liquid mixture thus obtained is transferred into a processing reactor with the addition of more water and heated continuously under controlled temperature and pressure. The slurry is cooled and then passed through a fine filter to remove undesired material like waste particles and fibres. The decoction obtained is passed into a tray dryer and dried at low temperatures until a target moisture content is obtained. The final product obtained is in powdered form, which is further subjected to a micro-pulverizer, resulting in a fine free-flowing powder. This powder is then put in a vibro-super to obtain the final powder of the desired particle size.

At the end of processing, samples are collected and in-house microbiological analysis, heavy metal analysis and phytochemical analysis are carried out. The extract is assessed for microbials, heavy metals, and pesticide levels and their absence and the tests were conducted as per the guidelines of United States Pharmacopeia. The quality of the KSM-66® extract is compliant with the limits set by United States Pharmacopeia (USP) and British Pharmacopeia. The standardised root powder extract contains approximately 5.3 % withanolides and is confirmed by HPLC. KSM-66® extract is standardized to the following withanolides: withanolide A, withanone B, withaferin A, withanoside IV, withastromonolide A, and withanone. KSM-66® Ashwagandha Root Extract contains negligible amounts of withaferin-A and withanone (below detection level). Also, the alkaloid content is below the detection level.

The batch-to-batch consistency of KSM-66® is within the recommended regulatory standard (5-10% variation) and the chemo profile of every batch also is confirmed by a third party, an independent laboratory. The certificates of analyses of KSM-66® are available upon request.

Storage
General guidelines are followed for proper storage of botanical medicines. The powder is packed into a double polyethylene food-grade plastic. The entire manufacturing process is carried out in a clean-room facility following Good Manufacturing Practices. Inspections and testing are carried out at various points during the manufacturing of KSM-66®. Protection from light, moisture, air and heat is considered.

3 Responses to the report "Risk assessment of the root from *Withania somnifera*" prepared by DTU Food Institute (Fødevareinstituttet)

In 2020, the Danish National Food Institute (DTU) published a risk assessment for *Withania somnifera*. After that a few other risks assessments have been published (e.g. RIVM letter report 2024-0029) at least partially based on the DTU's conclusions. It is very important to note that the DTU Food Institute's report does not directly apply to KSM-66®. KSM-66® is significantly different from the most ashwagandha extracts or plant parts on which DTU Food Institute's report (2020) is based. That is the reason we provide the responses to DTU report here.

In our view, one of the primary issues in the DTU Food Institute's report is that it is not taking into consideration the different properties concerning safety between different plant parts of ashwagandha. Basing a safety evaluation on the entire plant, given the varying levels of active substances in different parts, is inaccurate and can be seriously misleading. The importance of distinguishing between different plant parts when doing a safety assessment is generally known (e.g. EFSA, 2009).

In addition, another main weakness in the DTU Food Institute's report is that DTU Food Institute makes the conclusions based on all different extracts of ashwagandha. It is generally known that extraction techniques (e.g. solvent extraction, acidic/alkaline treatment) can substantially affect the composition of an extract. KSM-66® is extracted with water, therefore, the composition of active substances is different from ethanol/methanol extracts of *Withania somnifera* which DTU Food Institute referred in their report.

Our view is that by referring to animal studies on alcohol extracts of leaves and stems of ashwagandha, DTU Food Institute incorrectly concludes that also KSM-66®, an aqueous extract from root poses a safety risk. Our view is fully in line with EFSA's Scientific opinion on Guidance on Safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements (EFSA, 2009) which clearly states that the presence of a substance in a botanical does not mean that this substance will also be present in the botanical preparation. This depends largely on the plant part used, as well as the preparation method (EFSA, 2009).

Our view is also supported by the Opinion of the independent researcher, Dr. Senia Johansson, Ph.D. in Pharmacognosy. She concluded that the content of several substances varies significantly between the root and aerial parts of *Withania somnifera*. The recent results of an internal validation report[1] on identification of ashwagandha root and leaf by HPTLC point to the same direction: Withaferin A is present at a higher concentration in *Withania somnifera* aerial part materials and is only present in *Withania somniferia* root material at very low concentrations. Therefore, to ensure the safety of food supplements / dietary supplements that contain plant extracts, it is crucial to conduct a comprehensive safety evaluation on the specific part of the plant used in the supplement, such as the root extract. Dr. Johansson's opinion on ashwagandha is presented in Appendix 1.

In summarizing the differences between KSM-66® and other ashwagandha extracts mentioned in the DTU Food Institute's report, we conclude that, unlike many of the other extracts referenced:

1. KSM-66® is extracted only from the roots of the ashwagandha plant with no addition of leaves.

2. KSM-66® is a well-defined and standardized aqueous extract, derived from ashwagandha root. The extraction process of KSM-66® provide a different composition of active substances compared to ethanol/methanol extracts referred in the report, e.g. content of withaferin A is below the detection limit.

3. Analyses of KSM-66® show that the content of alkaloids is below the limits of detection. (DTU Food Institute report refers to analyses showing that the content of alkaloids in root ranges from 0.16–0.96% whereas in leaves, the content of alkaloids is indicated to be 2–4%.)

[1] The report is available upon request.

4. KSM-66® is the only ashwagandha extract standardized on 5 different withanolides. (DTU Food Institute assumes that the plant is poorly described, and that level of active substances vary).

5. KSM-66® does not contain withaferin A or withanone. The content of withaferin A and withanone is below the detection limit. This is ensured from every batch. (The DTU Food Institute's risk assessment report mentions that Indian samples of ashwagandha root and leaf contain both withaferin A and withanones).

6. All KSM-66® production takes place under GMP and quality, traceability, purity, etc. is carefully ensured. The batch-to-batch consistency of KSM-66® is within the recommended regulatory standard (5-10% variation) and the chemo profile (e.g. content of withanolides and withaferin A, physico-chemical analysis, sieve analysis, density, heavy metals, aflatoxins, pesticide residue analysis) of every batch also is confirmed by a third party, an independent laboratory.

As described below, unlike the extracts referred in the DTU Food Institute's report, KSM-66® is a well-defined and standardized extract with numerous studies assessing safety. The safety documentation of KSM-66® contains such information DTU Food Institute has not evaluated in their report. As an example, the Danish authors recommend a 90-day subchronic toxicology study, which has been done on KSM-66®. The derived No Observed Adverse Effect Level (NOAEL) in this 90-day study is used to calculate the acceptable daily intake (ADI) for KSM-66® based on the default factors used by EFSA (2012). In addition, there are several recent clinical studies assessing the safety of KSM-66® ashwagandha. For further information, please see the section 5 Safety of KSM-66® ashwagandha root extract.

4 History of use of Ashwagandha (*Withania somnifera*) root

4.1 Traditional use

Ashwagandha (*Withania somnifera*) root has a long history of use, it is one of the prominently used Ayurvedic ingredients in India. Generally, plant-based Ayurvedic products are used after rigorous evaluation following the Ayurvedic practices. Moreover, such products are time-tested, ashwagandha root has a history of use for more than 4000 years in different age and user groups, also during pregnancy (Mirjalili *et al*. 2009).

The ancient texts of the Indian traditional health care system describe multiple uses of ashwagandha root. It is mentioned in Vedas (a large body of texts originating in ancient India) as an herbal tonic and health food. The traditional uses of ashwagandha are based on claims on normal physiological functions such as aphrodisiac and rejuvenate properties (Williamson, 2002; API, 2001; Upton, 1999). The roots are also used as a nutrient in pregnant women and elderly people (Mirjalili *et al.* 2009). The decoction of the root boiled with milk and ghee is recommended for supporting fertility in women.

Ashwagandha use is described primarily for its root and not the aerial parts of the ashwagandha plant in national standard references such as the United States Pharmacopoeia, British Pharmacopoeia,

Indian Pharmacopoeia Commission, the Health Canada monograph, and the World Health Organization's monograph for ashwagandha.

Despite having a long history of use, no serious concerns have been reported and ashwagandha is considered the safe botanical in Ayurveda (Indian system of Medicine). The Ministry of AYUSH, Government of India (2021) has recently issued an advisory to the manufacturers of ashwagandha to refrain from the use of leaves for the preparation of ashwagandha products (ref. L-1 1O11/9/2021-DCC, dated October 06, 2021). The Indian authority advises the use of roots of ashwagandha for the preparation of single or compound formulations.

4.2 Ashwagandha root extracts in food supplements

In recent decades, the use of ashwagandha root extract as a food supplement has become popular around the world.

The root and root extracts from *Withania somnifera* have been marketed in the United States as a dietary supplement by different manufacturers under the common name ashwagandha. Various products and recommended daily intakes can be found in the FDA Dietary Supplement Label Database (Search | Dietary Supplement Label Database (DSLD) | NIH Office of Dietary Supplements). Based on the database, ashwagandha is currently on the market in the US as a dietary supplement with use levels of up to 1800 mg/day of ashwagandha root extracts, or 30 mg/kg body weight (bw) for an individual weighing 60 kg.

In the European Union, the root of *Withania somnifera* is not considered a novel food, it has a significant history of use in food supplements before 15 May 1997, while non-concentrated aqueous infusions from the *Withania somnifera* roots are not considered a novel food in any food products. Based on the article 13.1 health claim database, dose up to 3-6 g of dried root per day are used in the EU (Database of health claims submitted to EFSA for their evaluation).

4.3 The global presence of KSM-66® Ashwagandha

KSM-66® has significant market distribution all over the world. KSM-66® ashwagandha root extract has been on the market for over 14 years, and it is currently used in over 3000 products in 52 countries and in many major supplement companies across the globe like Pfizer, Walmart, Mars, Nature's Bounty, GNC, GSK, CVS, Nestle, Unilever, Bayer to name a few. KSM-66® is used in plenty of delivery formats, the most common being capsules, tablets, soft gels, and powders. It is also used as an ingredient in various functional foods and beverages like gummies, bars, chocolates, non-alcoholic beverages, tea, coffee, and processed fruit and vegetable juices. In the several last years alone, 6 billion doses of KSM-66® have been sold worldwide.

KSM-66® is self-affirmed GRAS (Generally Recognized as Safe) based on a critical evaluation and acceptance of an independent expert panel in 2019 (Ixoreal Biomed). As per the Ixoreal Biomed's post marketing surveillance worldwide, there is no report of the serious adverse events associated with KSM-66® ashwagandha usage. Ixoreal is in close contact with all its dominant customers like Bayer, Solary,

Pfizer, GSK etc concerning the quality aspects including side effects. Despite of a sale of 2 billion daily doses a year, no serious side effects have been reported cy customers. The few side effects that have been reported have been mild and transient like rash, upset stomach or headache.

4.4 Adulteration

According to market data, ashwagandha is in the top 5 herbal supplements in the US and the interest is growing. Such growth has led to an increased risk of adulteration, which threatens product quality and consumer safety. The risk can be considered like that of other popular food supplements.

To date a few papers assess the prevalence of adulterated *Withania somnifera*. Of these studies, relevant issues are high variability of chemical fingerprint of root extracts perhaps due to unregulated supplementation of the root (Sangwan *et al*. 2024). In an assessment of 10 commercial samples labelled as 'root extracts', 80% demonstrated adulteration with aerial parts (Mundkinajeddu *et al*. 2014). In contrast, of 28 whole root products selected from the Indian state of Kerala, Shalini *et al*. (2017) did not find evidence of adulteration, but there was a common occurrence of mould. In one of the largest studies on DNA identity testing, 584 commercial raw root samples were analysed by HPTLC (Singh *et al*. 2018). Of the 584 samples tested, 119 (20.4%) are found not to be composed solely of root material and 84 (14%) were leaf. In the most recent study, market samples were purchased from 17 manufacturers from 15 states and 5 union territories in India of the root (n = 33) and powder (n = 68). Of the samples, 18 of the powder and 1 of the root where identified as mixed samples containing other plant species (Amritha *et al*. 2020).

5 Safety of KSM-66® ashwagandha root extract

The next section summarizes the scientific preclinical and clinical data on KSM-66® ashwagandha root extract.

The studies have been carried out with representative test material of KSM-66®, i.e. the test material has been derived from the production process, has been in accordance with the compositional data and has met the specifications of KSM-66®.

5.1 Preclinical safety data of KSM-66®

It is not just historical or traditional use, but also toxicity studies conducted on KSM-66® that support its safety and tolerability.

All basic toxicological studies (i.e. genotoxicity, acute and subchronic toxicity studies among others) required for novel foods in the EU are available for KSM-66®, supporting its safety. The information provided in these studies does not raise any safety concerns as regards the toxicity of KSM-66®. The summaries of each study are presented in the subsections 5.1.1 – 5.1.7 below. Some of the studies are preprinted. The following are specific studies that have been conducted on KSM-66®, and all are peer

reviewed, other than one trial that is currently preprinted. Importantly, all studies, with the exception of the developing zebrafish embryos study, are OECD compliant (Organisation for Economic Co-operation).

5.1.1 Genotoxicity

In 2023, Kalaivani *et al*. (2023a) assessed the mutagenic potential of KSM-66® using a bacterial reverse mutation assay (OECD 471), a chromosome aberration assay (OECD 473) and an *in vivo* micronucleus test (OECD 474).

5.1.1.1 Bacterial Reverse Mutation Test with KSM-66® Ashwagandha root extract in Salmonella Typhimurium Strains

Study no: 1213/2020

Testing Laboratories: Centre for Toxicology and Developmental Research, Sri Ramachandra Institute of Higher Education and Research.

GLP Details: GLP Certified Facility (No. GLP/c-105/2017)

Strain: Salmonella typhimurium Lyophilized Disc - TA strains (TA 98, TA 100, TA 102, TA 1535, and TA 1537)

Concentration: 0.3125, 0.625, 1.25, 2.5 and 5 mg KSM-66®/plate both in the presence (5% v/v S9 mix)

Source of metabolic activity: S9 mix was prepared freshly before use by adding cofactor mix (1.65 M MgCl2, 0.4 M KCl solution, 0.1 M Glucose-6-phosphate and 1.0M NADP in 0.2M phosphate buffer (pH–7.4)).

Batch No: KSM/20/S067

Criteria of Positivity: There were several criteria for determining a positive result such as:

- The strains should yield spontaneous revertant colony plate counts within the frequency ranges expected from the laboratory's historical control data and preferably within the range reported in the literature.
- Untreated controls should be used unless there are historical control data demonstrating that no deleterious or mutagenic effects are induced by the chosen solvent.
- There is no requirement for verification of a clear positive response.
- Equivocal results should be clarified by further testing preferably using a modification of experimental conditions.
- Negative results need to be confirmed on a case-by-case basis by altering the test item spacing and increasing the S9 mix concentration.
- In those cases where confirmation of negative results is not considered necessary, justification should be provided.

Methods and Results: The study was conducted following 1. OECD Guideline number 471, Bacterial Reverse Mutation Test adopted: 21st July 1997, corrected on 26th June 2020. Test item formed suspension in RO water (50 mg/mL), while it was found to be soluble in dimethyl sulfoxide (DMSO) (50 mg/mL). Hence DMSO was selected as the vehicle for the study. Precipitation was not observed at the highest tested concentration of 5 mg/plate.

A preliminary cytotoxicity (Range finding - RF) study was carried out using the tester strain TA100 in triplicates to determine the non-cytotoxic concentration range for the mutagenicity assay with the test item at the concentrations of 5, 2.5, 1.25, 0.625, 0.3125 and 0.15625 mg/plate both in the presence (5%

v/v S9 mix) and absence of metabolic activation system. Cytotoxicity was not observed up to the tested concentration of 5 mg/plate both in the presence (5% v/v S9 mix) and absence of metabolic activation system. Normal background bacterial lawn was observed up to the tested concentration of 5 mg/plate both in the absence and presence (5% v/v S9 mix) of metabolic activation system.
Based on the results of RF, the main study (Trial I and Trial II) was conducted using the plate incorporation method with all the five tester strains TA98, TA100, TA102, TA1535 and TA1537.

In Trial I the tester strains were exposed to the test item KSM-66® ashwagandha Root Extract at the concentration levels of 0.3125, 0.625, 1.25, 2.5 and 5 mg/plate both in the presence (5% v/v S9 mix) and absence of metabolic activation system along with concurrent positive and negative controls. No positive increase in mean revertant colonies was observed in any of the tester strains at any of the tested concentrations as compared to concurrent vehicle control. Normal background bacterial lawn was observed in all the tester strains at all the tested concentrations as compared to concurrent vehicle control.

In Trial II the tester strains were exposed to the test item KSM-66® ashwagandha Root Extract at the concentration levels of 0.128, 0.32, 0.8, 2 and 5 mg/plate both in the presence (10% v/v S9 mix) and absence of metabolic activation system along with concurrent positive and negative controls. No positive increase in mean revertant colonies was observed in any of the tester strains at any of the tested concentrations as compared to concurrent vehicle control. Normal background bacterial lawn was observed in all the tester strains at all the tested concentrations as compared to concurrent vehicle control. The spontaneous reversion rates in the vehicle control were within the range of historical control data. The mean number of revertants obtained for the positive control was more than two-fold for tester strains TA98, TA100, TA102 and more than threefold for tester strains TA1537, TA1535as compared to the concurrent vehicle control, thus demonstrating the sensitivity and validity of the test procedure.

Conclusion: Based on the above results, it can be concluded that the test item KSM-66® ashwagandha Root Extract did not induce any point mutations by base substitutions or frameshift in the genome of Salmonella typhimurium tester strains and was found to be non-mutagenic up to the tested concentration of 5 mg/plate both in presence and absence of metabolic activation system under the tested experimental conditions.

The bacterial reverse mutation test (i.e. Ames test, OECD TG 471) is the most widely used assay to detect gene mutations. This test is one of the Tier I genotoxicity studies for novel foods, stated in the EFSA Guidance on the scientific requirements for an application for authorisation of a novel food in the context of Regulation (EU)2015/2283(2024).

5.1.1.2 In vivo Mammalian Erythrocyte Micronucleus Test with KSM-66® Ashwagandha root extract in Swiss Albino mice

Study no: 1215/2020

Testing Laboratories: Centre for Toxicology and Developmental Research, Sri Ramachandra Institute of Higher Education and Research

GLP Details: GLP Certified Facility (No. GLP/c-105/2017)

Animals: Male and Female (nulliparous and non-pregnant) Swiss Albino Mouse

Batch No.: KSM/20/S067

Dose: 500 mg/kg, 1000 mg/kg and, 2000 mg/kg

Methods: This study was conducted following the OECD Guideline for the Testing of Chemicals 474 (29th July 2016) was to assess the clastogenic potential of KSM-66® ashwagandha root extract in Swiss Albino Mice. 0.1% CMC was selected as the vehicle based on the solubility test of test item performed before dosing. In the dose range finding study, animals were grouped into 4 groups, 2 animals per sex per group. Negative control (vehicle, 0.1% CMC - 0 mg/ kg, b.wt., G1), Low dose - KSM-66® ashwagandha root extract (500 mg/ kg, b.wt., G2), Mid dose - KSM-66® ashwagandha root extract (1000 mg/ kg, b.wt., G3) and High dose - KSM-66® ashwagandha root extract (2000 mg/ kg, b.wt., G4).

All the animals were dosed by oral gavage for two days separated by 24 hours interval. The dose volume was 10 mL/kg body weight. Sampling of bone marrow was done within 23 - 24h of last dosing time. Animals were observed for clinical signs, mortality, morbidity, and body weight. Animals were euthanized and bone marrow was collected from both the femurs by flushing with 2 mL fetal bovine serum into a centrifuge tube. The suspension was centrifuged, and smear was prepared on two glass microscope slides per animal. The prepared smear was air dried and fixed with ethanol and stained with 5% Giemsa stain. Stained slides were air-dried and mounted using mountant DPX and examined under light microscopy with 100X oil objective. The cells were counted for PCE ratio among the total erythrocytes and 500 erythrocytes per animal were counted to determine PCE: TE ratio.

Based on the results of the DRF study, 500, 1000 and 2000 mg/kg, b.wt. of KSM-66® ashwagandha root extract were selected as low, mid, and high dose respectively for the main study.

Main study was conducted in 5 groups each comprising of 5 male animals. Negative control (vehicle-0.1% CMC - 0 mg/kg, b.wt. G1), Positive Control (Cyclophosphamide monohydrate, 40 mg/kg, b.wt., G5), Low dose - KSM-66® ashwagandha root extract (500 mg/kg, b.wt., G2), Mid dose - KSM-66® ashwagandha root extract (1000 mg/kg, b.wt., G3) and High dose - KSM-66® ashwagandha root extract (2000 mg/kg, b.wt., G4).

KSM-66® ashwagandha root extract was formulated with vehicle. Positive control was formulated using water for injection. Mice were dosed via oral gavage for negative control and test item groups for two days separated by 24 hours interval, while positive control animals were dosed intraperitoneally only on the second day of dosing. The dose volume was 10 mL/kg body weight. Bone marrow was collected within 23 - 24h of last dosing. Observations were carried out for clinical signs, mortality, morbidity, and body weight.

PCE (Polychromatic Erythrocyte) ratio among the total erythrocytes, PCE: TE ratio and percentage of MNPCEs were calculated for all test item treated groups and positive control and compared with the negative control.

Results: In DRF study, no mortality and morbidity were observed in the TI treated animals of either sex at all tested doses. No decrease in P/E ratio was observed in any of the tested dose levels as compared to negative control.

For the main study no toxicity to bone marrow (decrease in P/E ratio) was observed in any of the treatment groups as compared to negative control. Results of test item treated animals were comparable to concurrent negative control and well within the historical control values.

The number and percentage of micronucleated PCE were not increased in any of the test item treated groups as compared to negative control group. Positive control group yielded a statistically significant increase in micronucleated PCE as compared to negative control group thereby illustrating the validity of the experiment.

Conclusion: From the above results, it may be concluded that KSM-66® ashwagandha root extract under given experimental conditions, up to the guideline limit dose of 2000 mg/kg, body weight has been considered to be non-clastogenic and did not induce any cytogenetic damage to the chromosomes or mitotic apparatus of erythroblast in bone marrow of Swiss albino mice.

