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Physicochemical Stability and Biological Activity of *Withania somnifera* Extract under Real-Time and Accelerated Storage Conditions

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Key words

- *Withania somnifera*
- Solanaceae
- Ayurveda
- ashwagandha
- stability
- withaferin A
- withanolide A

Abstract

▼ Stability testing at preformulation stages is a crucial part of drug development. We studied physicochemical stability and biological activity of *Withania somnifera* (ashwagandha) dried root aqueous extract during six months real-time and under accelerated storage conditions. The characteristic constituents of ashwagandha roots include withanolides such as withaferin A and withanolide A. We modified and validated the HPLC-DAD method for quantitative measurement of withanolides and fingerprint analysis. The results suggest a significant decline in withaferin A and withanolide A content under real and accelerated conditions. The HPLC fingerprint analysis showed significant changes in some peaks during real and accelerated storage (> 20%). We also observed incidences of clump formation and moisture sensitivity (> 10%) under real-time and accelerated storage conditions. These changes were concurrent with a significant decline in immunomodulatory activity ($p < 0.01$) during the third

month of the accelerated storage. Thus, adequate control of temperature and humidity is important for WSE containing formulations. This study may help in proposing suitable guidance for storage conditions and shelf life of ashwagandha formulations.

Abbreviations

▼

AL:	anhydrous lactose
MCC:	microcrystalline cellulose
RPA:	relative peak area
RRT:	relative retention time
TIC:	total ion chromatogram
t_R :	retention time
WSE:	spray dried <i>Withania somnifera</i> root aqueous extract
(θ):	angle of repose

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Introduction

▼ Stability testing has remained an important aspect of drug development as it affects quality, safety, and efficacy of the product. It is carried out at distinct phases including preformulation, formulation development, product development, and postmarketing. Stability testing, especially of botanical drugs, is challenging because of the inherent physicochemical complexity. Further, botanical drugs are now considered as active substances in their entirety and there may be challenges in the selection of the assay or of the parameters and protocols that profile their stability characteristics. The regulatory agencies such as the United States Food and Drug Administration and the European Agency for the Evaluation of

Medicinal Products (EMA) advocate that the stability of botanical materials generally should not be based entirely on the assay of the active constituents or characteristic markers. The EMA has released a reflection paper to highlight special problems of herbal medicinal products and approach differences between medicinal products containing chemically defined active substances. Various crucial analytical challenges, cost factors, and the selection of appropriate stability protocols at different stages of herbal drug development are discussed in this document [1]. Earlier studies have used fingerprinting to assess herbal preparations in stability testing [2]. Studies on the chemical stability of botanicals such as *Andrographis*, *Hypericum perforatum*, *Zingiber officinale* have also been reported; however, a physi-

cochemical stability study and its correlation to biological activity at a preformulation stage has been rarely attempted [3–5]. We hypothesize that detailed stability investigations on herbal extracts at preformulation stages will enable rational selection of stability-indicating parameters, better excipients, adequate dosage forms and container closure systems. This may help in determining most suitable storage conditions and shelf life. As a model of such investigation, we report here the physicochemical and biological stability profile of spray dried *Withania somnifera* root aqueous extract (WSE).

Withania somnifera (WS) (L.) Dunal, family Solanaceae, commonly known as winter cherry, Indian ginseng or ashwagandha is one of the popular herbs used in Ayurveda for therapeutic and health benefits and is mentioned in Indian and American herbal pharmacopeia [6]. Aqueous-alcoholic extracts of WS are reported to have adaptogenic, antioxidant, adjuvant, and immunomodulatory activities with therapeutic benefits in cancer, arthritis, immunopathological, and stress related conditions [7–10]. Ashwagandha contains steroidal compounds known as withanolides including withaferin A and withanolide A. These have been reported to be responsible for significant biological activities and are recommended as active markers for standardization [11–13]. Aqueous extract of WS is commercially available and is used either singly (Stresscom®) or in combination (Abana®, Mentat®, Fleximuv®) for various therapeutic indications. Many studies have reported chemical and biological properties of WS extracts; however, sufficient data on their stability is not available. We report here the effect of real and accelerated storage conditions on the physicochemical and biological stability of WSE. In the present work, flow properties, moisture content, withanolide content (withaferin A and withanolide A), HPLC-DAD fingerprint, and the *in vivo* immunomodulatory activity of WSE were determined at monthly intervals under real and accelerated storage conditions for six months.

