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Chemical, microbial and safety profiling of a standardized *Withania somnifera* (Ashwagandha) extract and Withaferin A, a potent novel phytotherapeutic of the millennium

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ABSTRACT

Background: *Withania somnifera* (L.) Dunal, popularly known as Ashwagandha, is an ethnomedicinal plant with multiple pharmacotherapeutic applications. The diverse medicinal properties of the plant are largely due to the presence of withanolides, a group of C28 ergostane based steroidal lactones, with several sites of unsaturation and oxygenation. Withaferin A, a major withanolide present in Ashwagandha plant accounts for its emerging new roles to treat cancer, arthritis, inflammatory responses, immunomodulatory properties, and neuronal disorders. The root and leaf extracts are specifically important constituent materials for the development of phytotherapeutics, mostly intended for oral consumption. Several studies have been carried out to delineate the toxic manifestations of the extract for human consumption.

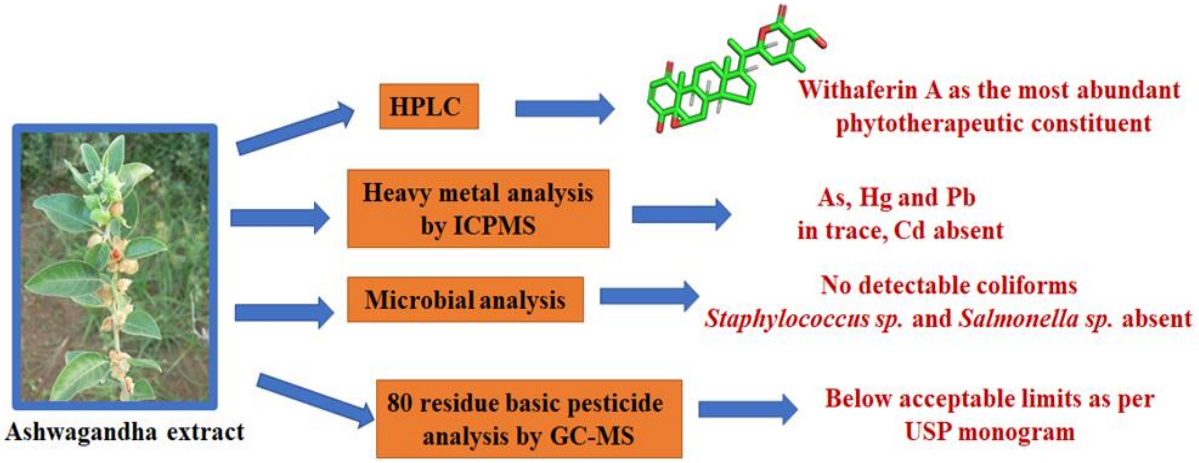
Objective: Establish the broad-spectrum safety of W-ferinAmax ashwagandha (WFA).

Study: This investigation demonstrated a novel, standardized W-ferinAmax ashwagandha (WFA) extraction technology from the whole herb of *Withania sominfera,* conducted HPLC analysis to identify the constituents, detected the heavy metals, microbiological contaminants, pesticides contaminants, and safety profile.

Results: A novel extraction technology was employed to obtain WFA from the whole plant of *Withania sominfera.* HPLC analysis revealed that WFA contains a total of 15.4% Withanolides. In particular, Withaferin A, Withanoside IV, and Withanolide A contents were 6.469%, 1.022%, and 0.073%, respectively. The extract contained only 0.403 ppm of heavy metals out of which traces of arsenic, mercury and lead were detected, and cadmium was absent. USP recommended 80 residue basic pesticide screening indicated that the extraction was safe for human consumption. It was also found to be free from pathogenic microbes as assessed by the absence of *E. coli* and other coliforms, *Salmonella* and *Staphylococcus* species.

Conclusion: The data generated cumulatively indicated that WFA is safe for further downstream processing and for human consumption.

Keywords: Ashwagandha, Withaferin A, phytotherapeutics, material safety; heavy metals; pesticides



INTRODUCTION

The use of *Withania somnifera* (also called Ashwagandha or Indian winter cherry) as an herbal medicine for the treatment of multiple ailments related to regulation of blood sugar, cardiac health, reduction of inflammation and pain, management of stress and improvement of cognitive function have been reported since ancient times especially in Indian subcontinent and southeast Asia [1-3]. The plant contains class of steroidal lactone metabolites known as withanolides, a C28 steroidal backbone with structural variations arising from differential oxidation at various sites [4-6]. Its main constituent, Withaferin A is implicated in plant defence against pathogens as well as protective drought or low temperature. Chemically, it is 5,6:22,26-diepoxyergosta-2,24-diene-1,26-dione substituted by hydroxy groups at

positions 4 and 27. Withaferin A has emerged as an effective therapeutic application and acts as an anti-cancer agent by promoting cellular apoptosis through a variety of mechanisms [7]. It is believed to be a potent inhibitor of angiogenesis [8]. Simultaneously, it also possesses strong immunomodulatory as well as cardio-protective activity [9,10]. Furthermore, it is being considered as a novel candidate for drug developments to treat neuronal disorders due to its ability to cross Blood-Brain Barrier [11]. Most of the immune-modulatory and anti-cancer activity of the compound is based on its inhibitory effect on NF-κβ mediated signalling pathway [12]. It also acts as an inhibitor of myocardial ischemia by upregulating AMP-Activated Protein Kinase-Dependent B-Cell Lymphoma2 signalling [10].