The test is commonly used methods to assess the genotoxic potential of substances in vivo. This test (an in vivo micronucleus test (OECD TG 474), is one of the common genotoxicity studies for food and feed safety assessment, stated in EFSA's Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment (2011).

5.1.1.3 In vitro Chromosomal Aberration Test with KSM-66® Ashwagandha root extract in cultured human lymphocytes

Study no: 1214/2020

Testing Laboratories: Centre for Toxicology and Developmental Research, Sri Ramachandra Institute of Higher Education and Research.

GLP Details: GLP Certified Facility (No. GLP/c-105/2017)

Strain: Human peripheral blood lymphocytes

Concentration: 0.3125, 0.625, 1.25, 2.5 and 5 mg/plate both in the presence (5% v/v S9 mix)

Source of metabolic activity: Rat liver S9 mix procured from Krishgen Biosystems, Mumbai and stored in deep freezer, set at -75±5°C was used in the study. An adequate amount of fresh S9 mix was prepared before start of the experiment by adding 10% Rat liver S9 fraction to cofactor mix for Concentration Range Finding and Main Study.

Batch No: KSM/20/S067

Criteria of Positivity: The following criteria were used:

Concurrent vehicle control values are considered acceptable for inclusion in historical control data when the data falls within 95% control limit.

- Concurrent positive control values fall within the laboratory historical control data and produce a statistically significant increase with concurrent vehicle control.
- Cell proliferation criteria in the solvent control were fulfilled to define the concentrations used in the main study.
- All three experimental conditions (short term treatment with metabolic activation and without metabolic activation, long term treatment without metabolic activation) were tested unless one resulted in positive results.
- Adequate number of cells (300 well spread metaphase) were analysed in minimum three different concentrations.
- Criteria for selection of top dose was consistent with the guideline and no reduction of 45±5% in mitotic index as compared to vehicle control was observed up to the highest tested concentration of 2 mg/mL.

Methods: This study was performed to identify whether the test item, KSM-66® Ashwagandha Root Extract causes any structural or numerical chromosomal aberrations (clastogenicity) in cultured human lymphocytes when exposed to both, with and without an exogenous metabolic activation using the invitro chromosomal aberration test in cultured human lymphocyte cell culture. The experiment was conducted following the OECD Guideline for the Testing of Chemicals 473 (Adopted on 29 July 2016) and mutually agreed protocol between CEFTE and the Sponsor.

Clastogenicity of KSM-66® Ashwagandha Root Extract was conducted in cultured human lymphocytes with vehicle control and positive control. Four test item concentrations (0.25, 0.5, 1 and 2 mg/ml) were selected for concentration range finding study in presence and absence of metabolic activation system to determine the cytotoxicity of KSM-66® Ashwagandha Root Extract. KSM-66® Ashwagandha Root Extract at 0.5, 1 and 2 mg/ml was selected as the concentrations for the main study (Short term treatment with and without metabolic activation S9, long term treatment without S9). Duplicate lymphocyte cultures were used for both, concentration range finding and main study. DMSO was used as vehicle control and positive controls such as Mitomycin-C (0.5µg/ml) for without S9 and Cyclophosphamide monohydrate (3µg/ml) for with S9 were used, respectively.

Results: KSM-66® Ashwagandha Root Extract at 0.25, 0.5, 1 and 2 mg/ml was not found to be cytotoxic both in the absence and presence of metabolic activation system as compared to concurrent vehicle control. The mean percent reduction values were 90.68, 88.14, 88.14, 77.12 in the presence (1% v/v) and 90.44, 91.30, 86.08, 81.74 in the absence of metabolic activation system at 0.25,0.5, 1, 2 mg/ml, respectively.

The mean percent reduction in mitotic index for short term exposure with metabolic activation S9 of KSM-66® Ashwagandha Root Extract at concentrations of 0.5 mg/ml, 1 mg/ml and 2 mg/ml were 90.18, 87.50 and 83.93 and was comparable to vehicle control. The mean percent reduction in mitotic index of positive control Cyclophosphamide monohydrate was 88.39 as compared with the vehicle control. The mean percent reduction in mitotic index of short-term exposure without metabolic activation S9 of KSM-66® Ashwagandha Root Extract at concentrations of 0.5 mg/ml, 1 mg/ml and 2 mg/ml were 90.76, 88.24 and 84.03 and was comparable to vehicle control. The mean percent reduction in mitotic index of positive control

Mitomycin C was 84.87 as compared with the vehicle control. No cytotoxicity was observed at the test item; KSM-66® Ashwagandha Root Extract concentrations of 0.5, 1 and 2 mg/ml. Similarly, the mean percent reduction in mitotic index of long-term exposure without metabolic activation S9 of KSM-66® Ashwagandha Root Extract at concentrations of 0.5 mg/ml, 1 mg/ml and 2 mg/ml were 97.54, 90.98 and 81.15 and was comparable to vehicle control. The mean percent reduction in mitotic index of positive control Mitomycin C was 82.79 as compared with the vehicle control. No cytotoxicity was observed in the test item; KSM-66® Ashwagandha Root Extract at concentrations of 0.5, 1 and 2 mg/ml. No cytotoxicity was observed in the test item; KSM-66® Ashwagandha Root Extract at any of the tested concentrations of 0.5, 1 and 2 mg/ml both in the short-term exposure (4 h) as well as in continuous exposure (24 h). No significant difference in the chromosomal aberration frequency was observed between the vehicle controls and at any of the test concentrations of 0.5 mg/ml, 1 mg/ml, and 2 mg/ml of KSM-66® Ashwagandha Root Extract. Significant increase in chromosomal aberrations was observed in both the positive control groups of Cyclophosphamide monohydrate and Mitomycin C confirming the sensitivity of the test system, the effectiveness of the S9 mix and the validity of the clastogenicity assay.

Conclusion: Based on the above observations, it may be concluded that KSM-66® Ashwagandha Root Extract was considered to be non-clastogenic up to the tested concentration of 2 mg/ml under the above-mentioned experimental conditions.

The *in vitro* mammalian chromosomal aberration test (OECD 473) is commonly used method for assessing the genotoxic potential of substances. This test (an in vivo micronucleus test (OECD TG 473), is one of the common genotoxicity studies for food and feed safety assessment, stated in EFSA's Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment (2011).

5.1.1.4 Summary

A battery of *in vitro* (Bacterial Reverse Mutation Test and Mammalian Chromosomal Aberration Test) and *in vivo* (Mammalian Erythrocyte Micronucleus TesT) tests to detect potential mutagenic activity have been carried out. The testing also included assessment of DNA damage and micronucleus formation at the chromosome level, as well as indicators of clastogenicity and aneugenicity. The negative results from the battery of *in vitro* and *in vivo* tests signify that there is no genotoxic concern with KSM-66® ashwagandha root extract.

5.1.2 Acute toxicity

5.1.2.1 Acute Oral Toxicity Study of KSM-66® Ashwagandha root extract in Wistar Rats

Reference: Kalaivani *et al*, 2023a

Study No: 1208/2020

Testing Laboratories: Centre for Toxicology and Developmental Research, Sri Ramachandra Institute of Higher Education and Research.

GLP Details: GLP Certified Facility (No. GLP/c-105/2017)

Animals: Female nulliparous and non-pregnant Wistar Rats.

Batch No: KSM/20/S067

Dose: 2000mg/kg

Methods: This study was conducted according to the OECD test guideline 423, Acute Toxic Class Method adopted on 17th December 2001. The dose formulation of test item, KSM-66® Ashwagandha Root Extract was freshly prepared in 0.1 % CMC sodium (vehicle) prior to administration. The dose was administered once orally to overnight fasted female Wistar rats at a dose of 2000 mg/kg body weight in two steps. The dosing was performed based on their individual body weight taken on study day 1 and 10.0 mL/kg b.wt. was maintained as dose volume. In first step, three female animals were administered with a single dose of 2000 mg/kg b.wt. of test item, by oral route. The animals were observed for clinical signs of toxicity, mortality, and morbidity for 14 days. As all the animals survived and no clinical signs of toxicity were observed up to 72 h in first step, three more animals were administered with same single dose of 2000 mg/kg b.wt. of test item by oral route as second step. In both the steps, body weights of animals were recorded on day 1 (before dosing), 8 and 15. Mortality and morbidity was observed twice daily from acclimatization to till necropsy. Clinical signs were observed approximately at 30 min, 1, 2 and 4 h on day 0 (after test item administration) and thereafter once daily till necropsy. All animals were euthanized for gross pathology on day 15.

Results: No mortality and morbidity were observed during acclimatization and following test item administration till the day of necropsy. All animals appeared normal and did not show any clinical signs of toxicity at 30 minutes, 1, 2 and 4 h on day 1 and from day 2 to 15 following test item administration. All animals showed gain in body weight on day 8 and 15 in comparison to their day 1 body weight. Body weight of both the step animals was found to be normal throughout the experimental period. No gross lesions were observed in all animals of both the steps during the necropsy.

Conclusion: Ashwagandha root extract was found to be well tolerated in a single dose of 2000 mg/kg body weight administered orally in Wistar rats.

5.1.2.2 Acute Oral Toxicity Study of KSM-66® Ashwagandha root extract in Wistar Rats

Study No: 1279/2024

Testing Laboratories: Centre for Toxicology and Developmental Research, Sri Ramachandra Institute of Higher Education and Research.

GLP Details: GLP Certified Facility (No. GLP/C-215/2023)

Animals: Female nulliparous and non-pregnant Wistar Rats.

Batch No: KSM/VG/24/S1007

Dose: 5000mg/kg bodyweight

Methods: This study was conducted according to the OECD test guideline 423, Acute Toxic Class Method adopted on 17th December 2001. The dose formulation of test item, KSM-66® Ashwagandha Root Extract was prepared by grinding by mortar and pestle. 5 g of the test item was dissolved in 10 ml of a vehicle. The preparation was transferred to a beaker and mixed gently to ensure uniformity. The dose of 5000 mg/kg body weight was administered orally to fasted (≤16 h) female Wistar rats. The dosing was performed based on their individual body weight taken on study day 1 and 10.0 mL/kg body weight was maintained as dose volume. In first step, three female animals were administered with a single dose of 5000 mg/kg body weight of the test item, by oral route. The animals were observed for clinical signs of toxicity, mortality, and morbidity for 14 days. As all the animals survived and no clinical signs of toxicity were observed up to 72 h in the first step, three more animals were administered with the same single dose of 5000 mg/kg body weight of test item by oral route as the second step. In both the steps, body weights of animals were recorded on day 1 (before dosing), 8 and 15. Mortality and morbidity was observed twice daily from acclimatization to till necropsy. Clinical signs were observed approximately at 30 min, 1, 2 and 4 h on day 0 (after test item administration) and thereafter once daily till necropsy. All animals were euthanized for gross pathology on day 15.

Results: No mortality or morbidity were observed during acclimatization and following test item administration till the day of necropsy. All animals appeared normal and did not show any clinical signs of toxicity at 30 minutes, 1, 2 and 4 h on day 1 and from day 2 to 15 following test item administration. All animals showed gain in body weight on day 8 and 15 in comparison to their day 1 body weight. No gross lesions were observed in all animals of both the steps during the necropsy.

Conclusion: Ashwagandha root extract was found to be well tolerated in a single dose of 5000 mg/kg body weight administered orally in Wistar rats.

Based on the observations, the LD50 cut-off value of KSM-66® Ashwagandha Root Extract was found to be greater than 5000 mg/kg body weight and classified as Category-5 or unclassified, as per Globally Harmonized Classification System (GHS) for Chemical Substances and Mixtures.

5.1.3 Subacute toxicity

5.1.3.1 Two Weeks Dose Range Finding Study of KSM-66® Ashwagandha Root Extract by Oral Route in Wistar Rats – A Toxicity Study

This study assessed potential toxic effects associated with repeated oral administration of Ashwagandha root extract (ARE) for two weeks in Wistar rats (Kalaivani *et al*, 2024). This study was conducted according to the OECD test guidelines. Twenty male and female Wistar rats were assigned to four groups viz., control, ARE (500/1000/2000 mg/kg body weight per day). Animals were observed for

mortality/morbidity, clinical signs of toxicity (daily cage side observation), clinical examination (gait, mobility, arousal level, respiration, clonic/tonic movement, stereotype, bizarre behaviour, defecation, urine pools, vocalization, and rearing) prior to dosing and before necropsy day, body weight (weekly), and feed consumption. After 14 days, the animals were euthanized (CO2) and subjected to detailed gross necropsy (examination of external orifices, cranial, thoracic, and abdominal cavities, and their contents). Organs (liver, kidney, heart, spleen, brain, adrenal, thymus, ovaries, uterus with cervix, testes, and epididymis) were removed and weighed. No clinical signs of toxicity were observed with Ashwagandha treated groups, and it had no significant ($p>0.05$) effect on body weight and feed consumption by animals compared to control. No differences ($p>0.05$) were observed between ARE treated animals and control with respect to the absolute and relative organ weight and gross pathological findings. Ashwagandha root extract was found to be well tolerated in doses up to 2000 mg/kg body weight per day administered orally for 14 days in Wistar rats.

5.1.3.2 28 Days Repeated Oral Dose Study of KSM-66® Ashwagandha in Rats

Sub-acute toxicity of Ashwagandha was done as per the OECD-407 guidelines and was carried out for 28 days where satellite group was observed for 43 days (Langade *et al*, 2023). Wistar rats, 30 male and 30 females, were included in the study with 10 [5 M, 5 F] animals per group. Laboratory procedures were performed in accordance with CPCSEA guidelines. Animals were housed in standard laboratory conditions and were administered drugs orally- vehicle to control group and Ashwagandha 200, 400, 800 mg/kg body weight/day to study group.

General parameters were noted, blood collection was done for haematological and biochemical parameters. All the animals were sacrificed, dissected and observed for gross necropsy and organs of high dose groups from control and Ashwagandha groups were sent for Histopathological examination.

Result: Gradual weight gain was observed in all the animals. No signs of intoxication and no changes in blood biochemistry were observed. Histopathological changes in organs were within normal limits.

Conclusion: Under the conditions of this study and based on the toxicological endpoints evaluated, the test item KSM-66® Ashwagandha Root Extract was not observed to have any adverse effects when administered orally for a period of 28 days in Wistar rats at the doses of 400, 400, and 800 mg/kg body weight/day.

5.1.4 Subchronic toxicity

5.1.4.1 90-Day Repeated Dose Oral Toxicity Study of KSM-66® Ashwagandha root extract in Wistar Rats

Reference: Kalaivani *et al*, 2023b

Study no: 1210/2020

Testing Laboratories: Centre for Toxicology and Developmental Research, Sri Ramachandra Institute of Higher Education and Research

GLP Details: GLP Certified Facility (No. GLP/c-105/2017)

Animals: Male and Female (nulliparous and non-pregnant) Wistar Albino Rats

Batch No.: KSM/20/S067

Dose: 500 mg/kg, 1000 mg/kg, and 2000 mg/kg bodyweight/day

Methods: This study was conducted according to OECD test guideline 408 repeated dose 90-day oral toxicity study in rodents (adopted: 25th June 2018). One hundred animals (50 male and 50 female) rats were used for the study. Animals were assigned to six groups viz G1-Control, G1R-Control recovery, G2-500 mg/kg, b.wt, G3-1000 mg/kg, b.wt G4 and G4R-2000 mg/kg, b.wt.

Main group comprises 10 animal/sex/group and Recovery group comprises for 5 animal/sex/group. G1 andG1 Control Recovery group animals were administered 0.1%CMC vehicle, treated groups G2, G3, G4 and G4R were administered orally (once in a day) in for a period of 90 days to determine No-Observed Adverse Effect Level (NOAEL). Recovery group animals (G1R and G4R) were observed for 14 days without test item administration to identify reversibility or persistence of any toxic effects. All the animals from treated, control, recovery control and recovery groups were observed for clinical signs of toxicity once daily, detailed clinical examination every week after dosing day and before necropsy day. Mortality/Morbidity was observed twice daily. Weekly body weight and daily feed consumption were recorded for all the treated and vehicle control animals. At the end of treatment period, the animals were euthanized by CO2 and subjected to detailed gross necropsy which includes gross examination of external orifices, the cranial, thoracic, and abdominal cavities, and their contents. On completion of the gross pathology examination, the selected organs were weighed for all animals.

Results: Mortality and morbidity were not observed in any of the animals in control, treated, and recovery groups of animals during the study. No clinical signs of toxicity were observed in the control, treated, and recovery groups of animals from the day of test item administration till the end of the observation period. No test item related changes were observed in ophthalmoscope examination when compared to control, control recovery, treated and high dose recovery group of animals. No test item related changes in sensory reactivity and motor activity were observed in control, control recovery, treated and high dose recovery group of animals.

There were no test item-related changes observed in the control, control recovery, treated and high dose recovery group animals in haematology, electrolytes, and biochemistry parameters. In all the Control and treated groups of animals in day 91 and Control Recovery and High dose Recovery group of animal's thyroid hormones T3, T4, TSH were analysed on the end of the observation period, and no test item related changes were observed. No external gross pathological findings were observed in any of the animals at all treated dose levels including control group animals. No test item related internal gross pathological findings were observed in any animals of all treated groups when compared with control. All other gross pathological findings observed were either related to physiological, to spontaneous or incidental changes.

No test item related gross pathological findings were observed in any organs or tissues with the control, control recovery, treated and high dose recovery groups animals. There was no statistically significant difference observed in absolute and relative organ weight of all treatment groups when compared to control group animals of both sexes. There were no significant changes in the recovery group during and post treatment observation. No signs of delayed toxicity were also observed after treatment period for 14 days.

Conclusion: Under the conditions of this study and based on the toxicological endpoints evaluated, the test item KSM-66® Ashwagandha Root Extract was not observed to have any adverse effects when administered orally for a period of 90 days in Wistar rats at the doses of 500, 1000, and 2000 mg/kg body weight/day.

This study, a 90-day toxicity test in rodents (OECD TG 408), is one of the basic studies for novel foods, stated in guidance of EFSA NDA Panel Novel food application guidance (2024).

5.1.5 Reproductive toxicity

Reference: Preprinted. Centre for Toxicology and Developmental Research, 2023.
Study no: 1240/2022
Testing Laboratories: Centre for Toxicology and Developmental Research, Sri Ramachandra Institute of Higher Education and Research
GLP Details: GLP Certified Facility (No. GLP/C-158/2021)
Animals: Male and Female (nulliparous and non-pregnant) Wistar Albino Rats
Batch No.: KSM/22/S340
Dose: 500 mg/kg, 1000 mg/kg, and 2000 mg/kg bodyweight/day
Toxicity Evaluation on Reproduction and Development: Ashwagandha (*Withania somnifera*) Root Extract Oral Administration in Wistar Rats

Objectives: This randomized controlled study investigated the effects on body weight, reproductive organ weight, and thyroid hormone levels in pups of Wistar rats (10M/13F per group) who received oral Ashwagandha extract (500, 1000, or 2000 mg/kg/day body weight) or carboxymethylcellulose (control). Males were dosed for a period of up to 4 weeks, including the premating and mating period. Females were dosed for 63 days, including premating, mating, gestation and lactation up to PPD 13.

Methods: Animals were housed in polypropylene cages with males and females together during the pre-exposure and pre-mating periods. During the mating phase, one male and one female were kept together until confirmation of pregnancy. Pregnant females were housed individually during the post-mating period and lactating phase, while male rats were returned to their group cages. The procedure used was based on OECD Guideline 421 (OECD, 2016).

Results: The no observed adverse effect level (NOAEL) in adult rats was 2000 mg/kg. The mean body weights of pups and parental animals were similar ($p > 0.05$) in the treatment and control arms. No treatment related changes in thyroid hormones were observed for pups or parental animals ($p > 0.05$). Also, the weight of reproductive organs in parental male and female rats was similar ($p > 0.05$) in all groups. No other treatment related effects were observed in the pups and parental animals.

Conclusion: Standardized Ashwagandha root extract did not show any toxicity to the reproduction and development of the rats in this screening study over a 4-week period.

5.1.6 Other

Effect of Ashwagandha (*Withania somnifera*) on Thyroid functions in a Zebra Fish (Danio rerio) model

Reference: Naseem *et al*, 2023.

Background: Extract from the roots of Ashwagandha plant has been used through generations for its positive effect as an anti-stress, anti-tumor, anti-arthritic, and neuro-regenerative agent. The compounds have shown considerable effect as a thyroid disrupting agent also. Extremely contradictory findings have been shown in both animal and human studies regarding the effect of Ashwagandha on

thyroid levels, and hence it is important to monitor the safety and efficacy in preclinical models before we shift to clinical trials for the same.

Objective: To evaluate the thyroid disrupting potential of the Ashwagandha root extract (ARE) on Zebra Fish (Danio rerio) model.

Methodology: A total of 105 (7 groups of 15 each) transgenic Zebra fish embryos (tg: mCherry) at 2 days post-fertilization stage were included to study the effect of ARE on the developing thyroid gland. Potassium perchlorate (KOCl), a known thyrotoxic substance was used as a positive control and dimethyl sulfoxide (DMSO) was the vehicle control. The effect on thyroid glands was assessed using a thyroglobulin reporter gene fluorescence assay. Five serial concentrations from 1 mcg/ml to 50 mcg/ml of ARE were used. On day 5 post-fertilization, images were analyzed under a fluorescence microscope for intensity variations.

Result: The result from this study showed no differences in the expression of thyroglobulin reporter gene with Ashwagandha root extract.

Conclusion: The Ashwagandha root extract did not show any effect on the thyroid gland in developing zebra fish embryos.

5.1.7 Summary and conclusions of pre-clinical safety studies

Ashwagandha root has undergone multiple *in vitro* and *in vivo* toxicity studies to evaluate its safety. Despite the vast array of testing methods and endpoints, no adverse effects were found after exposure to KSM-66® (i.e. pulverised water extract of ashwagandha root). Previous studies have shown potential concerns on the effect of ashwagandha root on the thyroid, however, no changes were observed in thyroid weight or hormones in rats in reproductive and subchronic studies on KSM-66®. Additionally, zebra fish embryos exposed to KSM-66® did not show any changes in the thyroid gland. Recent authority reports, based on a citation distortion of an AHP monograph, have mentioned a concern that ashwagandha may be unsafe during pregnancy, due to questions about potential abortifacient activity. However, in a reproductive and developmental screening toxicity test, doses of up to 2000 mg/kg body weight/day did not result in any adverse effects among the parental rats and pups.