Many studies have been conducted earlier to assess the safety of Ashwagandha as a therapeutic for oral consumption and all of them have demonstrated that the plant is free from any toxic side effects in all practical dosages used thus far in various formulations. However, there have been some studies which have indicated that uncontrolled and high doses may cause bowel irritability, diarrhoea, and vomiting. Few reports are available on human trials, which indicated that *W. somnifera* may exhibit neurosedative effects and at times can also bring about acute respiratory distress causing a sharp fall in blood pressure and cardiac arrhythmia [13-16].

We developed W-ferinAmax ashwagandha (WFA), a methanol-water extract from the whole herb of Withania somnifera (Ashwagandha), in our laboratories and the total Withanolide content was found to 15.4%. HPLC analysis was conducted to detect total Withanolides, Withanoside IV, Withaferin A and Withanolide A. The bioaccumulation of heavy metals was also assesses using Shimadzu inductively coupled plasma mass spectrometry ICMPS-2030.

According to the guidelines issued by US Pharmacopeia (USP), any material of botanical origin meant for human consumption must be either completely free or contain trace pesticides within the specified and recommended tolerable limits. Accord-ingly, detection of 80-compound basic pesticide screening with GC/MS-MS was carried out, using a com-prehensive quantitative analysis following the AOAC Official Method 2007. Residual solvents were analyzed as per ICH/Food Industry guidelines.

MATERIALS AND METHODS

Extraction and manufacturing of W-ferinAmax ashwagandha (WFA) from the whole Ashwagandha (Withania somnifera) herb: The phytochemical auth-entication and verification of the *Withania somnifera* (L.) Dunal whole herbs was performed by the in-house herbalist/botanist with authentic herbarium samples. Furthermore, to revalidate, phytochemical profiling was done using an HPLC. *Withania somnifera* whole herbs were freshly harvested and dried under shade at 30°C to minimize the loss/degradation of the active constituents. Moisture content at this stage was found to be about 9% as analysed according to guidelines of USP 43. Thereafter, it was extracted using methanol: water in an NSF-GMP certified facility. Thereafter, the solvent was evaporated by fractional distillation and the extract was defatted with hexane. at a solvent to sample ratio of 1:10. Samples were stirred for 12 h and the hexane was removed by filtering, then replaced every 12 h for a total time of 48 h. Finally, it was concentrated, ground, and sieved to obtain a homogenous powder containing the active con-stituents. This was stored in a desiccated chamber until further analysis. Figure 1 demonstrates the flow chart of the manufacturing procedure.

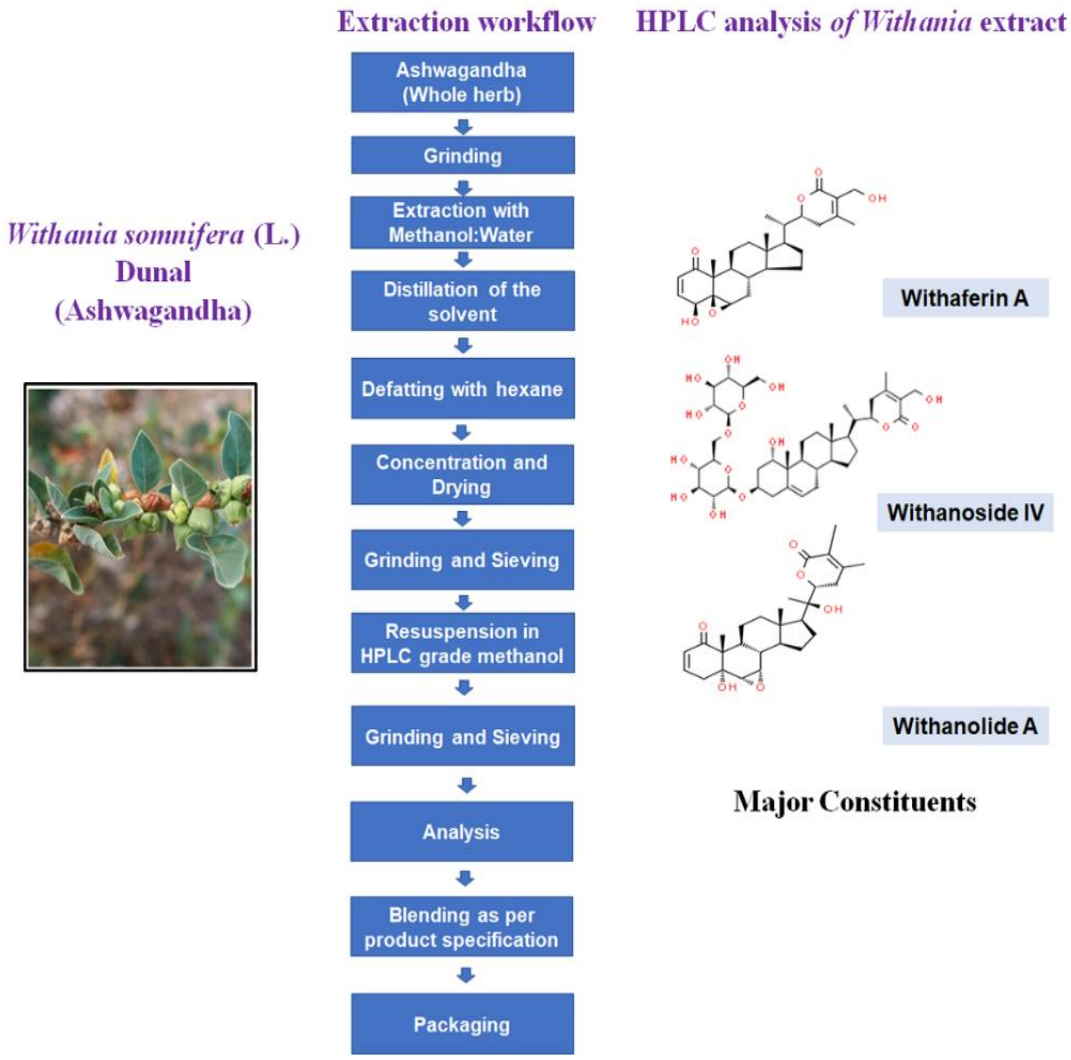


Figure 1: Major phyotherapeutic constituents of Withania somnifera (L.) Dunal (Ashwagandha). An extract from the whole herb was prepared as indicated in the flow chart before analysis by HPLC.

Analysis of W-ferinAmax ashwagandha (WFA) by HPLC: The recommended methodology of USP (USP 43, Ashwagandha/Dietary supplements) was used for the analysis of WFA by HPLC. The HPLC system (Shimadzu, LC-2010CHT /LC-2030 C) was equipped with a quaternary pump and vacuum degasser, temperature control column compartment, autosampler, and UV detector. Briefly, about 100 mg of WFA was dissolved in about 7 ml of HPLC grade methanol, heated gently in a water bath for 20 minutes, cooled and diluted with methanol. The HPLC was equipped with a reverse-phase L1 C18 HPLC column (4.6 mm x 25 cm, end capped; 5 µm packing LI) (Phenomenex Luna, Torrance, California, USA) at a flow rate of 1.5 ml/min and the column temperature was maintained at 27°C. The mobile phase consisted of Solution 1: Potassium dihydrogen phosphate (0.14 g) was dissolved in 990 ml of HPLC grade water, 0.5 ml of phosphoric acid was added, mixed well, and diluted with HPLC grade water to 1000 ml. The solution was filtered through a 0.45 µm membrane filter and degassed in a sonicator for 3 minutes; Solution 2: filtered and degassed HPLC grade acetonitrile solvent. The pumps were programmed to run a gradient of 95% Solution 1, 5% Solution 2 to 5% Solution 1, 95% Solution 2 over a period of 40 minutes (Table 1).

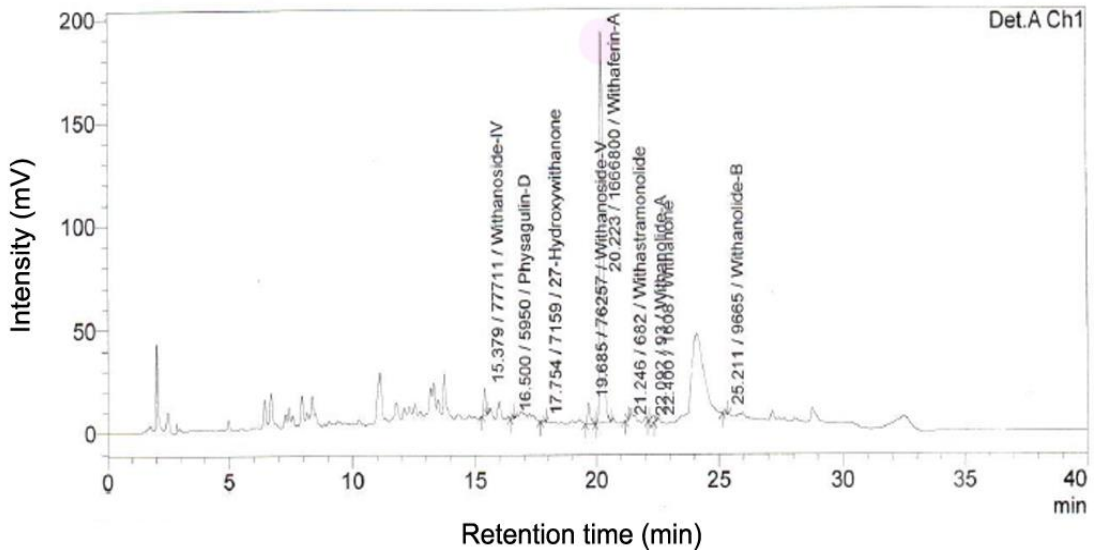


Figure 2: HPLC Chromatogram of W-ferinAmax ashwagandha, a powdered herb extract from Withania somnifera (L.) Dunal (Ashwagandha) showing Withaferin A as the major component (pink shaded) along with other withanolides, withanosides, withanones, withastramonolide and physagulin D.