The available studies, suggest that repeated oral exposure to KSM-66® is not expected to be of concern to human health. Nevertheless, based on the available information, the most relevant study for determination of the Acceptable Daily Intake (ADI) is the 90-day subchronic toxicity study. It was noted that no dose response relationship was observed in the subchronic rat study. Therefore, the highest dose (2000 mg KSM-66®/kg body weight/day) was considered as a NOAEL. No test-item related changes or adverse effects were observed in this study,

When doing the extrapolation from subchronic to chronic study duration in rodents, the EFSA Scientific Committee recommends the use of an uncertainty factor of 2, considering the extent of investigations usually performed in 90-day studies.

The ADI is derived by dividing the overall NOAEL (mg/kg body weight) by an appropriate uncertainty factor and multiplying by the mean human body weight of 70 kg (EFSA Scientific Committee, 2012). EFSA Scientific Committee has also defined the uncertainty factors used in establishing health-based guidance values. In the absence of chemical-specific data on kinetics and/or dynamics, the

Committee recommends using the overall default UF of 100 (10 for inter-species variability x 10 for intra-human variability.

Therefore, calculating the acceptable daily intake (ADI) by applying a UF of 200 (10 for inter-species variability x 10 for intra-species variability x 2 for subchronic to chronic), the ADI will be 2000 mg/kg/day / 200 x 70 kg = 700 mg/day of KSM-66®.

5.2 Clinical safety data of KSM-66®

5.2.1 Summary of clinical data

So far 29 clinical studies on KSM-66® food supplement have been published in the scientific journals or preprinted for different health areas including safety. From these studies, 27 studies were placebo-controlled and double-blinded. In addition, several studies have been completed and under peer review for publication, and numerous studies on KSM-66® are ongoing. A summary table of studies of studies is provided in Appendix 2.

In the studies, the most common KSM-66® dose was 600 mg/day, but in a high dose safety study the dose of 2000 mg/kg/day was given for 12 weeks. The treatment time was 8 weeks in 27 studies, 12 weeks in 6 studies, 10 weeks in two studies and 24-week interim data was presented of a study scheduled for 12 months.

The most studies were conducted on healthy but stressed individuals and are closely monitored concerning adverse events. In addition, the studies also included assessment of safety parameters, such as assessment of vital signs, analysis of clinical chemistry and/or hematology parameters, including liver and kidney function, analysis of thyroid hormones and sex hormones in some studies. The overall outcome of these studies was that KSM-66® is very well tolerated, values of reported parameters were within normal limits, and no serious adverse effects were reported.

The clinical studies related to safety, sexual health, liver function and thyroid hormones are briefly discussed below.

5.2.1.1 Clinical studies assessing the safety as the primary endpoint

Safety of KSM-66® is supported by four clinical studies on primarily on safety parameters:

1. *A published clinical safety study in 80 healthy volunteers for 8 weeks with the dose of 600 mg/day (Verma et al. 2021)*

   The safety outcomes considered were laboratory assessment of haematological parameters, serum biochemistry analysis including hepatotoxicity evaluation, and thyroid function parameters. No significant changes or abnormalities were observed in the measured parameters. No adverse events were reported by any of the participants in this study.

2. *Long term safety study in subjects consuming KSM-66® with the daily dose of 600 mg daily (Salve et al. 2024, preprint, under peer review; on-going)*

   The primary objective of this study was to evaluate the clinical safety of KSM-66® ashwagandha on long-term administration over 12 months. The study is still on-going; the interim results (6 mo) are available. After six months of intervention, all adverse events reported were of mild severity and resolved without any intervention. Minor changes from baseline were seen during the 6 months in some parameters measured. All laboratory parameters assessed before and after the treatment were found within normal reference ranges.

3. *A global, multi-center, multi-national, prospective, randomized, double-blind placebo-controlled study (Pakhale et al. 2024, preprint, under peer review)*

   The objectives of the study were to compare the safety and tolerability of KSM-66® ashwagandha root extract with daily dose of 600 mg in healthy adults across different populations versus placebo for 8 weeks (n=1000). The primary outcome was the safety profile assessed through laboratory parameters and adverse events (AEs), while tolerability was assessed by the patients on a 7-point Likert scale of Global Assessment of Tolerability to Therapy (GATT). As a result, no serious adverse events were reported; a total of 74 adverse events (7.4%) were documented, with 46 events (9.2%) in the placebo group and 28 events (5.6%) in the KSM-66® group. There were no differences in any of the mean values of the laboratory parameters between the intervention and placebo groups at the week 8 (PP dataset (n= 934)). Tolerability ratings showed no statistically significant difference between the groups (p=0.487), and most of the patients rated the ARE therapy as “Good” or “Excellent”.

4. *High-dose safety study in healthy adults consuming KSM-66® with the daily dose of 2000 mg (this dose is about 3-times higher than ADI from the toxicity/preclinical studies) (Movva et al. 2024, preprint, under peer review)*

   The primary objective of this study was to evaluate the clinical safety of KSM-66® ashwagandha on administration over 12 weeks based on complete blood count, renal function test, liver function test, lipid profile, and thyroid function test. As reported earlier, KSM-66® at a dose level of 600 mg/kg for 8 week is safe, this longer-term study at higher dose was planned. Based on the preliminary study results, there were no significant changes in the laboratory parameters between the baseline levels compared to post-intervention levels.

For further information, please see the study summaries and the Appendix 2. The summary of each study is presented in the Section 5.2.2 Clinical studies assessing safety as a primary endpoint (the study summaries).

5.2.1.2 Clinical studies assessing safety as the secondary endpoint

In addition to the safety studies, there are numerous of clinical studies observing the health effects of KSM-66® (Appendix 2). In the clinical efficacy studies, the safety has been usually assessed based on the frequency of adverse events reported by the participants. Also, Patient's Global Assessment of Tolerability to Therapy (PGATT) has been in use. Mild adverse events were reported both in KSM-66® and the placebo groups of participants. According to the investigators and authors of the studies, these adverse events reported have been minor and no relation to sexual health, sex hormones, hepatotoxicity or liver injury and they do not raise any suspicion of toxicity with daily consumption of KSM-66®. Thus, it can be concluded that KSM-66® was well-tolerated among the participants of the studies.

In addition, various laboratory parameters have been evaluated in the studies. A summary of the findings on thyroid hormones, sex hormones, and liver function from the clinical safety and efficacy studies is presented in the next section.

In our systematic literature search, we found over 100 clinical studies published on different ashwagandha products (including different extracts and root powders) for different health areas. Since the extracts may contain different active substances depending on plant parts and extraction method used, those studies do not directly apply to KSM-66® and are not included in the report.

5.2.1.2.1 Summary of results

5.2.1.2.1.1 Thyroid hormones

Five studies have evaluated the effect of KSM-66® supplementation on the thyroid hormone levels. Three of these studies were conducted in subjects with normal thyroid function (Verma *et al*. 2021, Salve *et al*. 2024 (long-term study), Movva *et al*. 2024 (high-dose study) whereas two studies in subjects with subclinical hypothyroidism (Sharma *et al*. 2018, Wankhade *et al*. 2024).

The study of Verma *et al*. 2021 showed no change in thyroid levels in healthy volunteers (males and females) with the daily dose of 600 mg of KSM-66® during 8 weeks of treatment. The data of long-term safety study with the same daily dose showed similar results: no effect in thyroid parameters after 6 months of treatment (Salve *et al*. 2024).

Based on the preliminary study results of the high-dose study (KSM-66® 2000 mg per day, which is about 3-times higher than the ADI from the toxicity/preclinical studies), the laboratory parameters on thyroid function assessed before and after the treatment were within normal reference ranges and no difference was seen between the baseline and post-intervention values (Movva *et al*. 2024). The study was an open label, non-comparatives study and contains not a placebo group for a comparison.

In subjects with impaired thyroid function, KSM-66® treatment with the same daily dose adjusted thyroid hormone levels toward normal levels after 8 weeks of administration (Sharma *et al*. 2017). Similarly, the adjusting the thyroid stimulating hormone (TSH) levels closer to normal level was seen in the most recent study in subjects with subclinical hypothyroidism based on persistently elevated TSH levels (Wankhade *et al*. 2024). In that study, KSM-66® treatment did not affect triiodothyronine (T3) or thyroxine (T4) levels. The all measured thyroid hormone levels after using KSM-66® were in normal reference range and therefore considered physiology safe.

For the study summaries of Verma *et al*. 2021, Salve *et al*. 2024 (long-term study), and Movva *et al*. 2024 (high-dose study), please see section 5.2.2 Clinical studies assessing safety as a primary endpoint (the study summaries). The study summaries of the studies by Wankhade *et al*. (2024) and Sharma *et al*. (2018) are presented in the section 5.2.3 Clinical studies of KSM-66® on thyroid function (the study summaries).

5.2.1.2.1.2 Sexual health and sex hormones

By today, a total of 13 studies have evaluated the effect on sexual health (7 on women and 6 on men) from which 11 studies included on laboratory assessment of sex hormones: 5 studies in women (Gobal *et al*. 2021, Amit et. al. 2024a, and 3 recent, completed, unpublished studies) and 5 studies in men (Chauhan *et al*. 2022, Wankhede *et al*. 2015, Ambiye *et al*. 2013, Amit at al. 2024b, 1 recent unpublished study). In addition, the testosterone levels were measured in men and women in a long-term safety study (Salve *et al*. 2024) and in the study by Verma *et al*. 2023, both conducted in healthy subjects.

Based on the study results, KSM-66® intervention has not shown any harmful effects on sexual health in either sex. On the contrary, KSM-66® seems to support some normal sexual functions in both men and women (Ajgaonkar *et al*., 2022, Chauhan *et al*., 2021, Gopal *et al*., 2021, Dongre *et al*., 2015, Wankhede *et al*., 2015, Ambiye *et al*., 2013). This is in alignment with the traditional Ayurvedic use of ashwagandha root promoting a normal hormonal balance and overall reproductive health in men and women.

There are four clinical studies evaluating the effect of daily dose of 600 mg of KSM-66® on sexual health in women with sexual dysfunction/ hypoactive sexual desire disorder (Ajgaonkar *et al*., 2022, Dongre *et al*., 2015, 2 recent, unpublished studies). In all studies, KSM-66® improved sexual satisfaction with normal limits (at least in some subscales of different questionnaires) and no significant adverse effects were noted in 8 weeks of study duration. The sex hormone levels were measured in the latest, yet unpublished studies. Based on the results, the daily dose of 600 mg of KSM-66® has no effect on sex hormone levels (follicle stimulating hormone (FSH), luteinizing hormone (LH), estrogen-E2, progesterone, or testosterone) in health subjects with women with sexual dysfunction/ hypoactive sexual desire disorder in 8 weeks of supplementation.

Concerning perimenopausal women, there are 3 studies available. A study in women, aged 45-60 years, showed that KSM-66® (daily dose 600 mg) decreased the severity of normal perimenopausal and aging symptoms in 8 weeks (Gopal *et al*., 2021). The observed beneficial changes in the estradiol and serum follicle stimulating hormone levels were within the normal reference range indicating the safety of KSM-66® and can be considered physiologically comfortable in perimenopausal women. Furthermore, a recent, unpublished study also found some beneficial changes in normal hot flash events in menopausal women with the same daily dose. In contrary to the earlier study by Gopal *et al*., no clinically significant effect on serum sex hormones (estradiol, follicle stimulating hormone (FSH), luteinizing hormone (LH)) was seen in this study at the end of 8-week intervention. Based on the preliminary study results of another unpublished study in the same population with the same daily dose of KSM-66®, no significant differences were observed in serum hormone levels of estradiol and testoterone. Instead, the level of follicle stimulating hormone (FSH) and luteinizing hormone (LH) were lower in the KSM-66® group compared to placebo group at 12 weeks (23.18 vs 37.22 m/u/ml; 45.43 vs 61.78 m/u/ml, respectively). All the values are in normal limits in perimenopausal women indicating the safety of KSM-66®.

Total of five clinical studies evaluated the reproductive and sexual health or testosterone levels in men with or without low sexual desire (Chauhan *et al.*, 2021, Wankhede *et al.*, 2015, Ambiye *et al.*, 2013, Amit at al. 2024b, a recent study under review). The intervention with the daily dose of 600-675 mg of KSM-66® was shown to have beneficial effect on sexual health in males after 8-12 weeks of treatment. All effects could be classified as beneficial changes in normal physiological functions. Moreover, the testosterone levels observed in these studies were all within the normal limits, also after KSM-66® treatment, indicating the safety of KSM-66® (Chauhan *et al.*, 2021, Wankhede *et al.*, 2015, Ambiye *et al.*, 2013, Amit at al. 2024b, a recent study under review). Furthermore, luteinizing hormone level were within the normal limits (Ambiye *et al.*, 2013). No change in serum prolactin levels was observed (Chauhan *et al.*, 2021).

The testosterone levels were measured in men and women in a long-term safety study (Salve *et al*. 2024), and in the study by Verma *et al*. 2023, both in healthy subjects. The small increases in serum testosterone levels remained within the normal reference range, indicating the safety of ashwagandha on reproductive health and sex hormones.

The summary of each study is presented in the section 5.2.4 Summaries of clinical studies of KSM-66® on sex hormones / sexual health. For the study of Salve *et al*. 2024 please see section 5.2.2 Clinical studies assessing safety as a primary endpoint (the study summaries).

5.2.1.2.1.3 Liver function

Thirteen of the studies evaluated liver function (such as Verma *et al*. 2021, Salve *et al*. 2024 (long-term-study), Movva *et al*. 2024 (high-dose study), Pakhale *et al.* 2024; please see also Appendix 2). They show that KSM-66® does not negatively affect liver values in healthy women, healthy men, children aged 5-12 years, frailty elderly with the daily dose of 600 mg after 8 weeks of treatment. Based on the interim results of long-term safety study, there were some minor, clinically insignificant changes in serum alanine transaminase (ALT) aspartate transaminase (AST), but the parameters stayed in normal limits (Salve *et al*. 2024). The level of alkaline phosphatase did not change during the 6-month treatment (Salve *et al*. 2024). Based on the preliminary study results of the high dose study (2000 mg per day), the laboratory parameters on hepatic function assessed before and after the treatment were within normal reference ranges even though the levels showed some decrease after 12-week treatment. The study was an open label, non-comparatives study and contains not a placebo group for a comparison.

The results of the studies do not raise safety concerns related to the use of KSM-66® in different study populations.

The summaries of these studies are presented in the different sections 5.2.2-5.2.5 below.

5.2.2 Clinical studies assessing safety as a primary endpoint (the study summaries)

5.2.2.1 Verma, N., Gupta, S. K., Tiwari, S., & Mishra, A. K. (2021). Safety of Ashwagandha Root Extract: A Randomized, Placebo-Controlled, study in Healthy Volunteers. Complementary therapies in medicine. 57, 102642

This was an 8 week, randomized, double-blind, placebo-controlled clinical study conducted to assess the safety of KSM-66® ashwagandha root extract on healthy adults (18-45 y).

Study product: KSM-66®, daily dose 600 mg

Results: The present study revealed that the consumption of KSM-66® for 8 weeks was safe in both males and females. The mean body weight and the BMI of the volunteers did not show significant change throughout the study. The demographic characteristics did not differ significantly between the KSM-66® and the placebo groups. Gender-wise analysis of the demographic data was done for the participants according to their respective group. There was no significant deviation observed for both the gender groups in the placebo and the treatment arm at the end of the study.

All the vital parameters assessed before and after the treatment were found within normal reference ranges with no statistically significant changes for their respective mean values (height, weight, temperature, pulse, respiratory rate, systolic and diastolic blood pressure, BMI).

During the comparison of the outcomes between the study group and the placebo group, there were no significant changes observed for the hematological and other parameters such as hemoglobin, neutrophil count, platelet count, or biochemical markers of the hepatic function: alanine transaminase, aspartate transaminase and alkaline phosphatase. All values were within the normal reference range.

In addition, there were no statistically significant changes observed in the thyroid function test outcomes for the study group in comparison to the placebo group. Thyroid function tests at baseline showed that the mean values of the study group of thyroid-stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4) were 2.29 ± 0.79 µIU/mL, 1.39 ± 0.29 ng/mL, and 7.92 ± 1.75 µg/dL, respectively. After 56 (±4) days of intervention, the mean serum levels of TSH, T3, and T4 were noted as 2.19 ± 0.89 µIU/mL, 1.39 ± 0.28 ng/mL, and 8.12 ± 1.91 µg/dL, respectively.

During the comparison of the outcomes between the study group and the placebo group, no significant changes were observed for haematological and other parameters.

The participants administered with KSM-66® over 8 weeks did not show any adverse reactions.

Laboratory parameters at baseline and at 8 weeks after start of KSM-66® ashwagandha are presented in a table below:

	Active / Treatment Group		Control / Placebo Group		
Parameters	**Mean Baseline (SD or SE or 95% CI)**	**Mean End of Study (SD or SE or 95% CI)**	**Mean Baseline (SD or 95% CI)**	**Mean End of Study (SD or 95% CI)**	**p value (Between)**
Triiodothyronine (T3)	1.39 (0.29)	1.39 (0.28)	1.32 (0.35)	1.42 (0.35)	>0.05
Thyroxine (T4)	7.92 (1.75)	8.12 (1.91)	8.18 (1.97)	7.98 (1.81)	>0.06
Thyroid-stimulating hormone (TSH)	2.29 (0.79)	2.19 (0.89)	2.24 (0.78)	2.23 (0.69)	>0.07
Alkaline Phosphatase	89.32 (19.17)	88.75 (17.57)	90.57 (18.21)	91.02 (17.25)	>0.08
SGOT/AST	24.62 (7.80)	24.57 (6.83)	25.07 (9.09)	25.47 (7.92)	>0.09
SGPT/ALT	28.70 (12.58)	28.95 (11.64)	25.8 (9.26)	25.87 (8.33)	>0.10

5.2.2.2 Salve, Jaising; Kale, Anjeev; Ademola, John; Langade, Deepakkumar; Prajapati, Banwarilal (2024). Safety of 24-Week Therapy with Ashwagandha (Withania Somnifera) Standardized Root Extract in Healthy Adults: A Prospective, Observational Study. Preprint (6 mo interim data)

The ultimate primary objective is to evaluate the clinical safety of KSM-66® Ashwagandha on long-term administration over 12 months. The full study results are not yet available, this is an interim report summary of 24-week treatment period data.

The primary outcome of the study was clinical safety based on the number and proportion of treatment emergent adverse event (TEAE) due to ashwagandha root extract during the 24 weeks study period. The adverse events, either spontaneously reported by the patient, or noticed by the clinician were recorded during the study.

The secondary outcomes of the study were laboratory safety, i.e. mean change in laboratory values for hepatic parameters, renal parameters, creatinine phosphokinase (indicator of effects on skeletal muscle), thyroid parameters, serum cortisol and serum testosterone (free and total testosterone) from baseline to the week 24.

This report includes data of 3 sites from India, and one was Italy.

Study product: KSM-66®, daily dose 600 mg

This study enrolled men and women patients aged 18-65 years who visited the study sites and were prescribed ashwagandha root extract for improvement of general health. Those who had used any drugs which influence stress and anxiety (e.g., corticosteroids, antidepressants) within four weeks preceding the enrolment, or were currently using herbal extracts such as Ginkgo Biloba or Omega-3 were not included. Additional exclusions encompassed anyone with depressive episodes, suicidal ideation, panic disorders, social anxiety, obsessive-compulsive disorder, alcohol dependence, schizophrenia, mania, diagnosed post-traumatic stress disorder, or clinically severe unstable conditions impacting the liver, kidneys, heart, or lungs. Individuals exhibiting hypersensitivity to ashwagandha, those who had participated in prior clinical trials within the preceding three months, or any individuals with conditions considered dangerous by the investigator were eliminated. Participants were asked to continue their routine diet and physical activities during the study period.

Results:

A total of 18 adverse events were reported, the most frequently reported event being nausea reported by 6 (3.1%) patients. Other adverse events included abdominal pain (1.6%) and headache (1.6%). No common adverse events were diarrhea, dizziness, and vomiting reported by 2 patients each (1.0%). All events were of mild severity and resolved without any intervention.

All laboratory parameters assessed before and after the treatment were found within normal reference ranges. When comparing baseline values to 6 months, there was no change in laboratory values for bilirubin, alkaline phosphatase (ALP), serum creatinine, blood urea nitrogen (BUN), thyroid parameters (serum TSH, T3 and T4), serum cortisol, blood glucose or lipid profile.

Even though there were some minor changes in serum alanine transaminase (ALT), aspartate transaminase (AST), creatine phosphokinase- myocardial band (MB)) and testosterone values, all these parameters stayed in the normal limits.

The parameters will be analyzed again after 12 months of intervention, expected in early 2025.

Results (6 months): Laboratory parameters at baseline and at 6 months after start of KSM-66® ashwagandha are presented in a table below.