Table 1. Chromatographic parameters

Mobile Phase	Solution 1: Potassium dihydrogen phosphate; Solution 2: Acetonitrile			
Column	Reverse-phase L1 C18 HPLC column (4.6 mm x 25 cm)			
Wavelength	227 nm			
Flow rate (ml/min)	1.5 ml/minute			
Gradient	Time (Min)	Flow (ml/min)	Mobile phase Solution A (%)	Mobile phase Solution B (%)
	0.0	1.5	95	5
	18.0	1.5	55	45
	25.0	1.5	20	80
	28.0	1.5	20	80
	30.0	1.5	95	5
	40.0	1.5	95	5
Injection Volume	20 µl			
Run Time	40 minutes			
Column Temperature	27°C			
Sample Temperature	Ambient			

Sample Solution: 100 mg of accurately weighed WFA was dissolved in 7 ml of HPLC grade methanol, heated gently in a water bath for 20 minutes, cooled and diluted with methanol. A 20 µl of aliquot of the sample was injected through a HPLC column (as described above) at a flow rate of 1.5 ml/min with a column temperature of 27°C in

a gradient of the two solutions (see gradient program as above) over a period of 40 minutes, using a UV detector at 227 nm.

Preparation of Standard Solutions: Stock solutions of all marker constituents were prepared at a concentration of 1.0 mg/mL in methanol. The calibration curves were prepared using solutions of different concentrations ranging from 10 to 550 μg/mL. Composite standard solution containing 0.1 mg/ml of USP Withanolide A RS and 0.1 mg/ml of USP Withanoside IV RS in HPLC grade methanol along with pure standard (1000 ppm) of Withaferin A (99.99% purity) and Withanolide A (99%) were also run parallelly under the same set of conditions.

The percentage of withanolide aglycones was calculated as:

- = Ru/Rs x Cs x (V/W) x 100
- Where Ru = sum of the peak areas of Withaferin A, 12-deoxywithastramonolide, Withanolide A, Withanone and Withanolide B from sample solution
- Rs = peak area of Withanolide A from standard solution
- Cs = Concentration of USP Withanolide A RS in standard solution (mg/ml)
- V = Volume of the sample solution in ml
- W = Weight of the Ashwagandha extract (in mg)

The percentage of withanolide glycosides in the Solution was calculated as:

- = Ru/Rs x Cs x (V/W) x 100
- Ru = sum of the peak areas of withanoside IV, withanoside V, and withanoside VI from Sample solution
- Rs = peak area of withanoside IV from Standard solution
- Cs =concentration of USP Withanoside IV RS in Standard solution (mg/mL)
- V = volume of the Sample solution (mL)
- W = Weight of the Ashwagandha extract (in mg)
- Add the percentages of withanolide aglycones and withanolide glycosides.

Methodology Validation: USP 43 Validation of Compendial Procedures was followed, and as outlined in the ICH guidelines, HPLC method was validated in terms of precision, accuracy, and linearity. All the assay method and procedure were repeated and evaluated in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were ascertained by injecting serial dilutions of solutions of the ISRN Analytical Chemistry 3-4 standards with known concentrations. The LOD and LOQ were calculated based on the signal-to-noise ratio of more than 3-4 times for LOD and 10-12 times for LOQ, respectively.

Heavy metal analysis of W-ferinAmax ashwagandha (WFA) using Shimadzu inductively-coupled-plasma-mass spectrometry (ICPMS): Analysis of heavy metals was carried out using a Shimadzu ICPMS-2030 inductively coupled plasma mass spectrometer (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) coupled with an auto sampler AS-10. Certified individual reference standards from Sigma Aldrich 1000 ppm were employed for making the stock solution and calibration. The parameters used are shown in Table 2. Approximately 1-2 gm of homogenized WFA was completely digested under pressure with 6 mL of nitric acid (HNO3) and 1 mL of perchloric acid ($HClO_4$). After subsequent cooling, they were filtered using a 0.45 µm filter (Whatman Millipore, Clifton, NJ, USA) and adjusted to 50 mL with 1% HNO_3. Lead (Pb), cadmium (Cd), arsenic (As), and mercury (Hg) were detected in the final solution.

Table 2. Instrument parameters for Shimadzu ICPMS 2030

Instrument	Shimadzu ICPMS 2030
Acquisition type	Quantitative
Quantitation method	Calibration curve method
Replicates	3
Profile integration time	Mid
Sweeps/Replicate	10
Solvent rinse (Low and High)	45 Sec
Sample rinse (Low and High)	45 Sec
Rotation speed (Low)	20 RPM
Rotation speed (High)	60 RPM
Integration mode	Auto

Basic pesticide screening in W-ferinAmax ashwagandha (WFA) using an Agilent 7000D Triple Quadrupole GC/MS System: Pesticide detection was carried out according to USP guidelines using an Agilent 7000D Triple Quadrupole GC/MS System (Agilent Technologies, Santa Clara, California, USA). This method is applicable to the multi-residue analysis of 80 pesticides in various foods and dietary supplements. Briefly, about 100 ml of 10% (w/v) WFA in acetone was prepared and allowed to stand for 20 minutes. To it, 1 ml of toluene solution containing 1.8 mg of carbophenothion was added and mixed thoroughly. The solution was subsequently filtered and washed twice with acetone. It was then dried using a rotary evaporator set below 40°C until complete evaporation of the solvent was attained. The evaporation was repeated after dissolving the residue in minimal volume of toluene, filtered, and finally diluted in toluene to 10.0 ml. For detection of Organochlorine, Organo-phosphorus, and Pyrethroid Insecticide, a size-exclusion chromatography using a 7.8- mm × 30-cm stainless steel column with 5-mm packing L21 was used along with toluene as the mobile phase at a flow rate of about 1 mL/min. The column was calibrated using test solutions of the pesticides prior to analyses. An alkali flame-ionisation detector was used, and hydrogen was used as the carrier gas. The temperature of the injection port temperature was maintained at 250°C and that of the detector was maintained at 275°C. The column temp-erature was programmed in the following way: 80°C for the first minute and then increased to 150°C at a rate of 30°C/min, maintained at 150°C for 3 min, then increased to 280°C at a rate of 4°C/min and maintained at this temperature for 1 min. Carbophenothion was used as the internal standard. A measured aliquot of the sample was injected subsequently and the chromatograms were recorded. The content of each pesticide was determined from the peak areas of each pesticide. Typical limit of quantification (LOQ) estimated during the method validation are at or below 0.01 mg/kg for the majority of the analytes.