	Active / Treatment Group		Control / Placebo Group		
Parameters	Mean Baseline (SD or SE or 95% CI)	Mean End of Study (SD or SE or 95% CI)	Mean Baseline (SD or 95% CI)	Mean End of Study (SD or 95% CI)	p value (Between)
Total Bilirubin (mg/dl)	0.85 (0.26)	0.82 (0.29)	NA	NA	0,065
Direct Bilirubin (mg/dl)	0.30 (0.19)	0.29 (0.18)	NA	NA	0,576
Indirect Bilirubin (mg/dl)	0.55 (0.22)	0.53 (0.24)	NA	NA	0,08
ALT (IU/L)	30.81 (9.68)	31.72 (10.33)	NA	NA	0,018
AST (IU/L)	30.13 (9.90)	32.41 (12.84)	NA	NA	<0.001
ALP (IU/L)	83.28 (33.30)	83.31 (31.04)	NA	NA	0,974
Creatinine (mg/dl)	0.75 (0.66)	0.68 (0.26)	NA	NA	0,218
BUN (IU/L)	23.32 (21.25)	22.04 (8.45)	NA	NA	0,367
CPK-MB (IU/L)	18.83 (11.86)	17.83 (10.14)	NA	NA	0,005
TSH (uU/mL)	1.16 (1.02)	1.15 (0.92)	NA	NA	0,899
T4 (µg/dL)	10.93 (7.05)	10.61 (7.81)	NA	NA	0,605
T3 (ng/dL)	115.64 (71.91)	117.85 (67.80)	NA	NA	0,08
Cortisol (mcg/dL)	18.85 (20.19)	15.11 (13.58)	NA	NA	<0.05
Free Testosterone (ng/dl) - Males	463.19 (313.01)	528.40 (292.27)	NA	NA	<0.001
Free Testosterone (ng/dl) - Females	26.32 (21.54)	28.52 (19.46)	NA	NA	<0.001
Total Testosterone (ng/dl) - Males	444.84 (258.07)	513.16 (299.75)	NA	NA	0,024
Total Testosterone (ng/dl) - Females	34.27 (24.30)	37.67 (25.81)	NA	NA	<0.001
Plasma Glucose (mg/dL)	96.03 (10.76)	83.72 (10.65)	NA	NA	0,074
Total Cholesterol (mg/dL)	184.32 (41.19)	183.18 (48.00)	NA	NA	0,81
HDL Cholesterol (mg/dL)	61.80 (13.58)	61.16 (12.82)	NA	NA	0,653
LDL Cholesterol (mg/dL)	115.70 (30.40)	114.64 (31.10)	NA	NA	0,738
Triglycerides (mg/dL)	110.57 (45.22)	110.57 (42.99)	NA	NA	0,995

5.2.2.3 *Pakhale, K; Salve, J; Ademola, J; Paracca, J; Langade, Dk (2024). Safety of 8-Week Therapy with Ashwagandha (Withania somnifera) Root Extract (600mg/day) in Adults with Stress and Anxiety: Findings from a Prospective, Randomized, Multi-center, Double-Blind, Placebo-Controlled Study. PREPRINT.*

This study was a global, multi-center, multi-national, prospective, randomized, double-blind placebo-controlled study. This study compared the safety and tolerability of ashwagandha root extract in healthy adults across different populations.

Study product: KSM-66®, daily dose 600 mg

This study was conducted involving 1,000 men and women aged 18 to 65 years. Participants were randomly assigned to receive either 600 mg/day (two divided doses) of ashwagandha root extract (n=498) or a placebo (PL, n=502) orally for eight weeks. The primary outcome was the safety profile assessed through laboratory parameters and adverse events (AEs), while tolerability was assessed by the patients on a 7-point Likert scale of Global Assessment of Tolerability to Therapy (GATT).

Results: Demographic and baseline characteristics were similar between groups. No serious adverse events were reported; a total of 74 AEs (7.4%) were documented, with 46 events (9.2%) in the placebo group and 28 events (5.6%) in the ARE group. The most common AEs include nausea (3.2% in the placebo group vs. 2.0% in KSM-66® group), dry mouth (1.4% in both groups), and headache (2.2% in the placebo vs. 0.2% in the KSM-66® group).

Laboratory evaluations showed no significant changes in liver function tests, renal function markers, or hematological parameters between the KSM-66® and placebo groups. Both groups maintained normal ranges for key biomarkers, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, and hemoglobin levels throughout the study duration.

Tolerability ratings showed no statistically significant difference between the groups (p=0.487), and most of the patients rated the ARE therapy as “Good” or “Excellent”.

Laboratory parameters at baseline and at 8 weeks after start of KSM-66® ashwagandha are presented in a table below.

	Active / Treatment Group		Control / Placebo Group		
Parameters	**Mean Baseline (SD or SE or 95% CI)**	**Mean End of Study (SD or SE or 95% CI)**	**Mean Baseline (SD or 95% CI)**	**Mean End of Study (SD or 95% CI)**	**p value (Between)**
Red blood cell (lakh per cumm)	4.61 (0.47)	4.84 (0.49)	4.58 (0.41)	4.85 (0.48)	0,927
Haematocrit (%)	43.61 (16.35)	45.54 (4.68)	42.38 (5.57)	45.17 (5.29)	0,37
Haemoglobin (gm/dL)	13.63 (1.70)	27,96	13.50 (1.85)	13.78 (2.64)	0,305
Platelets (per cumm)	3.06 (0.73)	3.34 (2.72)	3.03 (0.77)	3.28 (2.06)	0,756
Aspartate aminotransferase (U/L)	29.00 (13.52)	25.91 (7.37)	27.85 (9.89)	26.33 (8.12)	0,504
Alanine aminotransferase (U/L)	31.13 (16.44)	29.14 (10.22)	31.11 (11.50)	28.30 (10.72)	0,32
Creatinine (mg/dl)	0.79 (0.19)	0.82 (0.20)	0.80 (0.20)	0.83 (0.20)	0,579

5.2.2.4 Movva, Navya; Salve, Jaising; Debnath, Khokan; Thakare, Vaishali; Langade, Deepakkumar (2024). Safety and Efficacy of Ashwagandha (Withania somnifera) Root Extract (2000 mg/day) in Healthy Adults: A Prospective, Non-comparative Study. PREPRINT.

Study Objectives: primary objective of the study was to evaluate the laboratory safety of Ashwagandha standardized root extract in healthy adults over 12-week therapy based on complete blood count, renal function test, liver function test, lipid profile, and thyroid function test.

Secondary objectives were to evaluate the clinical safety of Ashwagandha standardized root extract in healthy adults over 12-week therapy, and to evaluate the effect of the Ashwagandha standardized root extract on Quality of Life (QoL) using the SF-36 tool.

Study product: KSM-66®, daily dose 2000 mg (this dose is about 3-times higher than the ADI from the toxicity/preclinical studies.)

Study Population: 100 healthy adults

Inclusion criteria:

- Adults (male and female) aged between 18 and 65 years.
- Participants should be healthy and free from any chronic illness, such as diabetic, cardiovascular or any other condition that could affect the safety of the study.
- No plan to commence any other alternative treatment modality for their conditions.
- Willingness to sign an informed consent document and to comply with all study related procedures

Exclusion criteria:

- History of alcohol or smoking abuse.
- History of hypersensitivity to ashwagandha.
- Taking nutritional or energy supplements, medication, or steroids.

- Any history of drug abuse.
- Having any clinical abnormalities.
- Simultaneously participating in any other clinical trial or participated in the past three months.
- Participants who use medication for blood pressure, use beta blockers, inhaled any beta-agonists, use any hormonal contraceptives, having a history of corticosteroid use within three months, participants under psychotropic medication within last 8 weeks.
- Participants diagnosed with any heart disease, diabetes, stroke, neurological disorders or depression.
- Have clinically significant acute unstable hepatic, renal, cardiovascular, or respiratory disease that will prevent participation in the study.
- Patients with depressive episode, panic disorder, social phobia, obsessive-compulsory disorder, alcohol dependency; schizophrenia and mania.
- Patients with post traumatic disorder.
- Have an established practice of meditation for three or more months.
- Pregnant and lactating women.
- Participation in other clinical trials during previous 3 months
- Any clinical condition, according to the investigator which does not allow safe fulfilment of clinical trial protocol

Results (preliminary): Based on the preliminary study results, the laboratory parameters stayed in normal reference limits after a 12-week.

Concerning hepatic or thyroid function assessed before and after the treatment, the levels were within normal reference ranges even though the levels showed some decrease after 12-week treatment.

The laboratory parameters at baseline and change from baseline in laboratory values in PP dataset (n=86) are presented in a table below.

Parameters	Active / Treatment Group		Control / Placebo Group		p value (Between)
	Mean Baseline (SD or SE or 95% CI)	Mean End of Study (SD or SE or 95% CI)	Mean Baseline (SD or 95% CI)	Mean End of Study (SD or 95% CI)	
WBC count (X1000 cells per cmm)	4.92 (0.82)	4.89 (0.85)	NA	NA	>0.05
RBC (millions/cmm)	4.78 (0.32)	4.60 (0.49)	NA	NA	>0.05
HB (g/dL)	12.85 (1.30)	12.75 (1.36)	NA	NA	>0.05
Haematocrit (%)	42.65 (2.61)	43.95 (2.88)	NA	NA	>0.05
Platelet count(X10000 cells per cmm	18.28 (6.11)	20.29 (7.52)	NA	NA	>0.05
FBS (mg/dL)	91.31 (11.79)	86.27 (11.33)	NA	NA	>0.05
HbA1c (%)	5.13 (1.24)	4.77 (1.15)	NA	NA	>0.05
Total Bilirubin (mg/dL)	0.87 (0.24)	0.78 (0.31)	NA	NA	>0.05
Direct Bilirubin (mg/dL)	0.34 (0.26)	0.28 (0.18)	NA	NA	>0.05
Indirect Bilirubin (mg/dL)	0.49 (0.26)	0.40 (0.09)	NA	NA	>0.05
ALT/SGPT (U/L)	32.71 (6.71)	27.84 (3.54)	NA	NA	>0.05
AST/SGOT (U/L)	33.15 (6.37)	28.04 (3.22)	NA	NA	>0.05
Alkaline Phosphatase (U/L)	84.17 (33.49)	81.09 (18.30)	NA	NA	>0.05
Total Proteins (g/dL)	7.19 (0.95)	7.36 (1.43)	NA	NA	>0.05
Albumin (g/dL)	4.33 (0.51)	3.65 (0.86)	NA	NA	>0.05
Globulin (g/dL)	2.88 (0.51)	3.12 (0.82)	NA	NA	>0.05
Creatinine (mg/dL)	0.86 (0.25)	0.85 (0.22)	NA	NA	>0.05
BUN (mg/dL)	18.19 (4.71)	16.62 (3.60)	NA	NA	>0.05
Uric acid (mg/dL)	5.95 (1.42)	5.76 (1.35)	NA	NA	>0.05
Calcium (mg/dL)	9.02 (0.68)	8.59 (1.21)	NA	NA	>0.05
Total Cholesterol (mg/dL)	168.41 (29.36)	164.72 (28.81)	NA	NA	>0.05
HDL Cholesterol (mg/dL)	43.95 (7.57)	42.08 (7.54)	NA	NA	>0.05
LDL Cholesterol (mg/dL)	109.62 (26.37)	107.31 (25.61)	NA	NA	>0.05
Triglycerides (mg/dL)	113.37 (44.92)	111.06 (43.90)	NA	NA	>0.05
TSH (µIU/mL)	0.72 (0.88)	0.69 (0.70)	NA	NA	>0.05
T3 (ng/dL)	133.04 (55.67)	133.90 (51.24)	NA	NA	>0.05
T4 (ng/dL)	11.07 (7.06)	11.71 (51.24)	NA	NA	>0.05

5.2.3 Clinical studies of KSM-66® on thyroid function (the study summaries)

For the studies of Verma *et al*. 2021, Salve *et al*. 2024, and Movva *et al*. 2024 , please see section 5.2.2 Clinical studies assessing safety as a primary endpoint (the study summaries).

For the study of Verma *et al*. 2023, please see section 5.2.4 Summaries of clinical studies of KSM-66® on sex hormones / sexual health.

5.2.3.1 Sharma, A.K., Basu, I., Singh, S., 2018. Efficacy and Safety of Ashwagandha Root Extract in Subclinical Hypothyroid Patients: A Double-Blind, Randomized Placebo-Controlled Trial. J Altern Complement Med 24, 243-248

A prospective, randomized, double-blind, single-center placebo-controlled study was performed to evaluate the efficacy and safety of ashwagandha root extract in subclinical hypothyroid patients. Fifty subjects with elevated serum thyroid stimulating hormone (TSH) levels (4.5–10 μIU/L) aged between 18 and 50 were randomized in either treatment (n = 25) or placebo (n = 25) groups for an 8-week treatment period.

Study product: KSM-66®, daily dose 600 mg

Results: After eight weeks of treatment with KSM-66® serum thyroid stimulating hormone (TSH) ($p < 0.001$), triiodothyronine (T3) ($p = 0.0031$), and thyroxine (T4) ($p = 0.0096$) levels normalized compared to placebo. KSM-66® treatment normalized the serum thyroid indices (TSH, T3, T4) during the 8-week treatment (time-effects: TSH [$p < 0.001$], T3 [$p < 0.001$], and T4 [$p < 0.001$]). The levels were within normal limits.

KSM-66® treatment was found safe based on evaluated physical (monitoring of the cardiovascular, respiratory, musculoskeletal, genitourinary, and nervous system), hematologic (hemoglobin, hematocrit, platelet count, red blood cell and white blood cell counts) and vital parameters (systolic and diastolic blood pressure, pulse rate, respiratory rate, body temperature). No significant changes in any of these parameters were observed during the study.

Four subjects (8%) (KSM-66®: 1[4%]; Placebo: 3[12%]) out of 50 reported few mild and temporary adverse effects during this study.

5.2.3.2 Wankhade, Kalpana; Khanna, Aman; panchal, Parth; Kalashetti, Suhas (2024). Ashwagandha Root Extract (Withania Somnifera) in Subclinical Hypothyroidism: A Randomized, Double-blind, Placebo Controlled Study on Safety, Efficacy and Quality of Life (QoL). PREPRINT.

Randomized, double-blind, two-arm, placebo-controlled, parallel, comparative, prospective, 12-week intervention study was performed to evaluate the efficacy of the ashwagandha formulation on subclinical hypothyroidism in terms of changes in thyroid stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4) in adult subjects with diagnosis of subclinical hypothyroidism based on persistently elevated TSH levels (4.6–15.0 mU/L), with T4 within the reference range (n=54). Patients on current prescription of levothyroxine, antithyroid drug were excluded.

Study product: KSM-66®, daily dose 600 mg

All the patients will be asked to continue their routine diet and exercise during the whole study period.

Results: After 12 weeks of treatment with KSM-66®, no significant changes in Total T3 or T4 were noted between the groups at either week 8 or week 12, indicating no major impact on these parameters ($p > 0.05$). In contrast the serum TSH was lower in the KSM-66® group (mean 5.31 mIU/mL) versus a placebo group (mean 7.51 mIU/mL) ($p < 0.005$), i.e. KSM-66® adjusting the TSH value closer to the normal range (e.g. 0.5 mU/l - 4 mU/l in 15-59 y) after 12 weeks of KSM-66® treatment.

Treatment tolerability was recorded using the Patient's Global Assessment of Tolerability to Therapy (PGATT) scales in KSM-66® and PL groups. Tolerability of Ashwagandha was reported between good to excellent by most of the patients in the KSM-66® group compared to placebo in a placebo group ($p=0.005$). A total of 20 patients (71.4%) in the KSM-66® group assessed tolerability of ashwagandha as good to excellent. Only 8 patients (28.5%) in the KSM-66® group reported average to poor tolerability of ashwagandha while 18 patients (69.2%) in placebo group reported average to poor tolerability. No adverse events were reported during the course of the study.

Concerning the hepatic parameters, compared to the baseline values, KSM-66® administration demonstrated no statistically significant changes between the groups.

5.2.4 Summaries of clinical studies of KSM-66® on sex hormones / sexual health

In women:

5.2.4.1 Ajgaonkar A, Jain M, Debnath K (2022), Efficacy and Safety of Ashwagandha (Withania somnifera) Root Extract for Improvement of Sexual Health in Healthy Women: A Prospective, Randomized, Placebo-Controlled Study. Cureus 14(10): e30787. DOI 10.7759/cureus.30787

In this prospective, randomized, placebo-controlled study, 80 women between 18 and 50 years of age without any deviation from the normal serum levels for estrogen, progesterone and testosterone and having hypoactive sexual desire disorder (HSDD) with a Female Sexual Function Index (FSFI) score <26, or Female Sexual Distress Scale (FSDS) score >11 were randomized to receive either capsule containing standardized KSM-66® root extract 300` mg twice daily (n=40), or identical placebo (n=40) for 8 weeks.

Study product: KSM-66®, daily dose 600 mg

Results (safety): No significant changes were observed in the vital parameters (blood pressure, pulse rate, temperature, respiratory rate) in the two groups ($p>0.05$).

The safety outcomes were clinical safety recorded as the number and proportion of Treatment Emergent Adverse Events (TEAEs) and Treatment Emergent Serious Adverse Events (TESAE) during the study period. Three women from each KSM-66® (7.5%) and placebo (7.5%) group reported adverse events. Nausea was reported by two (5.0%) women from each KSM-66® and placebo group, while drowsiness was reported by two (5.0%) women from the KSM-66® group and one (1.5%) from the placebo group.

One (1.5%) woman receiving KSM-66® reported both nausea and drowsiness. All events were of mild severity, were not associated with the study treatments, and were self-limiting.

The level of sex hormones was not measured at the end of the study.

To briefly summarize the efficacy results: The results indicate improvement in female sexual function with both placebo and KSM-66®. The use of KSM-66® brought the measured scores closer to normal values compared to placebo.

5.2.4.2 Gopal, S., Ajgaonkar, A., Kanchi, P., Kaundinya, A., Thakare, V., Chauhan, S. and Langade, D. (2021), Effect of an ashwagandha (Withania somnifera) root extract on climacteric symptoms in women during perimenopause: A randomized, double-blind, placebo-controlled study. J. Obstet. Gynaecol. Research https://doi.org/10.1111/jog.15030

8-week, randomized, double-blind, placebo-controlled study assessing the efficacy and safety of an KSM-66® root extract in perimenopausal women (aged, 45-60 y) n=91) experiencing mild climacteric symptoms.

Study product: KSM-66®, daily dose 600 mg

Results related to hormonal levels: At the end of the study, there was an increase in the serum estradiol levels in the KSM-66® group compared to the placebo group. In addition, ashwagandha intake resulted in a statistically significant reduction ($p < 0.005$) in the serum follicle stimulating hormone concentration, compared with the placebo group. There were non-significant decreases in the luteinizing hormone (p 0.591) and testosterone levels (p 0.755). The mean estradiol levels of 46.78 pg./mL in the KSM-66® group can be considered physiologically comfortable and safe. The observed changes in the hormone levels were within the reference range indicating the safety of KSM-66® root extract.

No major adverse events were reported by the participants although mild adverse events were reported by seven participants during the study. Three participants in the KSM-66® group reported abdominal discomfort, abdominal pain, and nausea. Four participants in the placebo group reported abdominal discomfort, abdominal pain, insomnia, and nausea. These adverse events reported were mild and temporary.

To summarize the results on the menopause rating scale (MRS) which is a validated self-report questionnaire on the severity of perimenopausal and aging symptoms. A between the group analysis revealed that there was a statistically significant reduction in the total MRS score in the KSM-66® group ($p < 0.0001$), compared to the placebo group. The total score of the MRS was 10.96 in KSM-66® group (-3.37 reduction compared to baseline, and 13.04 in the placebo group (-1.60 reduction compared to baseline) after 8 weeks. (The total score of the MRS is between 0 (asymptomatic) and 44 (highest degree of complaints). Based on literature reviews, total score ≤ 11, 12-35 and ≥ 36 are considered as asymptomatic, mild to moderate and severe to very severe, respectively.)

5.2.4.3 Dongre, S., Langade, D., & Bhattacharyya, S. (2015). Efficacy and Safety of Ashwagandha (Withania somnifera) Root Extract in Improving Sexual Function in Women: A Pilot Study. BioMed research international, 2015, 284154. https://doi.org/10.1155/2015/284154

Efficacy and Safety of KSM-66® ashwagandha (*Withania somnifera*) Root Extract in Improving Sexual Function in Women: A Pilot Study

The purpose of the double blind, placebo-controlled, randomized, study was to determine the efficacy and safety of a KSM-66® supplementation for improving sexual function in healthy females (n=50) with female sexual dysfunction (female sexual arousal disorder, female orgasmic disorder, or hypoactive sexual desire disorder). The study duration was 8 weeks.

Study product: KSM-66®, daily dose 600 mg

Results: No adverse effects of intervention were observed in the ashwagandha group. All the subjects (n = 25) showed excellent tolerability to the study product.

Concerning the sexual function, the results indicate improvement in female sexual function with both placebo and KSM-66®. The use of KSM-66® brought the measured scores closer to normal values compared to placebo.

The level of sex hormones was not measured in the study.

5.2.4.4 Amit Shrenikraj Mutha, Sonali Amit Mutha, Anupama Hem Tejuja et al. Efficacy and safety of Ashwagandha Root Extract on Sexual Health in Healthy Women: Findings of a Prospective, Randomized, Double-Blind, Placebo-Controlled Study, 17 October 2024a, PREPRINT (Version 1)

This prospective, randomized, double-blind, placebo-controlled study investigated the role of KSM-66® in enhancing sexual function in healthy women with Female Sexual Dysfunction (FSD).

Study product: KSM-66®, daily dose 600 mg

Sixty-two healthy women aged 18-50 years were randomly assigned to receive KSM-66® Ashwagandha root extract 600mg/d (n=31), or identical placebo (n=31) for 8 weeks.

The levels of the hormones follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were determined by Electrochemiluminescence Immunoassay (ECLIA), whereas the levels of the hormones estrogen-E2, progesterone, and testosterone were determined using Chemiluminescence Immunoassay (CLIA).

Results: No changes or differences between the groups were observed for hormones (FSH, LH, estrogen-E2, progesterone, or testosterone). No differences emerged between the groups for parameters (hepatic parameters (AST, ALT, ALP), bilirubin, Hb, creatinine, protein, albumin, globulin, and A-G Ratio).

The participants demonstrated good tolerance to the treatment, as evidenced by the occurrence of only one documented adverse event of dizziness (in a placebo group), with no reports of any serious adverse events.

To summarize the results on the primary outcome of the study i.e. Female Sexual Function Index (FSFI) scores with 6 different domains: desire, arousal, lubrication, orgasm, satisfaction, pain, FSFI total scores increased in both study groups, but the increase was higher in the KSM-66® group as compared

to the placebo group in week 8. No difference was observed in Female Sexual Distress Scale. No significant differences were found in the number of intercourse or non-sexual events (p=0.457) between the two groups throughout the study period. The quality-of-life score and perceived stress seemed to be lower in the KSM-66® groups compared to the placebo.