Analysis of the microbial load in W-ferinAmax ashwagandha (WFA): Presence of microflora in WFA was ascertained according to USP guidelines for safety ana-

lyses of samples of botanical origin. A 10% (w/v) WFA solution was prepared in sterile potassium phosphate buffer (pH 7.2). Then the sample was diluted suitably with the same buffer and passed through membrane filters for transferring the microbes of the sample to the membrane. In order to obtain the Total Aerobic Microbial Count (TAMC) and the number of CFUs per gram or millilitre of the product, the membrane filter was placed aseptically to the surface of a Soybean-Casein Digest Agar (in three replicates) and incubated at 37°C for 48-72 hrs. Total combined yeasts and moulds count (TYMC) was also obtained in a similar manner using Sabouraud Dextrose Agar. In order to ascertain the anaerobic flora, the pour plate method was used. 1 ml volume (3 replicates for each dilution) was taken for each dilution over which molten Soybean-Casein Digest agar was poured and incubated at 37°C for 48-72 hrs. In order to detect coliforms, the same membrane filtration test was performed using filter pads saturated with lauryl sulphate Tryptose broth, a selective media used for detection of coliforms. Subsequently, the filter pads were transferred into the base of petri-dishes with a sterilised pad dispenser and incubated at 37°C for 24 hrs for enumeration of total coliforms. For specific detection of fecal coliforms (*E. coli*), the incubation temperature was set at 44.5°C. Lysine-Mannitol-Glycerol agar was used for detection of *Salmonella sp.* [17] whereas Tryptic Soy broth Agar was used to detect *Staphylococcus sp.* using the same technique [18].

Statistical analysis: The results are expressed as mean ± S.D. All the data were analyzed using one-way analysis of variance followed by Tukey's post-hoc test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

W-ferinAmax ashwagandha (WFA), a standardized *Withania somnifera* extract: The standardized W-ferinAmax ashwagandha (WFA), extracted from the whole Ashwagandha (Withania somnifera) herb, appeared as a brown-colored powder with a bulk density ranging between 0.5 to 0.75 gm/ml. It was subsequently dried and subjected to HPLC analysis for estimation of the major chemical constituents. Loss in mass after drying was not more than 6 percent, while the particle size (minimum 95% passes through 40 mesh), residual solvents (not more than 3000 ppm methanol and 290 ppm n-hexane) and elemental impurities (not more than 10 ppm) conforms to USP 43.

Determination of Total Withanolides, Withanoside IV, Withaferin A, and Withanolide A content in W-ferinAmax ashwagandha (WFA): The content of different withanolides in WFA was detected by HPLC analysis (Figure 2). Three significant peaks corresponding to Withanoside IV, Withaferin A and Withanolide A were identified according to their retention times (Fig 2). It was found that total Withanolide content in WFA was 15.4%, while the concentration of Withaferin A was 6.469%. Also, the Withanoside IV and Withanolide A contents were found to be 1.022% and 0.073%, respectively (Table 3).

Table 3. Key constituents in W-ferinAmax ashwagandha (WFA) as determined by HPLC

Constituents	Amount (%)
Total Withanolides	15.4%
Withanoside IV	1.022%
Withaferin A	6.469%
Withanolide A	0.073%

Assessment of W-ferinAmax ashwagandha (WFA) for heavy metal and pesticide screening: Leaching of heavy metals in the soil has increased unprecedentedly over the last few decades both due to natural as well as anthropogenic reasons. Consequently, their bioaccumulation in the plant body has also gone up to levels which are deemed unfit for subsequent human consumption. Even higher has been the threat posed by uncontrolled application of pesticides for which often there is little or no mitigation. Pesticides not only persist in the environment for longer times, but they are also biotransformed to other potentially more toxic forms [19] thus making them more difficult to contain. In order to carry out a quantitative investigation regarding the safety of Ashwagandha extracts with regards to heavy metals and commonly used pesticides as per guidelines of USP, their contents were determined in the Ashwagandha extract.