5.2.4.5 Amelioration of Sexual Function with Ashwagandha (Withania Somnifera) Root Extract in Healthy Women: Findings from an 8-week, Prospective, Randomized, Double-Blind, Placebo-Controlled Study (not published, under peer review)

This was an 8-week prospective, double-blind, randomized, placebo-controlled, two-arm, parallel, comparative clinical study. The purpose of this study was to evaluate the efficacy and safety of KSM-66® in sexual function in healthy women (n=84) with hypoactive sexual desire disorder.

Study product: KSM-66®, daily dose 600 mg

Results: No significant differences were observed in serum hormone levels (estrogen-E2, progesterone) between the two groups at either time point. In both the groups, there were no adverse events reported.

To summarize the results on women's sexual health, the treatment of KSM-66® showed improvements in the Female Sexual Dysfunction (FSD) psychometric scales and subscales compared to the placebo group. These included the Female Sexual Function Index (FSFI), the Female Sexual Distress Scale (FSDS), and the Sexual Satisfaction Evaluation (SSE).

5.2.4.6 A Prospective, Double Blind, Randomized, Parallel, Single-Center, Two-Arm, Placebo Controlled Clinical Study to Assess the Efficacy and Safety of KSM-66 Ashwagandha® capsule (300 mg) on Menopause Symptoms (not published, under peer review)

A randomized, double-blind, placebo-controlled study was conducted for a duration of 8 weeks. Total 60 women with clinically diagnosed menopause were enrolled.

Study product: KSM-66®, daily dose 600 mg

Results: There were no differences in serum estradiol, luteinizing hormone (LH), or follicle-stimulating hormone (FSH) levels between the intervention and placebo groups at week 8. Instead, there was a minor difference in serum progesterone levels: the level was statistically higher in the KSM-66® compared to the placebo group, 0.433 ng/dL vs.0.300 ng/dL, respectively, but the clinical significance can be considered negligible. The total progesterone levels observed in this study were all within the normal limits for postmenopausal women (approximately less than 100 ng/dL).

There was no change in basic laboratory values (e.g. in hepatic parameters) during the study.

When compared the within group changes from baseline to 8 weeks, it was noted that the changes were significant for estradiol, LH and FSH in both study groups, in addition to progesterone levels after KSM-66® treatment.

Total 3 women (1 in KSM-66® group and 2 in placebo group) reported adverse events. One woman in KSM-66® group reported cough and cold while two women in placebo group reported stomachache and

indigestion respectively. All events were of mild severity, were not associated with the study treatments, and completely resolved with or without symptomatic treatment.

To summarize the results on menopause symptoms, there was statistically significant reduction in hot flashes events ($p<0.05$) in KSM-66® group while increased events were noted in placebo group. The results showed improvement in quality-of-life of menopausal women in Ashwagandha group reflected in statistically significant improvements in Mental Component Score (MCS-12) and a Physical Component Score (PCS-12) from Short Form survey (SF-12).

5.2.4.7 Efficacy and Safety of KSM-66® Ashwagandha (Withania somnifera) Standardized Root Extract for Treatment of Menopausal symptoms in women: A Prospective, Randomized, Double-Blind, Placebo Controlled Study (not published)

Prospective, randomized, double-blind, placebo-controlled 12-week intervention study investigated the role of KSM-66® in menopausal symptoms in women (n=53) aged 45-65 years with symptoms of menopausal syndrome. Improvement in the composite score of Menopause Rating Scale (MRS) from baseline to end of therapy was the primary endpoint in the study. The total score of the MRS ranges from 0 (asymptomatic) to 44 (highest degree of complaints). In addition, there were some secondary endpoints, e.g. changes in hormone levels from baseline to end of therapy.

Study product: KSM-66®, daily dose 600 mg

Results: No significant differences were observed in serum hormone levels of estradiol and testosterone between the groups. Whereas the level of follicle stimulating hormone (FSH) and luteinizing hormone (LH) were significantly lower in the KSM-66® group compared to placebo group at 12 weeks (23.18 vs 37.22 m/u/ml; 45.43 vs 61.78 m/u/ml, respectively). All the values are in normal limits in perimenopausal women.

Some mild adverse events were reported which were resolved, and no discontinuation of study treatment was required.

Concerning MRS score, there was significant reduction in MRS scores with KSM-66® as compared to the placebo at week 12 ($p<0.0001$).

In men and women:

5.2.4.8 Verma N, Gupta SK, Tiwari S, Mishra AK, Thakare V, Patil S. Effect of Ashwagandha Root Extract on Serum Testosterone and Muscle Recovery in Strength Training. International Journal of Medical and Pharmaceutical Research Website.Issue:5, Oct 2023; Page No: 371-381

The study aim was to investigate the effects of ashwagandha root extract on serum testosterone and inflammatory markers in healthy adults following resistance training.

Study product: KSM-66®, daily dose 600 mg

This was a prospective, randomized, double-blind, placebo-controlled study in 80 healthy male and female adults between 18–45 years of age. Enrolled participants were randomly allocated to receive capsule ashwagandha root extract 300 mg, or an identical placebo capsule containing starch twice a day. Secondary study assessments included, for example, serum testosterone (total and free) done at baseline and after 8 weeks. Clinical safety was assessed based on the frequency of adverse events reported by the participants.

Four participants in the placebo and 3 from ashwagandha group did not complete the study, and analyses were done on data of 73 (36 ashwagandha, 37 placebo) participants. Over the eight weeks, there was a significant change, increase in free testosterone level in the ashwagandha treatment group (1.11 ng/dL) relative to the placebo group (0.27 ng/dL) ($p<0.0001$) in males and (0.17 ng/dL vs. 0.06 ng/dL in females, respectively. To level of total testosterone increased more in KSM-66® group compared to the placebo group. The total testosterone levels observed in this study were all within the lower part of the normal limits in both men and women.

No significant changes were observed from baseline to week 8 in any of the laboratory tests conducted for hematology, renal function, liver function and thyroid function tests with both KSM-66® and placebo treated participants (supplementary data).

The participants did not report any adverse events during the study period.

In men:

5.2.4.9 *Chauhan, S., Srivastava, M. K., & Pathak, A. K. (2022). Effect of standardized root extract of ashwagandha (Withania somnifera) on well-being and sexual performance in adult males: A randomized controlled trial. Health science reports, 5(4), e741. https://doi.org/10.1002/hsr2.741*

In this 8-week randomized, double-blind, placebo-controlled study, we investigated the aphrodisiac property of KSM-66® in adult males (21 and 45 years, n=50). All recruited participants were required to have low sexual desire.

Study product: KSM-66®, daily dose 600 mg

Results: Concerning the hormonal function, serum testosterone and serum prolactin levels were evaluated as secondary outcomes at baseline and week 8; the results were within the normal range for all participants in both visits. The rise in the serum testosterone was 17% ($\Delta = 72$ ng/dl; $p < 0.0001$) in the KSM-66® group compared to 2% changes in the placebo group ($\Delta = 5.48$ ng/dl; $p = 0.39$). In contrast, both groups observed non-significant changes ($p > 0.05$) in serum prolactin levels. The total testosterone levels observed in this study were all within the normal limits, irrespective of group or time.

The treatment was well-tolerated among the participants. Overall, seven adverse events (AEs) were recorded, of which four were reported in subjects assigned the KSM-66® capsule (two subjects experienced sleepiness, one developed mild abdominal pain, and one low-grade joint pain), and three were noted in those allocated the placebo capsule (one subject had abdominal pain, while two subjects had mild diarrhea); only sleepiness may be related to the active intervention. There were no reports of

any serious adverse events. The routine physical examinations did not reveal any significant changes during the study and after 8 weeks of treatment. Also, the vital parameters were stable, with no significant differences ($p > 0.05$) between the study groups.

To summarize the efficacy results, when comparing the KSM-66® group to the placebo group, a between-group analysis demonstrated a statistically significant improvement in the total the derogates interview for sexual functioning-male (DISF-M) score in the KSM-66® group ($p < 0.0001$). All recruited participants were required to have low sexual desire as measured by a score of 15 or less on the sexual-desire domain (range: 0–24, with higher scores indicating greater desire) of the DISF-M questionnaire. After the KSM-66® treatment, the sexual desire had a score of 16.2 i.e. in the normal levels.

5.2.4.10 Wankhede, S., Langade, D., Joshi, K., Sinha, S.R., Bhattacharyya, S., 2015. Examining the effect of Withania somnifera supplementation on muscle strength and recovery: a randomized controlled trial. J Int Soc Sports Nutr 12, 43.

8-week, randomized, prospective, double-blind, placebo-controlled clinical study with 57 young male subjects (18–50 years old)

Study product: KSM-66®, daily dose 600 mg

Results: Concerning the testosterone levels measured as a secondary outcome in the study, over the eight weeks, there was a significant increase in testosterone level in the KSM-66® treatment group relative to the placebo group (Placebo: 18.00 ng/dL, 95 % CI, -15.83, 51.82 vs. KSM-66®: 96.19 ng/dL, 95 % CI, 54.86, 137.53; $p = 0.004$). The mean post-intervention level was notably higher in the ashwagandha group than in the placebo group (726 versus 693), but the levels are not as statistically significantly different. The total testosterone levels observed in this study were all within the normal limits.

No serious side effects were reported by subjects in either group. All subjects rated tolerability as either “good” or “excellent” on the Physicians Global Assessment of Tolerability to Therapy (PGATT) form. There was no statistically significant difference in PGATT scores between the 2 groups.

5.2.4.11 Ambiye, V.R., Langade, D., Dongre, S., Aptikar, P., Kulkarni, M., Dongre, A., 2013. Clinical Evaluation of the Spermatogenic Activity of the Root Extract of Ashwagandha (Withania somnifera) in Oligospermic Males: A Pilot Study. Evid Based Complement Alternat Med 2013, 571420

Total of 46 male patients between 22 and 40 years of age with semen factor infertility were enrolled to the 12-week, randomized and placebo-controlled study. Men with a total sperm count of <0.5million/mL or over 20 million/mL were not included.

Study product: KSM-66®, daily dose 675 mg

Primary outcome: semen parameters and serum hormone levels from baseline (Day 0) after 90 days of intervention (e.g. testosterone and luteinizing hormone (LH))

Secondary outcomes: The secondary efficacy outcome was the safety and efficacy of the study product. Safety was assessed based on the adverse events recorded during the study.

Results: Treatment with KSM-66® resulted in a significant increase in sperm concentration, sperm motility and semen volume after 90 days of intervention, as compared to the baseline value. No detailed information was given on the difference between the groups. According to the World Health Organization (WHO), normal sperm concentration ranges from 15–259 million sperm per milliliter. The result in a KSM-66® group of 25.61 106/mL after 12 weeks is within the normal limits.

Significant improvements were observed in serum hormone levels with the KSM-66® treatment. Serum testosterone increased by 17% (from 4.45 ± 1.41 ng/mL to 5.22 ± 1.39 ng/mL; $P < 0.01$). No detailed information was given on the difference between the groups. The total testosterone levels observed in this study were all within the lower part of the normal limits.

Luteinizing hormone increased by 34% (from 3.97 ± 1.21 mIU/mL to 5.31 ± 1.33 mIU/mL; $P < 0.02$), following treatment with Ashwagandha root extract, as compared to the baseline. No detailed information was given on the difference between the groups. The luteinizing hormone levels observed in this study were all within the normal limits.

5.2.4.12 Amit Shrenikraj Mutha, Sonali Amit Mutha, Anupama Hem Tejuja et al. Efficacy and Safety of Eight-Week Therapy with Ashwagandha Root Extract in Improvement of Sexual Health in Healthy Men: Findings of a Prospective, Randomized, Double-Blind, Placebo-Controlled Study, 14 October 2024b, PREPRINT (Version 1)

This 8-week prospective, double-blind, randomized, placebo-controlled study evaluated the efficacy and safety of KSM-66® on improving sexual health in healthy adult men between 30 to 50 years with poor sexual satisfaction and attending an infertility clinic (n= 100).

Study product: KSM-66®, daily dose 600 mg

Results: They observed some change differences in serum testosterone (p=0.012) compared to placebo, but no significant change was observed in dihydrotestosterone, prolactin, FSH, and LH levels at the end of the 8-week therapy. The testosterone levels were within the normal limits.

Furthermore, no differences were observed in any of the hematological, hepatic (alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP)), and renal parameters in per protocol dataset (n = 93) with KSM-66® and placebo groups at the end of the 8-week therapy.

During the study period, none of the participants reported any adverse events.

Related to questionnaires, the greater improvement in the number of sexual events (p=0.042) orgasms, and sexual desire scores were observed with KSM-66® compared to placebo. KSM-66® also led to a greater increase in semen volume (p=0.005), sperm count per ejaculate (p=0.007), and normal sperm morphology (p=0.007) compared to placebo. While no difference was seen in mean changes in sperm count per mL, sperm motility or semen vitality, number of satisfying sexual activities, erectile function, or intercourse satisfaction.

5.2.4.13 Ashwagandha Root Extract Improves Sexual Health in Healthy Men: Findings of a Prospective, Randomized, Double-Blind, Placebo-Controlled Study (not published, under review)

A prospective, double-blind, randomized, placebo-controlled, comparative 8-week study was conducted where total of 76 healthy men aged between 30 to 50 years of age, visiting the study sites for seeking interventions for their sexual problems were randomized in a 1:1 ratio to receive either KSM-66®capsule 300mg twice daily (n=38), or identical placebo (n=38) for eight weeks. The efficacy and safety analyses were done on all 76 participants.

Study product: KSM-66®, daily dose 600 mg

Results: No adverse events or serious adverse events were reported during study by the patients. The serum total and free testosterone levels were measured at baseline and at 8 weeks, no difference was observed between the groups. Similarly, no differences were observed between hepatic (ALT, AST, ALP, bilirubin) parameters. Related to renal parameters (blood urea nitrogen, creatinine) no difference was observed for blood urea nitrogen, however, the creatinine value was significantly lower in the KSM-66® group (0.50 mg/dL) compared to the placebo group (0.65 mg/dL) ($p<0.049$) at week 8.

Concerning the primary outcome, KSM-66® treatment tends to provide greater improvement in sexual activity and sexual desire inventory scores with men in KSM-66® group having significantly greater ($p<0.05$) improvement in the total number of sexual events compared to placebo.

5.2.5 Other clinical studies with relevant laboratory parameters measured (e.g. hepatic parameters)

Please, see the Section 5.2.2 Clinical studies assessing safety as a primary endpoint (the study summaries for summaries of studies by Verma *et al*. 2021, Salve *et al*. 2024 (long-term study), Movva *et al*. 2024 (high-dose study). The study summary of the study by Wankhade *et al*. (2024) is presented in the section 5.2.3 Clinical studies of KSM-66® on thyroid function (the study summaries). The study summaries of studies of Amit *et al*. 2024a, Amit *et al*. 2024b, 2 recent, completed, unpublished studies are presented in the section 5.2.4 Summaries of clinical studies of KSM-66® on sex hormones / sexual health.

5.2.5.1 Długołęcka B, Jówko E, Kotowska J, Gierczuk D. Effects of ashwagandha (Withania somnifera) supplementation on body composition and blood health indices in professional wrestlers. Pol. J. Sport Tourism 2023, 30(4), 26-32; DOI: 10.2478/pjst-2023-0022

The purpose of this randomized, double-blind, placebo-controlled study was to evaluate the effect of ashwagandha supplementation on the body composition, blood health and recovery indices, as well as the safety of ashwagandha supplementation in wrestling. To investigate this, body composition (using dual energy X-ray absorptiometry; DEXA) and blood parameters as indicators of athletes' health status (i.e., hematology, liver, kidney and muscle tissue function, lipid profile) were measured before and after 8-week supplementation with ashwagandha extract in professional national team wrestlers following an intensive training plan (n=21).

Study product: KSM-66®, daily dose 600 mg

Ashwagandha supplementation did not affect any of the body composition parameters, hematological or biochemical blood parameters (e.g. aspartate aminotransferase (AST), alanine aminotransferase (ALT)) ($p > 0.05$ for main effect of group and interaction of time and group). However, the increase in creatine kinase activity during the study seemed to be more pronounced in the placebo group (post-hoc; $p < 0.05$) than in the ashwagandha group (post-hoc; $p > 0.05$). All the values were observed within the normal reference ranges.

5.2.5.2 Naik, K. S., Muralidhar, G., Rao, B. S., & Wankhade, K. (2024). An 8-Week Oral Therapy with Ashwagandha (Withania Somnifera) Root Extract (600 mg/day) Improves Frailty and Quality of Life in Elderly: A Prospective, Randomized, Placebo-Controlled, Efficacy and Safety Study. PREPRINT

This placebo-controlled study assessed the efficacy and safety of a capsule containing 300mg of Ashwagandha Root Extract (ARE) administered twice daily orally for 8 weeks. Fifty elderly subjects with a frailty score ≥7 based on Frailty Assessment and Screening Tool (FAST) were randomized in a 1:1 ratio to receive either KSM-66® Ashwagandha (ARE, n=25) or placebo (PL, n=25) for 8 weeks.

Study product: KSM-66®, daily dose 600 mg

Results on safety parameters: KSM-66® was well tolerated with no adverse effects, and no changes in hepatic (AST, ALT, ALP) and renal parameters were observed at the end of the intervention period (8 wk).

5.2.5.3 Jain, Mukta; Naik, K Sunil; Gudla, Muralidhar; Bade, Srinivas Rao; Agarwal, Sanjay (2024). Ashwagandha Root Extract in Treatment of Mild Attention Deficit Hyperactivity Disorder (ADHD) in Children Aged 5–12 Years: A Prospective, Double-Blind, Randomized, Placebo Controlled Study. figshare. Preprint.

In this randomized, double-blind, placebo-controlled study, a total of 58 boys and girls (age 5-12 years) with a mild form of ADHD were randomly assigned to receive KSM-66® 150mg (n=29), or identical placebo (n=29) in a 1:1 ratio for 56 days. Children receiving any pharmacotherapy for ADHD prior to study screening were excluded.

Study product: KSM-66®, daily dose 300 mg

Results on laboratory parameters: No significant change was observed in laboratory parameters including liver or renal parameters (total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine) after 8 weeks of intervention.

All children completed the study as per protocol. One participant in the KSM-66® group reported mild cough and in the placebo group moderate fever. Both cough and fever were of mild to moderate severity, were not associated with the study treatments, and completely resolved with oral dextromethorphan and paracetamol respectively. None of the children stopped treatment due to adverse events and treatment compliance was more than 80% in both the groups.

6 Summary and conclusions

Ashwagandha (*Withania somnifera*) root is one of the prominently used Ayurvedic ingredients in India. Ashwagandha root has a history of use for more than 4000 years in different age and user groups, and also during pregnancy. Ashwagandha use is described by traditional Ayurveda texts. Ashwagandha is used primarily for its root in national standard references, such as many pharmacopoeias. Despite having a long history of use, no serious concerns have been reported in the past.

In recent decades, the use of ashwagandha root extract as a food supplement has become popular around the world. Ashwagandha root and extracts are currently on the market in the US as a dietary supplement with use levels of up to 1800 mg/day of root extract. KSM-66® ashwagandha is self-affirmed GRAS (Generally Recognized As Safe) based on a critical evaluation and acceptance of an independent expert panel in 2019 (Ixoreal Biomed). As per the Ixoreal Biomed's post marketing surveillance, there are no reports of serious adverse events associated with KSM-66® usage.

In the European Union, *Withania somnifera* root was marketed as a food ingredient and consumed to a significant degree before 15 May 1997 and, therefore, it does not fall under the Novel Food Regulation (EU) 2015/2283. The non-concentrated aqueous infusions from the roots are not considered novel foods, and all plant parts have a significant history of food use in food supplements.

KSM-66® has a significant market distribution all over the world. KSM-66® has been on the market for over 14 years, and it is currently used in over 3000 products in 52 countries and in many major supplement companies across the globe. KSM-66® is used in plenty of delivery formats, the most common being capsules, tablets, soft gels, and powders. In the last 6 years alone, over 6 billion KSM-66® ashwagandha doses have been sold worldwide.

It is not just historical or traditional use, but also toxicity studies conducted on KSM-66® that have assessed its safety and tolerability in various study models. In addition to numerous clinical studies, KSM-66® has been studied for several toxicological effects in various *in vitro* and *in vivo* models.

KSM-66® has undergone all basic toxicological preclinical studies required by the European Food Safety Authority (EFSA) for novel foods. Contrary to the DTU's report on ashwagandha extracts in general, KSM-66® has undergone a 90-day subchronic feeding study and thus, a safe intake limit can be calculated and established for its daily dose. In addition, the extract has undergone numerous clinical trials over the past several years, including in relation to safety. These studies were not considered by DTU at the time of the risk assessment.

The manufacturer presented data on a total of 36 clinical trials conducted with a food supplement containing ashwagandha extract KSM-66®. This includes 29 trials that have been published in scientific journals or preprinted, of which 27 were placebo-controlled and double-blinded. Most studies have been conducted with the daily dose of 600 mg of KSM-66®, but there is also data on safety with higher daily dose up to 2000 mg.

Treatment durations varied, with 27 studies lasting 8 weeks, 6 studies lasting 12 weeks, and 2 studies lasting 10 weeks. Additionally, 24-week interim data were presented for a study designed to last 12 months. The total number of participants in the presented placebo-controlled studies was about 3000

of which about half received KSM-66. When the two non-placebo-controlled studies are included, the total number of participants receiving KSM-66® was about 1700.

Mild adverse events have been reported for both KSM-66® and the placebo group by the participants. According to these studies, the adverse events reported were minor and no relation to sexual health, sex hormones, hepatotoxicity or liver injury and they do not raise any suspicion of toxicity with daily consumption of KSM-66®. In the long-term safety study, a total of 18 adverse events were reported during the first 6 months of administration, the most frequently reported event being nausea reported by 6 (3.1%) patients. All events were of mild severity and resolved without any intervention. Thus, it can be concluded that KSM-66® was well-tolerated among the participants of the studies published.

Multiple clinical studies have assessed the safety and impact of KSM-66® on various laboratory parameters, including sex hormones, thyroid function, and liver function. The measured values remained within normal reference ranges or adjusted toward normal levels in study subjects with baseline values outside the standard limits.

The supplier of KSM-66® is committed towards the goal of providing safe and effective products. For example, a study that evaluates the long-term safety of KSM-66® supplementation for one year is currently ongoing and the final report is expected to be available in 2025.

As a conclusion, taken together the very long history of use of ashwagandha root, several preclinical and clinical study safety data, we can conclude that the consumption of the KSM-66® dietary supplement at a dose of 700 mg/day in adults does not appear to pose a concern for human health.

7 References

[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.

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7.1 List of appendices (Available upon request from Ixoreal)

Appendix 1. Safety evaluation on the root part vs. the entire plant aerial parts
Appendix 2. Summary of clinical studies on KSM-66®

Final Report
21.03.2025

REPORT

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

Summary

Compiled by:

[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)).

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).

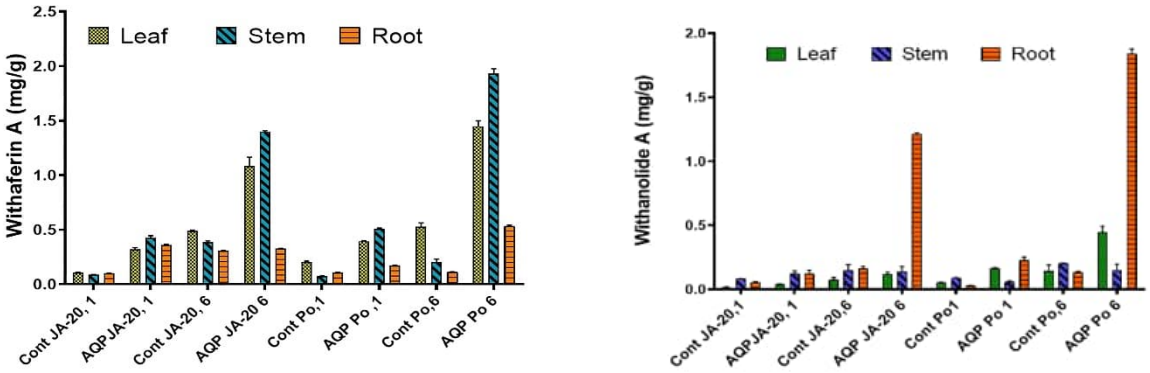


Figure 1. Differences in amounts of withaferin A and withanolide A between the roots, stems, and leaves of Ashwagandha.

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.

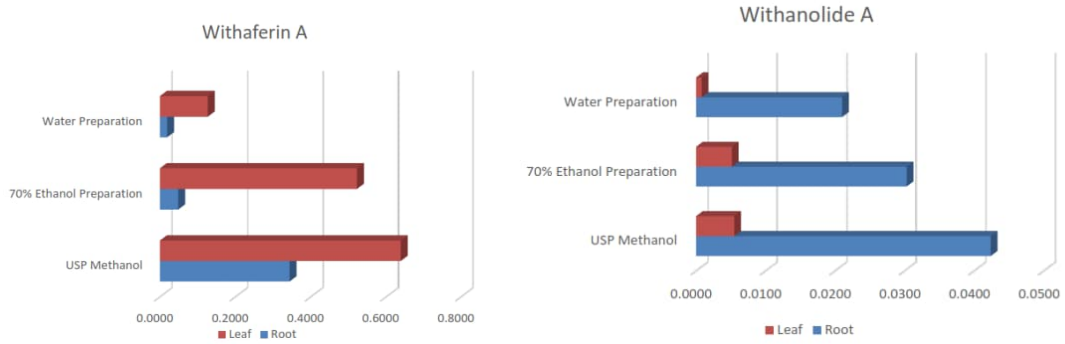


Figure 2. Differences in amounts of withaferin A and withanolide A between the roots and leaves of Ashwagandha.

[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.

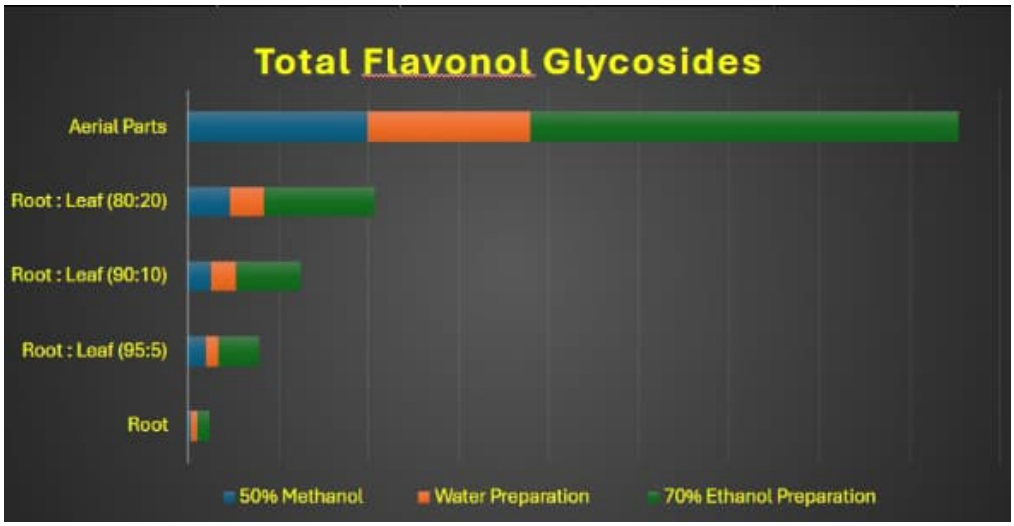


Figure 3. Differences in total flavonol glycosides between different extractions of Ashwagandha roots and leaves.

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.

Roots Vs Leaves
Adressing the Concerns on Ashwagandha Safety

By Ixoreal Biomed Inc

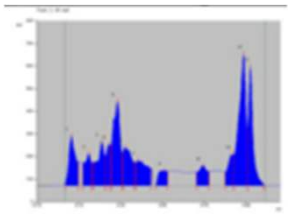
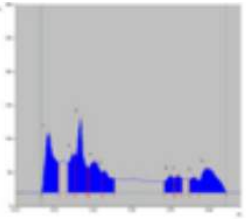
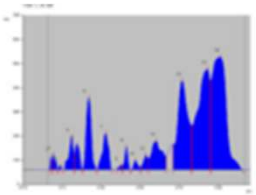
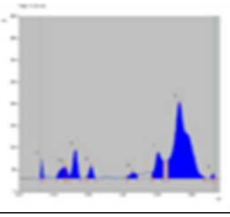
17th March 2025

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	
	
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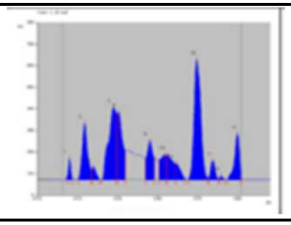
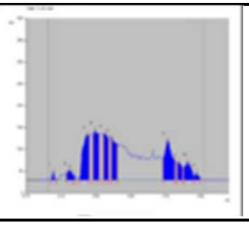
	

Roots Vs Leaves– Are these parts similar?

Qualitative Differences (Javed et al., 2021)

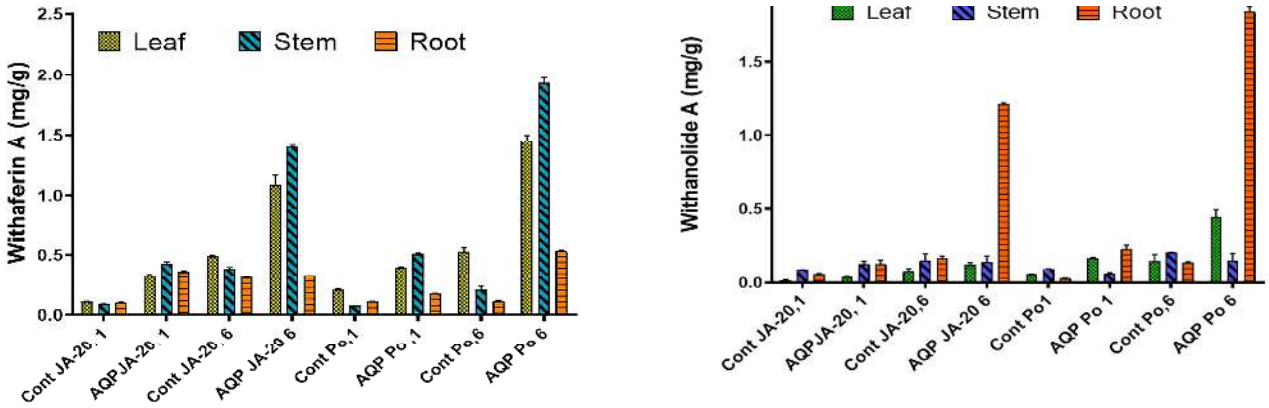
List of selected withanolides and other compounds identified from *Withania somnifera* (L.) Dunal.

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

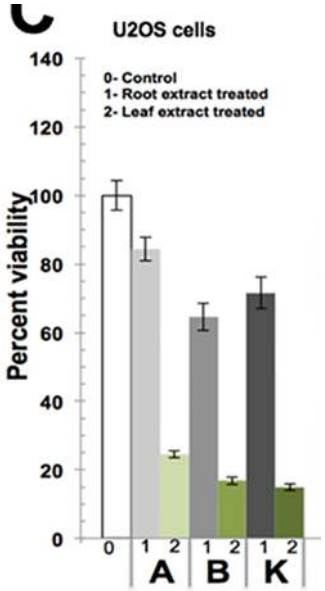
Substance	Roots	Leaves	Reference
Withaferin A (mg/g DW)	0.53	10.10	Kaul et al., 2016
	0.92	22.31	Chatterjee et al., 2010
Withanone (mg/g DW)	1.57	18.7	Kaul et al., 2016
	0.50	3.61	Chatterjee et al., 2010



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)

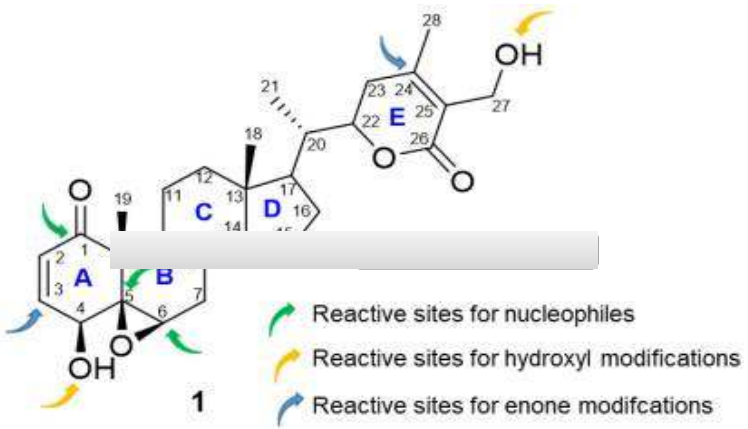
Roots Vs Aerial Parts– Are these parts similar?

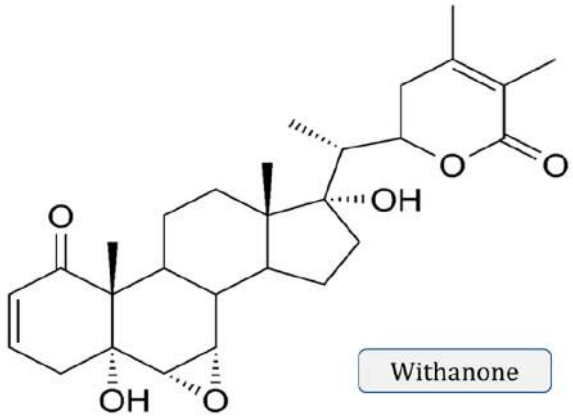
Bioactivity

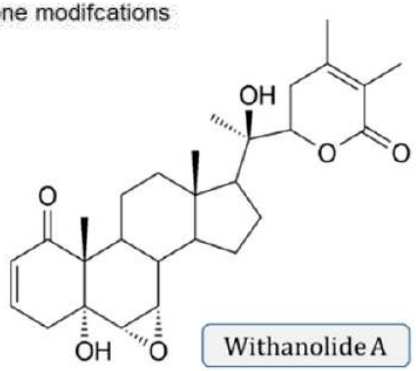
Plant Part	Effect on Sperms	Testing System	References
Roots	increase in sperm conc. Count, motility, ejaculate volume and serum test.	Rat Human	Kumar A et al., 2015; Ganu et al. 2010 Sahin et al., 2016 Ambiye et al, 2013 Sengupta et al., 2018
Fruit and Stem	Reduces primary and secondary spermatocytes,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

Roots Vs Leaves – Are these parts similar?

Chemical Reactivity



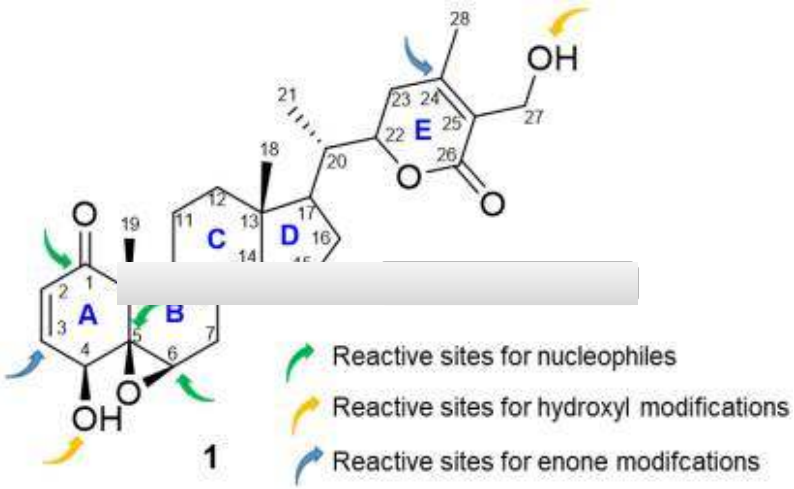


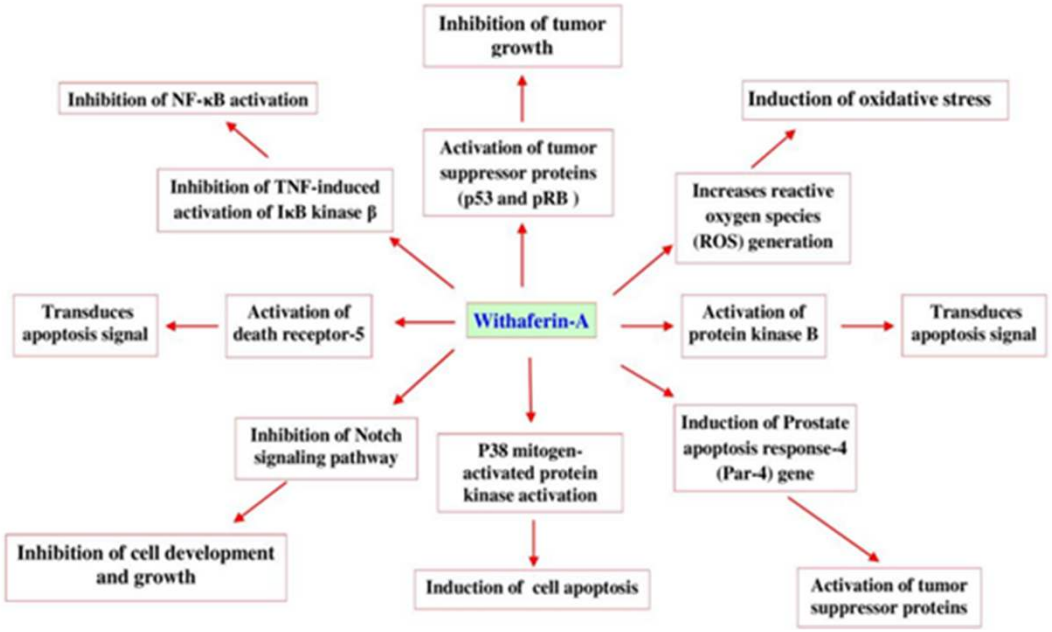


Withaferin A with oxidation at carbons 4, 5, and 6 is considered as an active type, especially as anticancer, whereas the withanolide A and withanone with oxidation at carbons 5, 6, and 7 rarely show any activity.

Roots Vs Leaves – Are these parts similar?

Chemical Reactivity

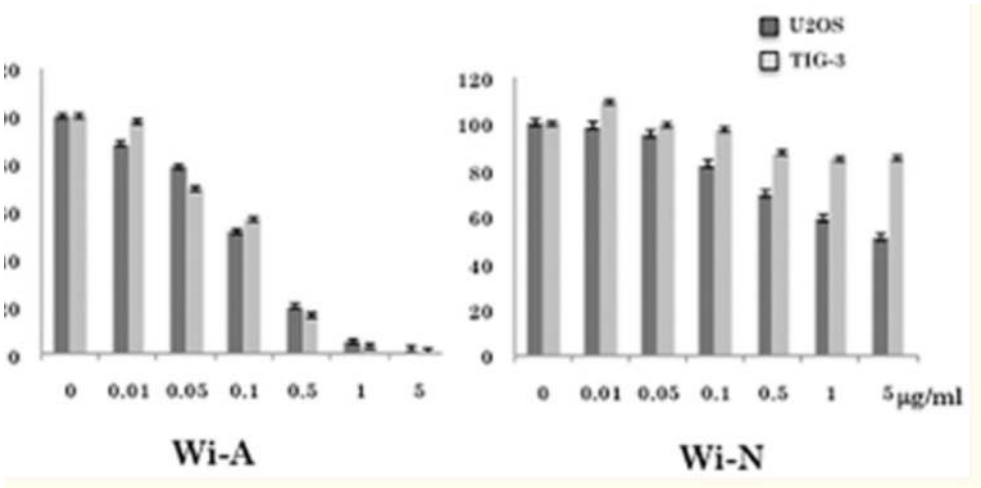




Kumar et al., 2015

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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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

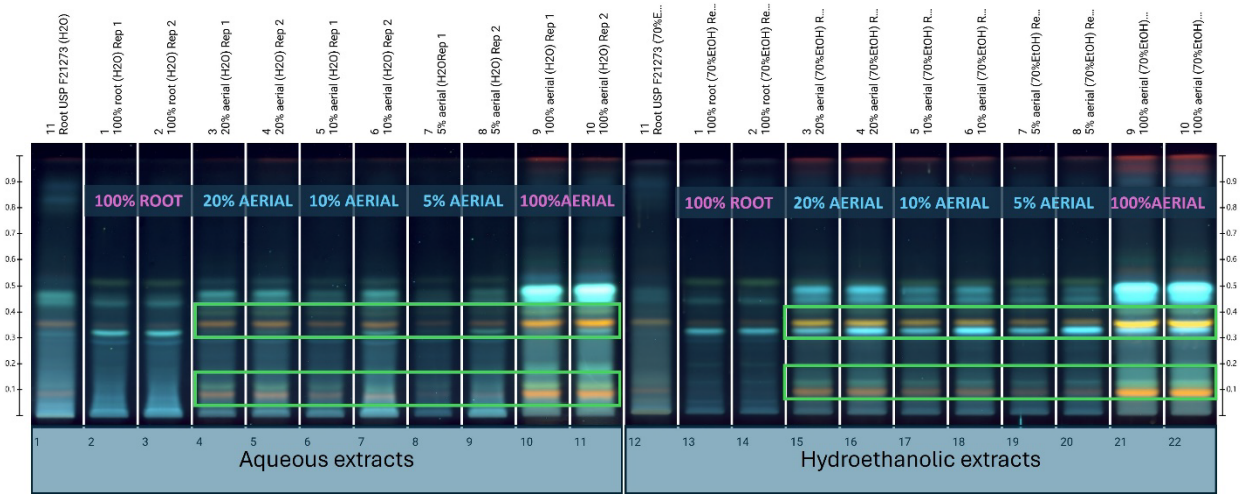
In Image 5, flavonoid compounds are detectable as low as 5% for both aqueous extracts (Lanes 8, 9) and hydroethanolic extracts (Lanes 19, 20). Flavonoid compounds are visible in the ranges of Rf 0.1-0.2 and Rf 0.3-0.45, as indicated by the green boxes.