Arsenic, mercury and lead were detected in trace limits within permissible levels (Table 4) but no cadmium was found in WFA. Ingestion of heavy metals has been associated with numerous adverse effects. It is well acclaimed that long term exposure to Arsenic increases the risk of many diseases including skin cancer, hyperpigmentation and diabetes [20]. Mercury is a potent neurotoxin, and its commonest form methyl mercury is associated with impaired senses, muscle weakness and lack of coordinated muscle movement [21]. Prolonged exposure to Pb blocks heme synthesis and hence leads to severe anaemia, additionally lead poisoning also leads to neurological disorders like dementia [22]. The Environmental Protection Agency (EPA) has set the pesticide limits in Withaferin A as mentioned in the Code of Federal Regulations (40 CFR Part 180) or the Federal Register (FR). In case of pesticides where EPA approved tolerance level has not been indicated, the limit is calculated by the formula: Limits (mg/kg) = AME/100B where A is the acceptable daily intake (ADI), as published by FAO-WHO, in mg/kg of body weight; M is body weight, in kg (60 kg); and B is the daily dose of the article, in kg and E is the extraction factor of the preparation method, determined experimentally. The 80-compound basic pesticide screen carried out by GC analyses as recommended by USP guidelines, Environmental Protection Agency, showed that the pesticide traces found in the preparation were within the acceptable limit for most of the compounds screened except for Coumaphos, Demeton-S and Disulfoton (Table 5). All three of these are popularly used organo-thiophosphate pesticides with acute neurotoxicity. They are strong inhibitors of acetyl-choline esterase [23] activity leading to severe loss of neuronal coordination and death in extreme cases [24]. The gross absence of pesticides above significant detection limits indicated that the extract was safe to be developed as a potent Withaferin A enriched phytotherapeutic.

Table 4. Heavy metal analysis in *W-ferinAmax ashwagandha (WFA)*

Heavy metal	Results
Arsenic	0.1159 ppm
Cadmium	Not detected
Mercury	0.2020 ppm
Lead	0.0851 ppm
Total Heavy Metals	0.4030 ppm

As per USP 43 guidelines, the allowable limits for each of the above is not more than 10 ppm.

Table 5: Basic pesticide screen in Ashwagandha root extract

Name	Amount in mg/kg (Limit in mg/kg)	Name	Amount in mg/kg (Limit in mg/kg)
Aldrin	<0.010 (0.05)	Hexachlorocyclohexane, alpha- (alpha-BHC)	<0.010 (0.3)
Bromopropylate	<0.010 (3)		
Cadusafos	<0.010	HCH, beta- beta-BHC)	
		HCH, delta- (delta- BHC)	
Carbophenothion	<0.010 (0.8)	Heptachlor, Heptachlor exoepoxide	<0.010 (0.05)
Chlordane (cis+trans)	<0.010 (0.05)		
		Hexachlorobenzene	<0.010 (0.1)
Chlorfenvinphos (E- and Z-isomers)	<0.010 (0.5)	Lindan (gamma-HCH, gamma-BHC)	<0.010 (0.6)
Chlorpyrifos-ethyl	0.068 (0.2)	Malathion	<0.010 (1)
Chlorpyrifos-methyl	<0.010 (0.1)	Methacrifos	<0.010 (0.05)
Coumaphos	<0.010 (0.00025)	Mevinphos (E- and Z-isomers)	<0.010 (0.1 mg/cubic metre)
Cyfluthrin (sum of isomers)	<0.010 (0.1)	Methidathion	<0.010 (0.2)
Cyhalothrin, lambda-	<0.010 (0.1)	Methoxychlor	<0.010 (0.05)
Cypermethrin (sum of isomers)	0.11 (1)	Mirex	<0.010 (0.01)
(Chlorthal-dimethyl, DCPA)	<0.010 (0.01)	Oxadiazon	<0.010
dichlorodiphenyldichloroethane, p, p'- Dichlorodiphenyldichloroethylene, DDT, o, p'- DDT, p,p'-	<0.05 (1)	Parathion-methyl, Parathion	<0.010 (0.2)
		Pentachloranisole	<0.010 (0.01)
		Pendimethalin	<0.05 (0.1)
Deltamethrin (0.5 mg/kg)	<0.010	Pentachloroaniline	<0.010
Demeton-S	<0.010 (0.0003)	Pentachlorobenzene	<0.01(0.012)
Diazinon	<0.010 (0.5)	Permethrin (sum of isomers)	<0.010 (1)
Dichlofenthion	<0.010	Pentachlorothioanisole	<0.010
Dichlorvos	<0.010 (1)	Piperonyl butoxide	<0.010 (3)
Dicloran (DCNA)	<0.010	Phorate	<0.010
Dieldrin	<0.010 (0.005)	Phosalone	<0.010 (0.1)
Disulfoton	<0.010	Pirimiphos-ethyl	<0.010 (0.05)

Endosulfan I (alpha-isomer), Endosulfan II (beta-isomer), Endosulfan sulfate	<0.010 (3)	Procymidone	<0.010 (0.1)
		Pirimiphos-methyl	<0.010 (4)
		Propetamphos	<0.010
Endrin	<0.010 (0.05)	Profenofos	<0.010 (0.1)
EPN	<0.010 (0.5)	Propargite	<0.05
Ethion	<0.010 (2)	Propyzamide (Pronamide)	<0.010
Ethoprophos (Ethoprop)	<0.010 (0.035)	Prothiofos	<0.010 (0.05)
Fenchlorphos (Ronnel)	<0.010 (0.1)	Quinalphos	0.42 (0.05)
Fenitrothion	<0.010 (0.5)	Quintozene (Pentachloronitrobenzene)	<0.010 (1)
Fenpropathrin	<0.010 (0.03)	Tecnazene	<0.010 (0.05)
Fensulfothion	<0.010 (0.05)	Tetradifon	<0.010 (0.3)
Fenthion	<0.010 (0.05)	Vinclozolin	<0.010 (0.4)
Fenvalerate/Esfenvalerate (sum of isomer)	<0.010 (1.5)	Fluvalinate, tau- (sum of isomers)	<0.010 (0.5)
Fipronil	<0.0050	Fonofos	<0.010 (0.05)

The acceptable limits are as per USP monogram (Chapter 561) and InChem guideline.