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*

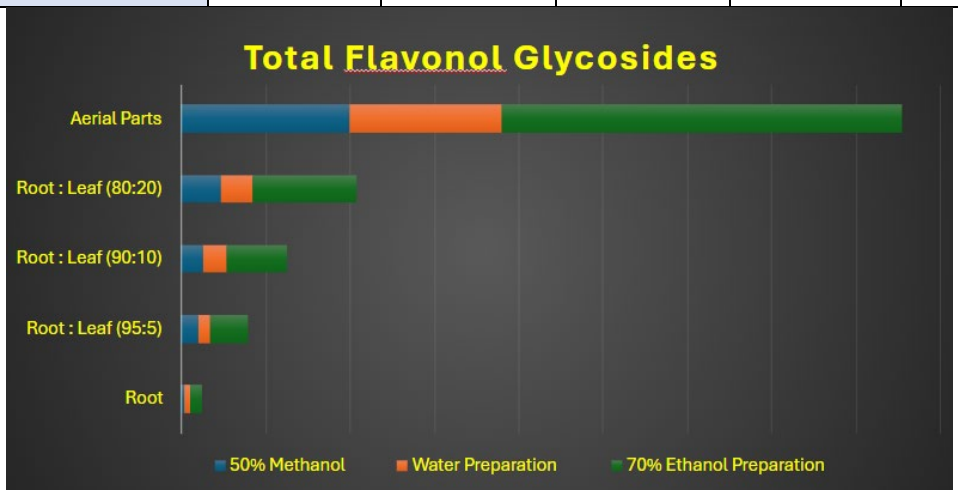
The content of withanolides and limit of flavonol glycosides derived from aerial parts were analyzed according to the HPLC conditions described in the USP Ashwagandha Root Powder Monograph (Appendix 4 for reference). Total Flavonol Gylcosides, Withaferin A, and Withanolide A were determined using three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

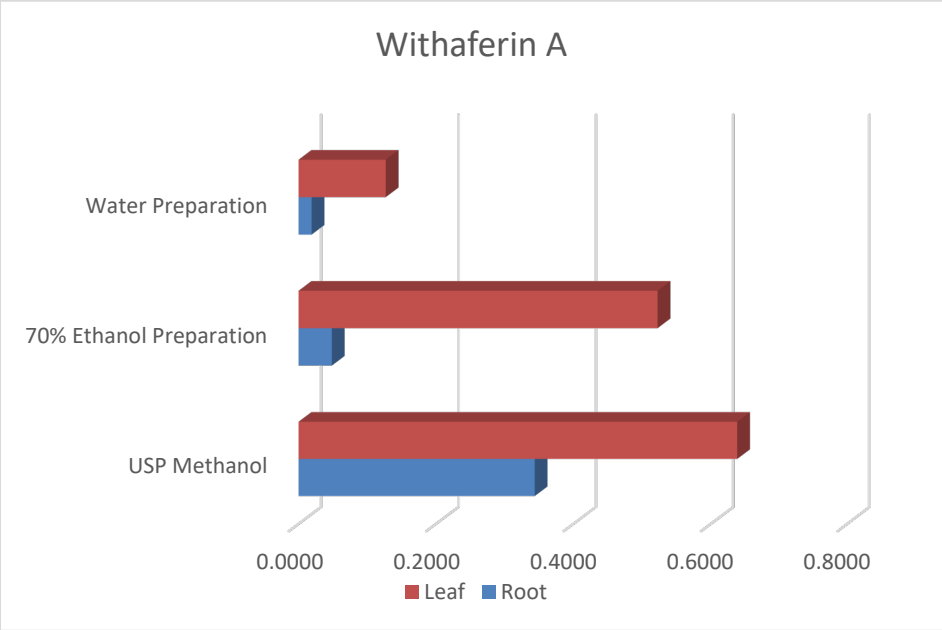
Total Flavonol Glycosides	100% Root	Root : Leaf (95:5)	Root : Leaf (90:10)	Root : Leaf (80:20)	100% Leaf
50% Methanol	0.0018	0.0107	0.0133	0.0238	0.1002
Water Preparation	0.0036	0.0064	0.0140	0.0191	0.0897
70% Ethanol Preparation	0.0074	0.0230	0.0357	0.0616	0.2376



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

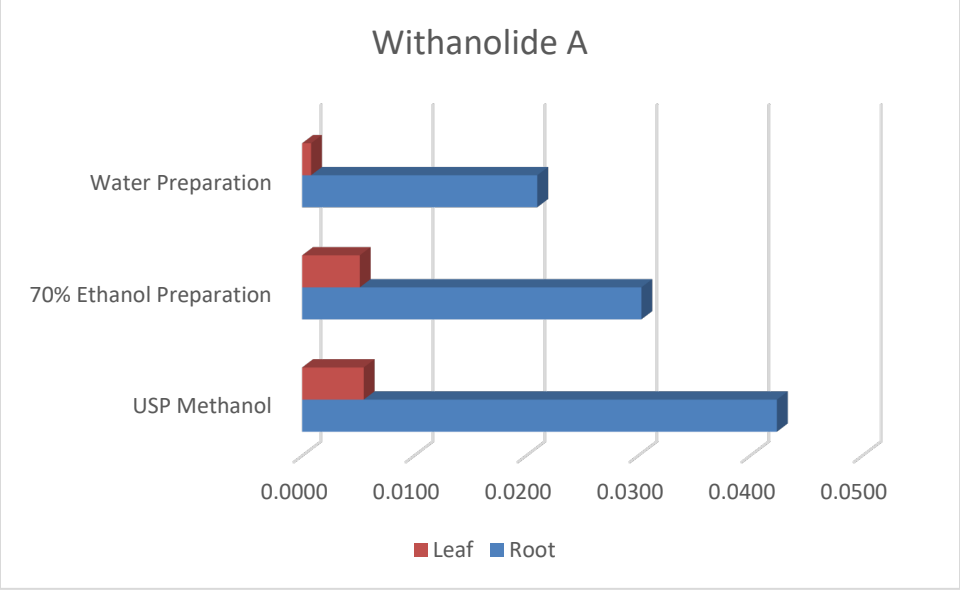
Withaferin A	Root	Leaf
USP Methanol	0.3437	0.6384
70% Ethanol Preparation	0.0484	0.5226
Water Preparation	0.0190	0.1268



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

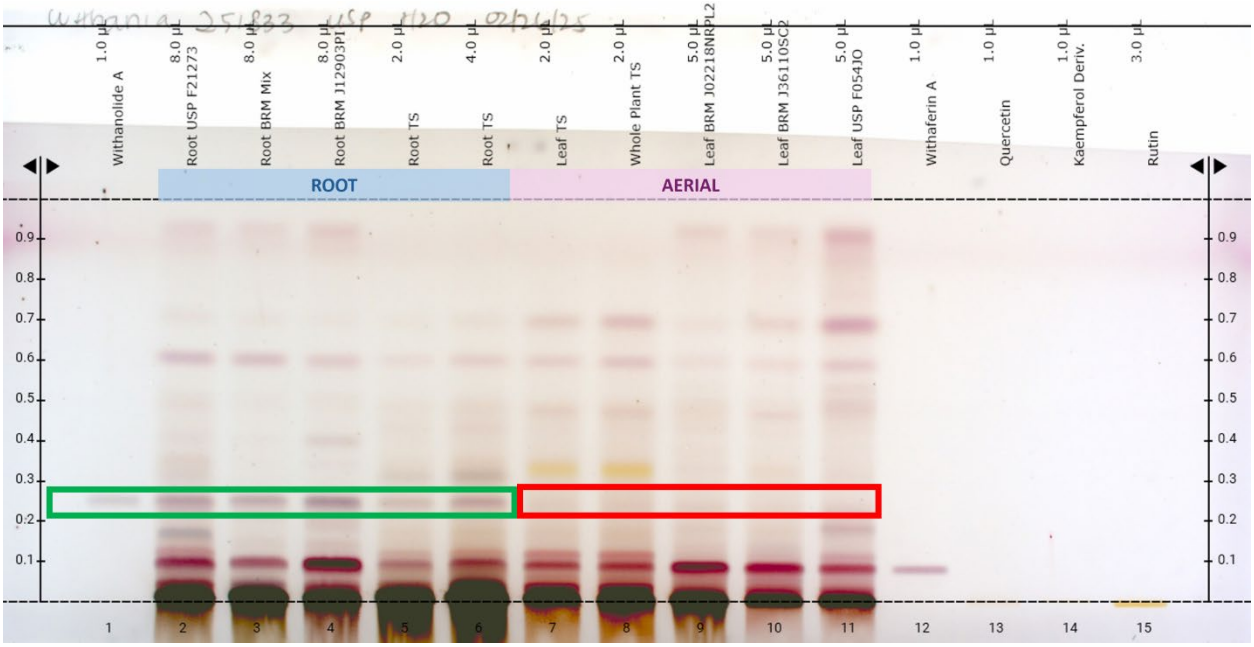


Image 1B:

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

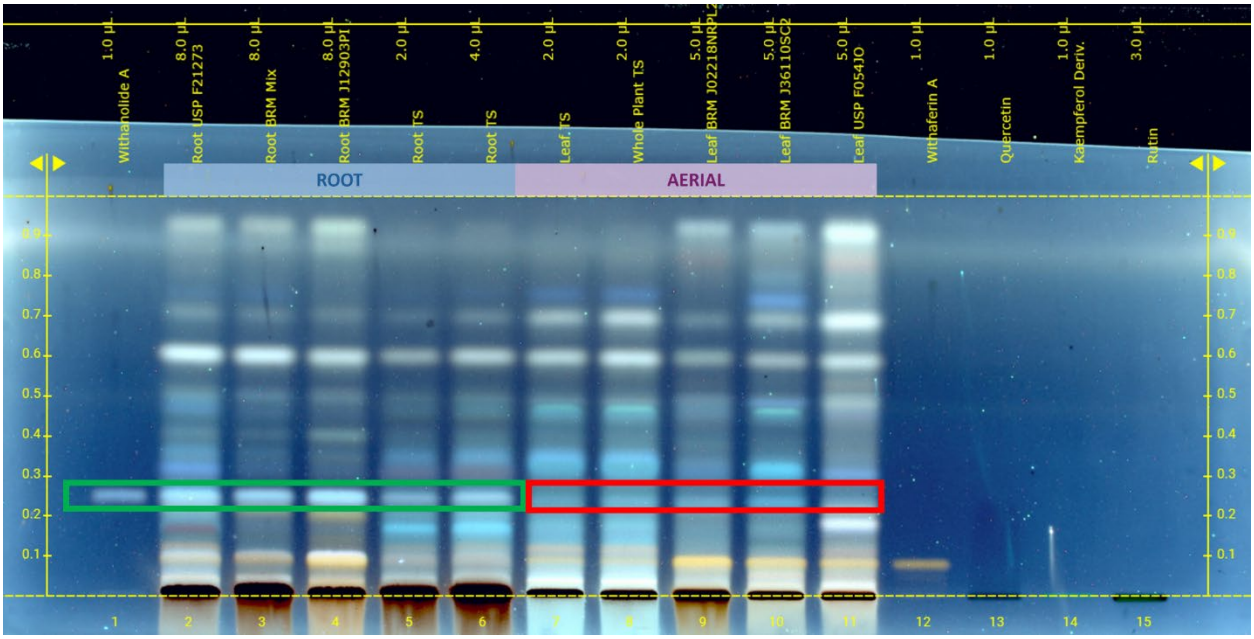


Image 2A:

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

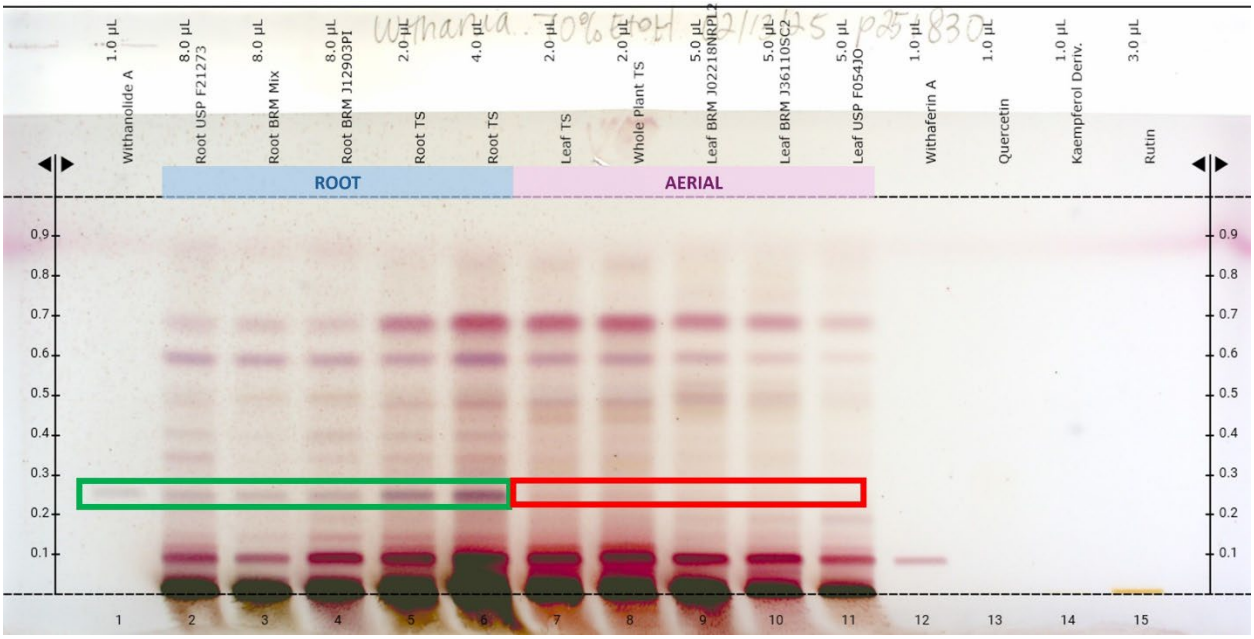


Image 2B:

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

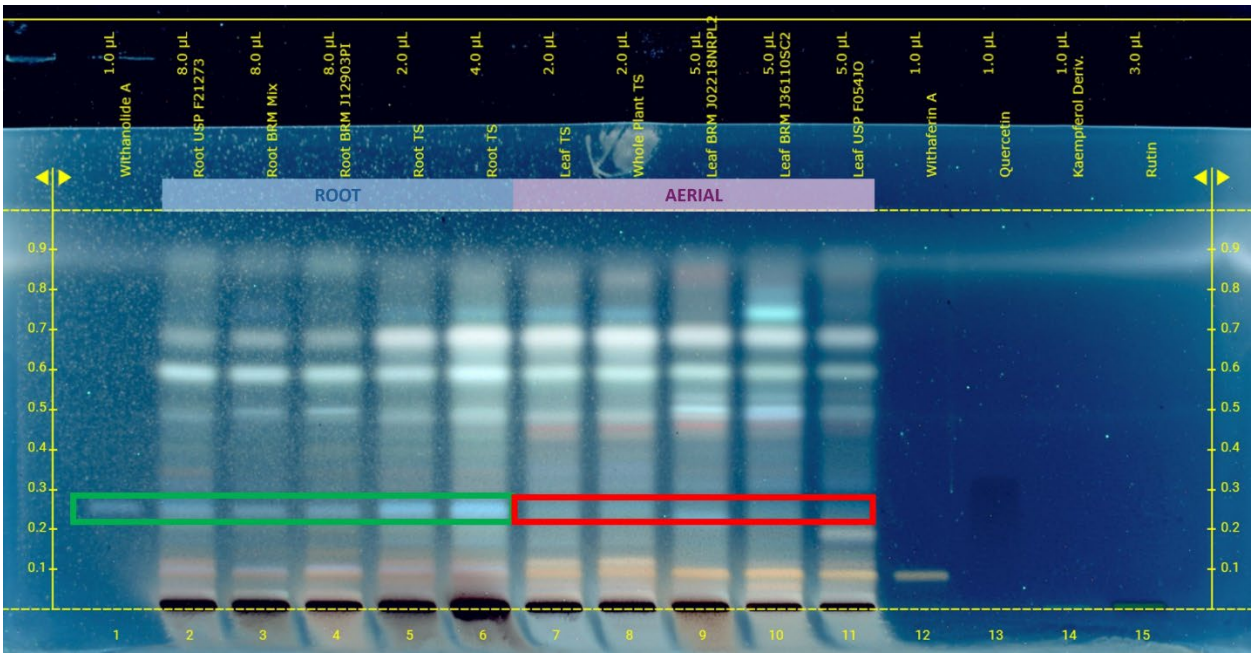


Image 3:

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

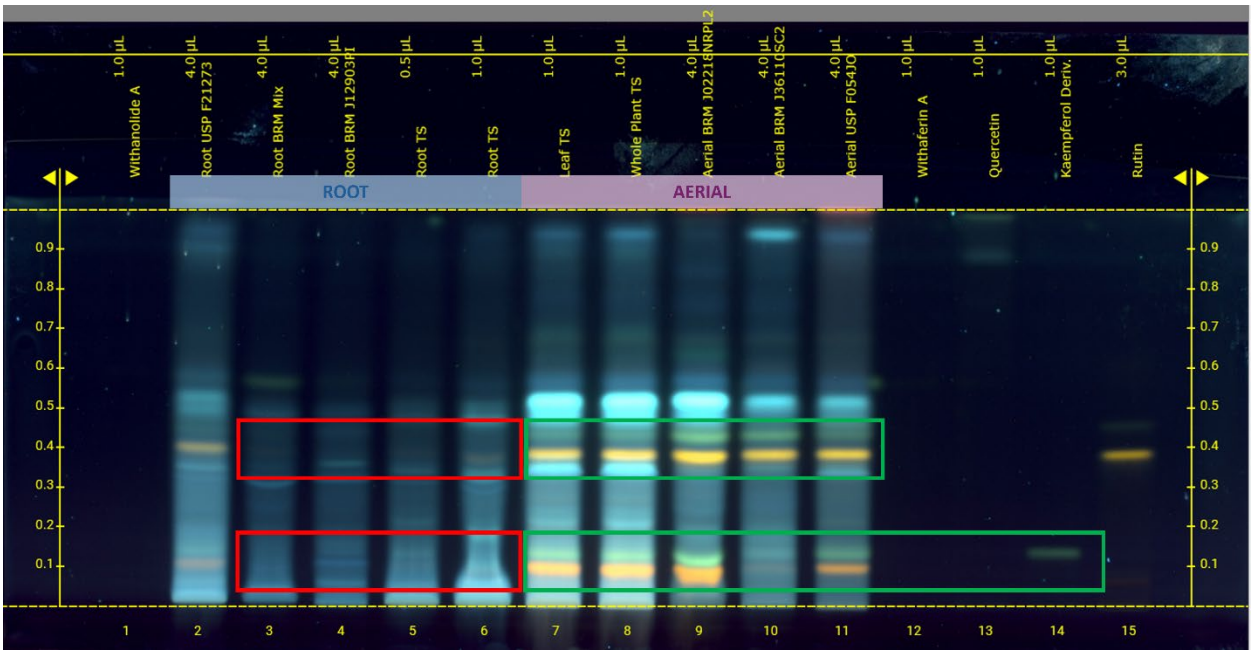
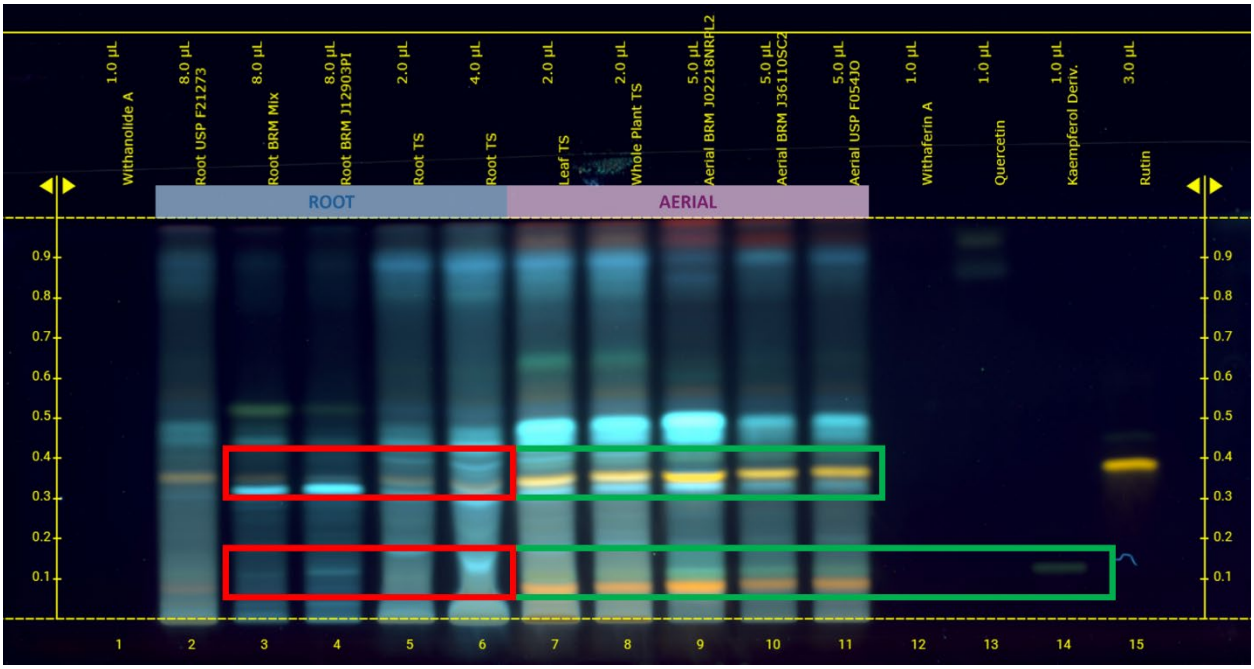


Image 4:

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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United States Pharmacopeia (2024). Dietary Supplement Monographs, Powdered Ashwagandha Root. USP-NF. Rockville, MD: United States Pharmacopeia.
DOI: https://doi.org/10.31003/USPNF_M2788_02_01
Doc ID: GUID-43057A36-34DF-4B91-9458-BF4CA58205D4_2_en-US

United States Pharmacopeia (2024). Dietary Supplement Monographs, Powdered Ashwagandha Root Extract. USP-NF. Rockville, MD: United States Pharmacopeia.
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-

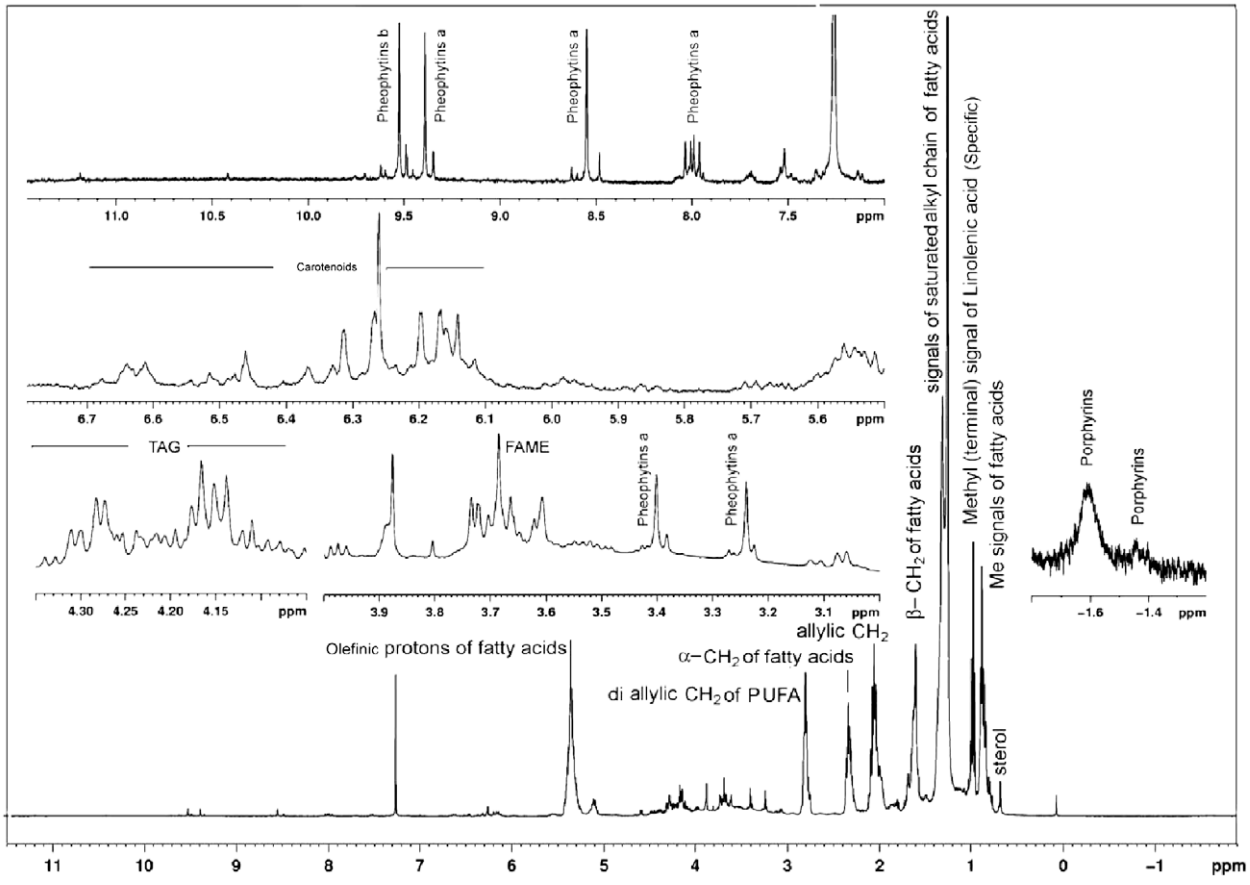

Fig. 1. ^{1}H NMR spectra of the hexane extract of *W. somnifera* leaves.