Analysis of Microbial Load in W-ferinAmax ashwagandha (WFA): Herbal extracts are often contaminated by microbial growth which comes from adjoining rhizospheres of the plant. Although presence of microbes symbiotically boosts production of a number of secondary metabolites of commercial importance [25], in many cases they are also responsible for spoilage of downstream production processes with a subsequent hefty production cost. Therefore, it is a mandate for industries to have a prior assessment of microbial load in the raw materials. According to the recommendations of USP 43 for materials of botanical origin, total bacterial count should be within 10^4 CFU/gm, and the total combined mold and yeasts count should be within 10^3 CFU/g. In addition, the sample should be free from all kinds of coliforms including faecal coliforms which are the APHA certified indicator organism, Staphylococci which are also potential indicators for the presence of pathogens (National Research Council (US) Subcommittee on Microbiological Criteria) as well as Salmonella, the potent human pathogens [26].

Safety of Withaferin A and W-ferinAmax ashwagandha (WFA): Several scientists around the world have assessed the safety and toxicological profile of the Ashwagandha extract and its constituents. Sharma et al. [27] have demonstrated that oral administration of aqueous Ashwagandha extract (100 mg/kg/day) to rats over a period of eight consecutive months didn't exhibit any toxicity. Prabu et al. [28] have also demonstrated that oral administration of methanolic extract of Ashwagandha (2,000 mg/kg/day) over a period of 28 consecutive days induce no toxicity.

Singh et al. [29] have reported the oral acute LD_{50} of Withania somnifera to be 1750 mg/kg body weight in albino mice. Sharada et al. [30] have determined the acute toxicity in Swiss Albino mice and sub-acute toxicity in Wistar rats. No deaths were reported following administration of a single intraperitoneal administration of

1100 mg/kg body weight within 24 hr. The oral LD_{50} was reported to be 1260 mg/kg body weight. Oral administration of 100 mg Withania somnifera extract/kg body weight/day over a period of 30 consecutive days didn't cause any mortality or significant changes in peripheral blood constituents or other parameters [30]. In another independent settings, Gupta et al. [31] conducted extensive toxicological evaluation including both acute and sub-acute toxicity of oral Withaferin A in mice. These researchers reported that withaferin A exhibited no toxicity or death up to 2000 mg/kg body weight in the acute toxicity study. The sub-acute toxicity study was conducted in mice following oral administration of 10-, 70-, and 500 mg/kg body weight/day of Withaferin A over a period of 28 consecutive days. Extensive toxicological analyses including mortality rate, serum chemistry, physiological, and hematological assessment exhibited no signs of toxicity at all the dose levels used in this study. The authors reported that Withaferin A is extremely safe even at high doses and the No Observed Adverse Effect Level (NOAEL) of Withaferin A to be a minimum of 500 mg/kg body weight [31].

Withaferin A is the one of the most potent chemical constituents' responsible for a major part of Ashwagandha's therapeutic potential. It is believed to evolve as a promising anti-cancer drug in near future owing to the versatile structural modifications which can be performed on its side chain leading to significant enhancement of its pharmacological activity [32]. Several researchers have demonstrated that both Withaferin A and Withanolide D exhibit anti-tumor efficacy [33-35]. Vaishnavi et al. (2012) have demonstrated that Withaferin A exhibited cytotoxic activities in both normal and cancer cells [36].

Analysis of standardized W-ferinAmax ashwagandha (WFA) exhibited that the total Withanolide content is 15.4%, out of which Withaferin A, Withanoside A and Withanolide A contents are 6.469%, 1.02% and 0.073%, respectively (Table 3). Recommended daily dose of WFA is 500 mg/day, which contains approximately 15% of total Withanolide. Thus, our recommended daily dose of WFA is quite safe for human consumption and possess a huge span of margin of safety.

Discussion: The medicinal plant Ashwagandha, scientifically known as *Withania somnifera* Dunal (family Solanaceae) and commonly known as Indian ginseng or Winter cherry, is a stout herb that reaches a height of 170 cm and carries yellow flowers and berry-like red fruit. Indigenous and Ayurvedic medicine have recognized Ashwagandha for more than 3000 years [1-6]. The old elementary foundational scripts in Ayurveda, *Caraka (Charaka)* and Sushruta Samhitas describe Ashwagandha as a medicinal plant exhibiting the enhancement of multiple vital physiological functions including (a) muscle and physical strength, (b) vitality and vigour, (c) nourishing, (d) spermatogenic, (e) sexual and repro-ductive performance [2-4].

Ashwagandha is a reservoir of biologically active alkaloids (isopelletierine, anaferine, cuscohygrine, anahygrine, etc.), steroidal lactones (withanolides including Withaferin A, Withanoside IV, Withanolide A, and others), flavonoids, phenolic acids, alkaloids, saponins, tannins, saponins and volatile oils conferring a host of therapeutic potential [1,2]. These novel phytonutrients have exhibited an array of health benefits including immune enhancing, adoptogenic, anti-aging, anti-diabetic, anti-arthritic, anti-anxiety, hepato-protective and cardioprotective activities, simultaneously offering protection against various neuro-degenerative diseases, impotency, amnesia, and several other ailments [1-4]. However, breast-feeding, and pregnant women, and people with autoimmune diseases with rheumatoid arthritis, type 1 diabetes, and lupus, and Hashimoto's

thyroid should avoid Ashwagandha for their daily consumption [1-3,7-10,37].

It has also been recently demonstrated by independent research groups that Withaferin A can effectively bind to the Spike protein of SARS-COV-2 thus effectively sequestering it from binding to ACE-2 protein [32, 37, 38]. In the process, it also does not downregulate cellular ACE-2 expression, thus also being compatible with diabetic patients. Gupta et al. reported that Withaferin A, being the major constituent in Withania somnifera, also exhibits multiple therapeutic benefits including immune enhancing, vitality, and vigour, hepato-, cardio- and neuroprotective properties [32-34, 38,39]. As discussed earlier, Ashwagandha and its constituents has multiple health benefits.

Maharia et al. (2010) had earlier reported the toxic manifestation induced following bioaccumulation of heavy metals by Ashwagandha root [40]. Accordingly, the accumulation of toxic heavy metals including arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb) was critically investigated using an ICPMS. Our investigational analysis clearly demonstrated that the total heavy metal load in the extract is way less than permissible limit of 10 ppm, as specified in the USP 43.

Pesticides accumulate in plants through indiscriminate use in agricultural fields, so much that they are sometimes also significantly bioabsorbed by non-crop plants. Therefore, despite their indispensability for commercial production of food-crops and aesthetic plants, pesticides continue to pose a serious threat to human health because of their non-perishability in the ecosystem and bioconversion to other more toxic forms. The major chemical groups of pesticides include the organochlorines (DDT, Endrin, Chlordane etc.), Organophosphates (Trichlorfon, glyphosate etc.), Carbamates (Carbaryl, Carbofuran etc.) and Pyrethroids (Permethrin, chrysanthemic acid) [41]. Coumaphos is not a popular and widely used pesticide, however it is frequently used in apicultures to confer protection against ectoparasites of bees. Based on recent evaluation of pesticide traces in veterinary products, the revised Maximum Residue Limit of Coumaphos has been fixed at 0.1 ppm in honey [42] which is much higher than ADL of 0.00025 ppm as set by ESDA. Therefore, the toxicity associated with the consumption of traces of Coumaphos with the Withania extract powder will be negligible, considering the fact that the extract will not be consumed be further processed to develop therapeutic formulations.

The extract was also found to be grossly free from any residual solvents. In parallel, we conducted extensive microbiological testing to determine the total bacterial and fungal counts, total coliform and *Escherichia coli* (*E. coli*)*, Salmonella sp.,* and *Staphylococcus aureus* (*S. aureus*). The results showed that the total bacterial count is less than 10^4 cfu/g and the total fungal count is less than 10^3 cfu/g, while *E. coli, Salmonella, S. aureus, and Coliform* were altogether absent in the sample.

Safety and toxicity analyses are now mandated for any nutraceutical product development. Owing to its growing interest and importance in the phyto-pharmaceutical sector, many upcoming therapeutics are believed to be based on purified Withaferin A instead of incorporating the wholesome Ashwagandha extract. Therefore, the present studies carried out on safety assessment of standardized ashwagandha extract have been meant to provide important insights for designing further downstream purification of Withaferin A.

CONCLUSION

Taken together, *Withania sominfera* plant derived and standardized W-ferinAmax ashwagandha (WFA) contains a total of 15.4% Withanolides as demonstrated by HPLC. Specifically, Withaferin A, Withanoside IV, and Withanoline A contents in WFA were 6.469%, 1.022%, and 0.073%, respectively. Furthermore, WFA contains only 0.403 ppm of heavy metals out of which traces of arsenic,

mercury and lead were detected, and cadmium was absent, which complies with USP 43 guidelines. Microbiological screening exhibited the absence of *E. coli*, *Salmonella*, *S. aureus* and *Coliform* in WFA, while it complies with the total bacterial and fungal counts. Furthermore, USP 43 recommended 80 residue basic pesticide screening was conducted, which complies with as per USP 43 guidelines. No Observed Adverse Effect Level (NOAEL) for Withaferin A was reported to be a minimum of 500 mg/kg body weight. These studies demonstrated that a daily recommended dose of 500 mg W-ferinAmax ashwagandha (WFA), containing a total of approximately 15% of total Withanolide, is safe for further downstream processing into therapeutics or health supplements for human consumption.

Abbreviations: ACE-2, angiotensin converting enzyme 2; ADI, acceptable daily intake; AOAC, Association of Official Analytical Chemists; APHA, American Public Health Association; CFU, colony forming unit; FR, Federal Register; GC-MS – gas chromatography-mass spectrometry; EPA, environmental protection agency; HPLC, high performance liquid chromatography; LOD – limit of detection; LOQ – limit of quantification; NOAEL - no observed adverse effect level (NOAEL); ppm: parts per million; SARS-COV-2, Severe Acute Respiratory Syndrome Coronavirus 2; TAMC, total aerobic microbial count; TYMC, total combined yeasts and moulds count; USP, United States Pharmacopeia; WFA, W-ferinAmax ashwagandha

Competing interests: PK and AG are employees of Chemical Resources (CHERESO), the study sponsor. DB, SPB, SC, and MB are independent consultants who drafted the manuscript. All authors critically reviewed and approved the manuscript. All authors have declared that they have no other conflict of interest.

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