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.

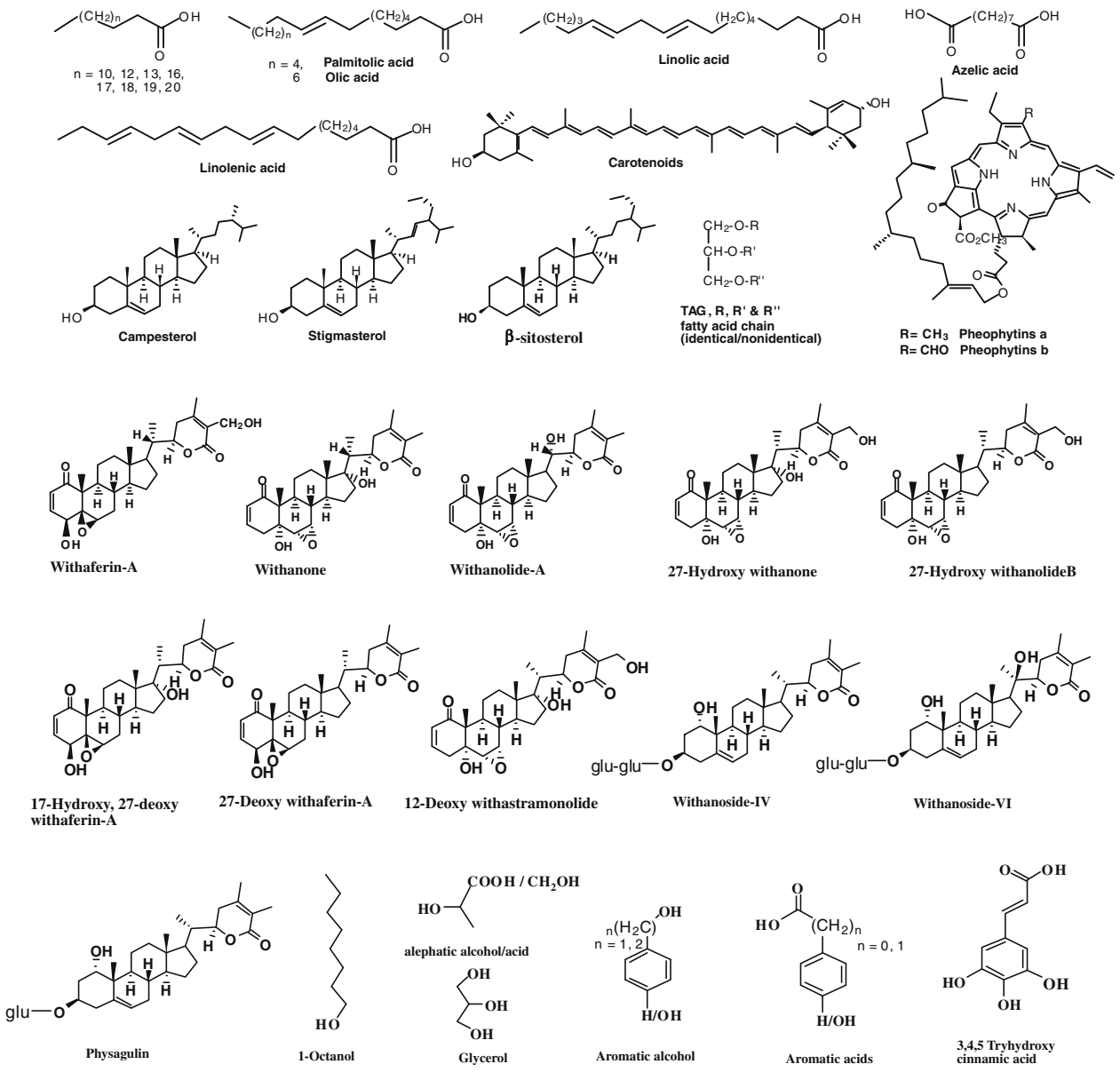

Fig. 2. Some of the phytochemicals identified in *W. sominfera*.

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

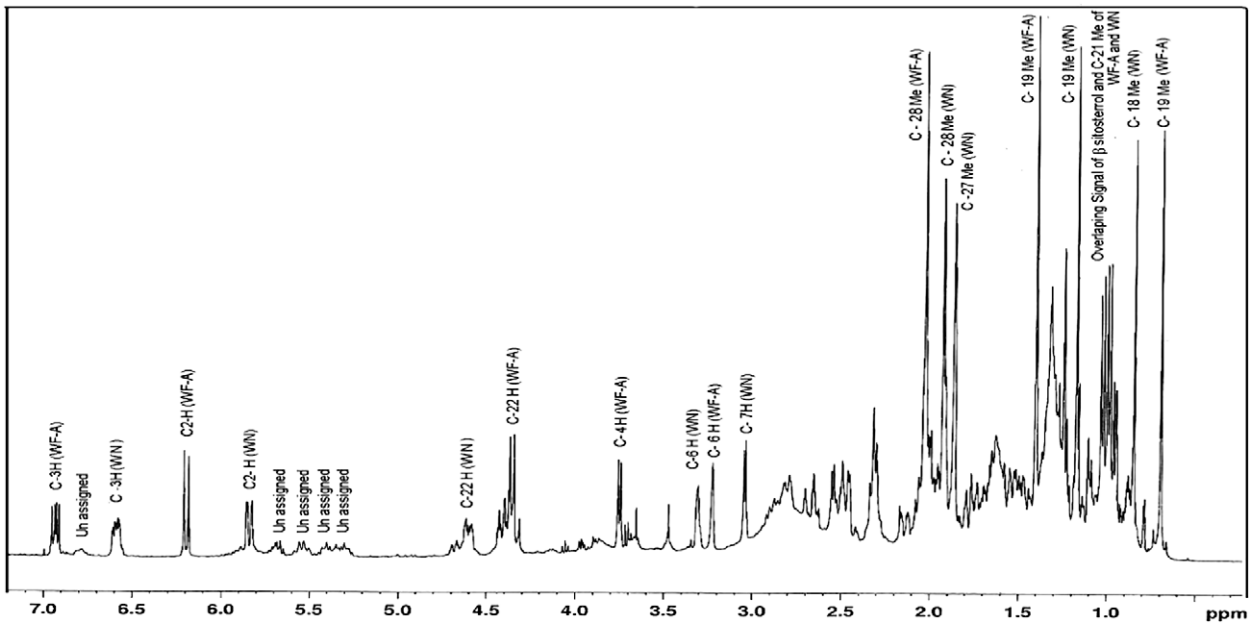

Fig. 3. ^{1}H NMR spectrum of the $CHCl_3$ partition of the *W. somnifera* leaves. WF-A represents withferin-A and WN represent withanone.

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.

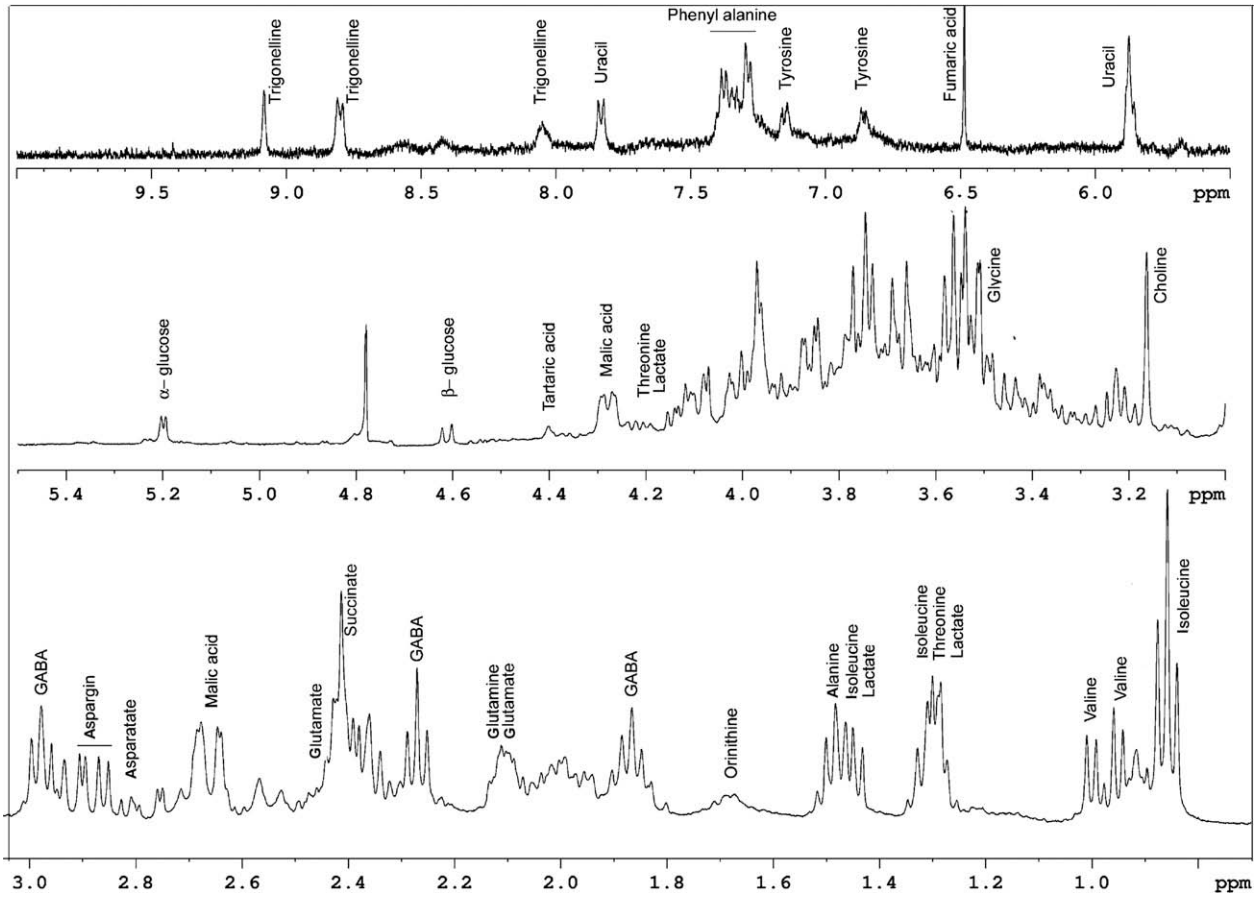

Fig. 4. ^{1}H NMR spectrum of the aqueous fraction of *W. somnifera* leaves.

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]

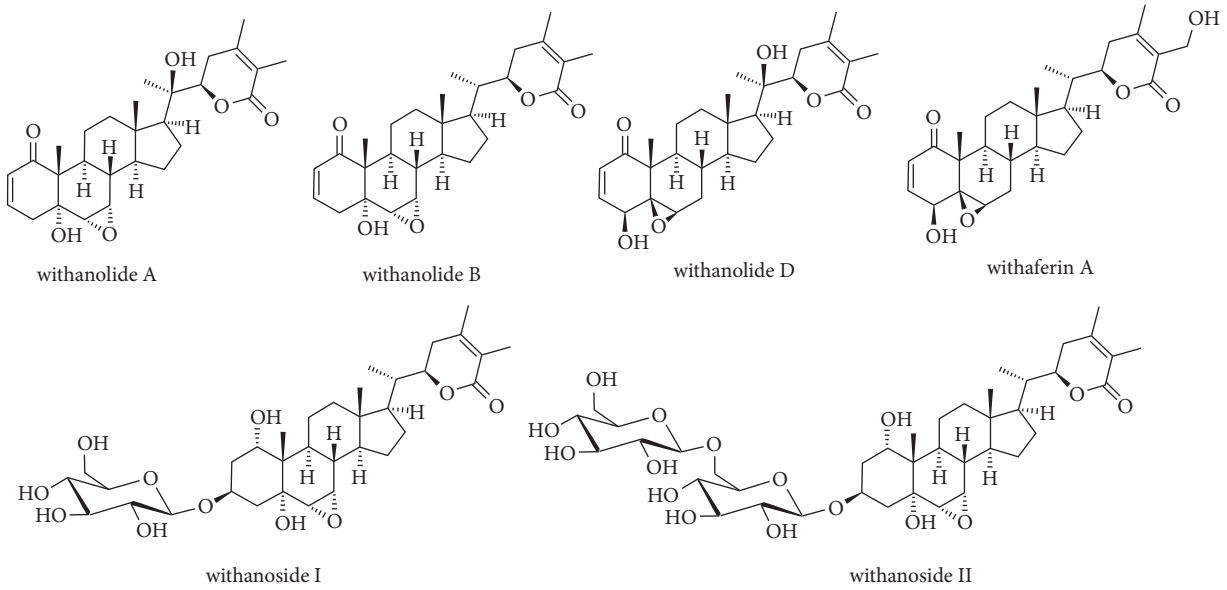

Figure 2: Chemical structures of some withanolide derivatives isolated from *Withania somnifera*.

(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

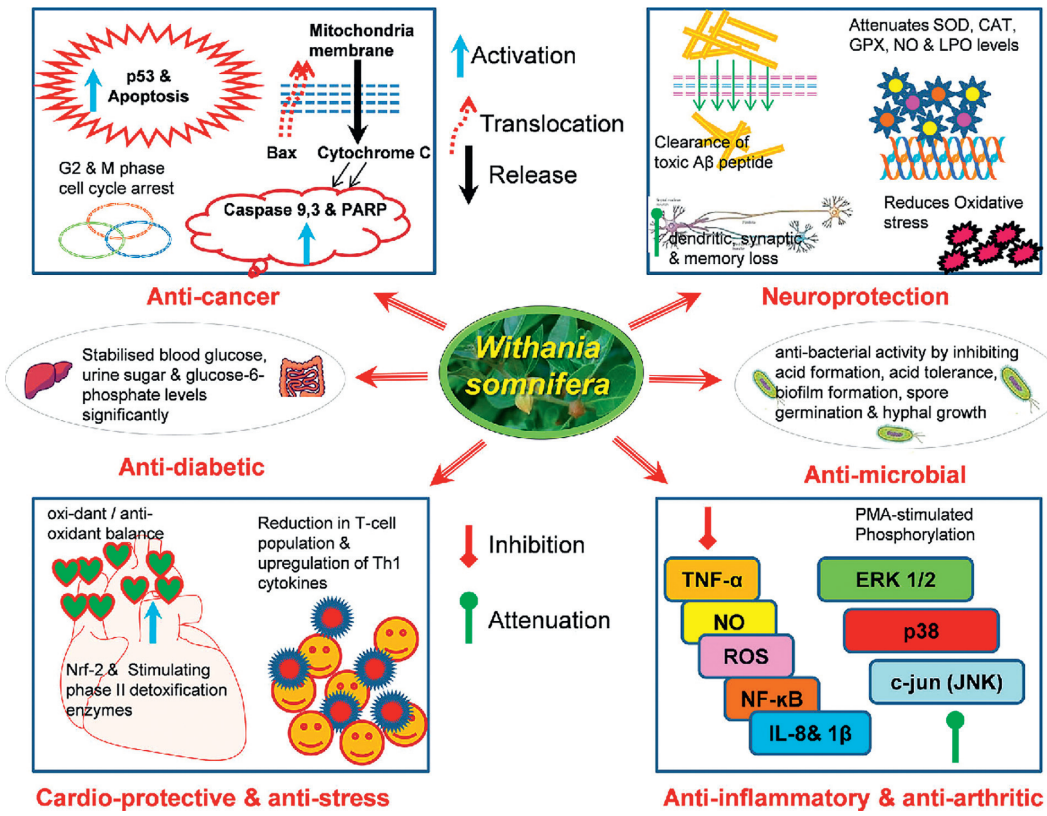

FIGURE 3: Major biological activities of *Withania somnifera*. Anticancer effects: *W. somnifera* exerts anticancer effects via multiple pathways, including nuclear factor (NFK-beta) and signal transducer and activator of transcription 3 (STAT3) signaling, PI3K (phosphoinositide 3-kinase)/AKT (a serine-threonine 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).

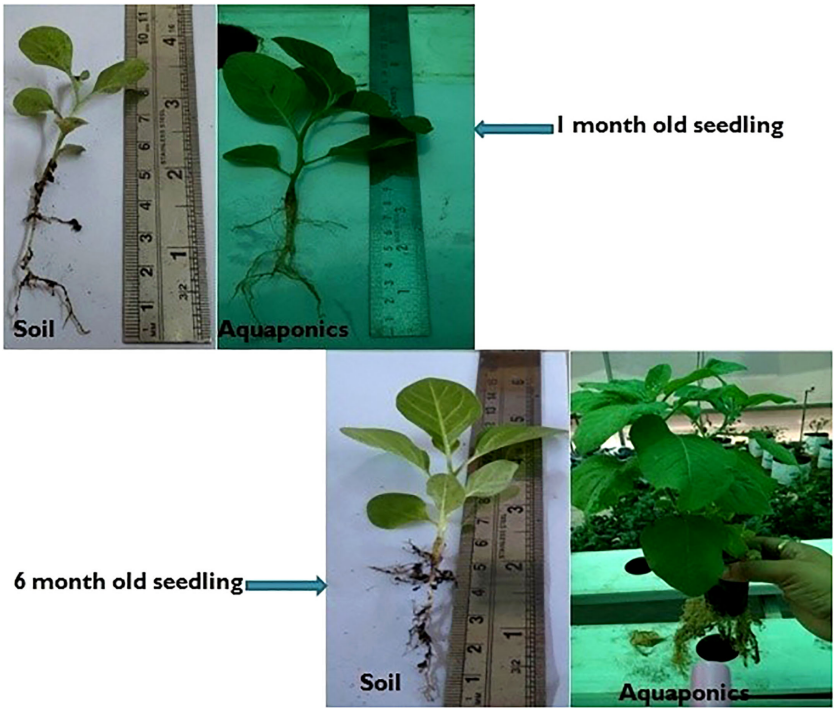

FIGURE 2
Comparison of seedlings of *Withania somnifera* grown in (i) Soil (ii) Aquaponics.

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).

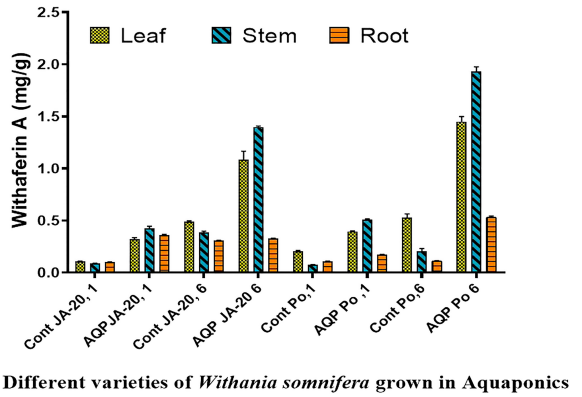

FIGURE 3
Withaferin A, content in the two varieties of *Withania somnifera* (Jawahar-20 and Poshita) grown in aquaponics (where Cont JA-20, 1 = 1 month Jawahar seedling grown in soil; AQP JA-20, 1 = 1 month Jawahar seedling grown in aquaponics; Cont JA-20,6 = 6 month Jawahar seedling grown in soil; AQP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

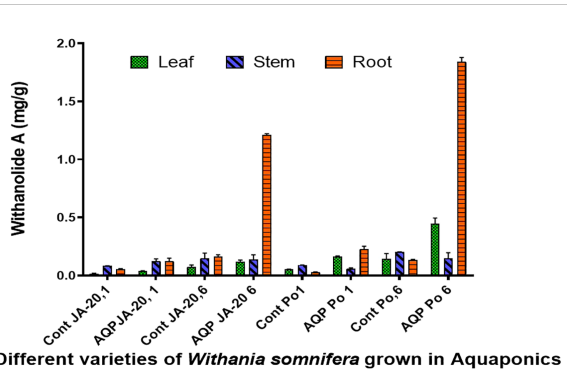

FIGURE 4
Withanolide A contents in Jawahar-20 and Poshita varieties of *Withania somnifera grown* in aquaponics (where Cont JA-20, 1 = 1 month Jawahar seedling grown in soil; AQP JA-20,1 = 1 month Jawahar seedling grown in aquaponics; Cont JA-20,6 = 6 month Jawahar seedling grown in soil; AQP JA-20,6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