Materials and Methods

Test material

Withania somnifera (Linn. Dunal, Solanaceae) dried roots were procured from market, powdered and used as a herbal drug (HD) in this study. The voucher sample of HD was registered and certified as authentic by the National Institute of Science Communication and Information Resources, New Delhi, India (vide NISCAIR/RHM/F-3/2003/413). Aqueous decoction of HD was prepared as per method described in the Ayurvedic Pharmacopoeia of India. The extract was spray dried as free flowing dark brown material (WSE) and was considered as a herbal drug preparation as per EMEA guidance. WSE preparation was ensured to be free from pathogens, aflatoxins, pesticide residues, and heavy metals to meet W.H.O. guidelines of purity and safety.

WSE samples were stored for six months in double polyethylene bags (transparent, food grade, conventionally used as packing material) in different calibrated chambers maintained under test conditions of $30 \pm 2^\circ\text{C}$ and $65 \pm 5\%$ relative humidity (RH) for the real-time testing and $40 \pm 2^\circ\text{C}$ with $75 \pm 5\%$ RH for the accelerated testing. These were the test conditions as per ICH recommendations on climatic zones III and IV [14]. WSE samples were withdrawn at monthly intervals and were examined for physical, pharmaceutical, chemical, and biological stability.

Chemicals

Methanol and 2- propanol (Merck), ethanol (BDH), withaferin A (purity 99.62%, Cat. no. 00023250), withanolide A (purity 98.43%, Cat. no. 00023251; Chromadex). Their purity was confirmed using HPLC. Levamisole (purity 99%; Sigma), microcrystalline cellulose (purity 99%, Avicel PH102), and anhydrous lactose (purity 99%, AL) were gift samples from Emcure Pharmaceuticals.

Physicochemical and pharmaceutical stability

Moisture content of WSE samples withdrawn at different intervals was determined by Karl Fischer titration method-A as per the Indian Pharmacopoeia. The size distribution of the dried product was determined by optical microscopy and the image analysis was made using a stereo microscope (Stemi 2000-C; Carl Zeiss) connected to an analogue camera. The images obtained were analyzed with Biovis Image Plus software (Expert Tech Vision). The angle of repose (θ) was determined by the fixed funnel method reported earlier [15]. The bulk and tapped densities were determined using the Density Tester USP (Model ETD 1020; Electro Lab) and the method I as per the United States Pharmacopoeia. The measurements were done in triplicate and data was further processed to obtain Carr's Compressibility using the following formula:

$$\text{Carr's compressibility index} = \frac{[(\text{Tapped Density} - \text{Bulk Density}) / \text{Tapped Density}] \times 100}{}$$

The chemical stability of WSE was evaluated using a modified reported High Pressure Liquid Chromatography – Diode Array Detector (HPLC-DAD) based method for detection of withanolides [16]. The HPLC system consisted of a modular Dionex instrument equipped vacuum degasser, quaternary pump (P680), autosampler (ASI-100), thermostatted column compartment (TSC-100), and a photodiode array detector (340 U). The data was recorded and processed by Chromeleon 6.70 software from Dionex Softron GmbH.

Separations were carried out using BDS Hypersil C-18 column (particle size, 5 μm ; $250 \times 4.6 \text{ mm}$; Thermolectron Corporation) with a guard column C18 BDS (particle size, 5 μm ; $50 \times 4.6 \text{ mm}$; Thermolectron Corporation) maintained at 50°C . The mobile phase consisted of water (A) and a mixture of methanol and reagent alcohol (B) in the ratio of 1 : 1. The reagent alcohol consists of ethanol, methanol, and 2- propanol in the ratio of 90.6: 4.5: 4.9. The analysis was done using gradient elution as 60A/40B to 55A/45B during the run time of 25 min. Each run was followed by a 5-min wash with 100 B and an equilibration period of 10 min. Flow rate was kept at 1 mL/min. WSE (4%) and marker compounds (0.1%) solutions in methanol were sonicated and filtered through a 0.2 μm membrane filter (Pall Corporation) prior to analysis. The volume of injection was optimized at 10 μL , and separations were monitored at 230 nm.

LC-MS analysis was performed using electrospray ionization on an API-2000 (Applied Biosciences/MDS SCIEX) equipped with triple quadrupole analyzer in the positive ion mode. For better LC-MS analysis, gradient conditions were modified as 65A/35B to 55A/45B in 25 minutes. The ionization voltage and temperature was kept at 4000 V and 450°C , respectively. The other conditions were: declustering potential 47 V, focusing potential 320, and exit potential 10. Scanning range was kept from m/z 100–1200 atomic mass units. A total ion chromatogram was obtained

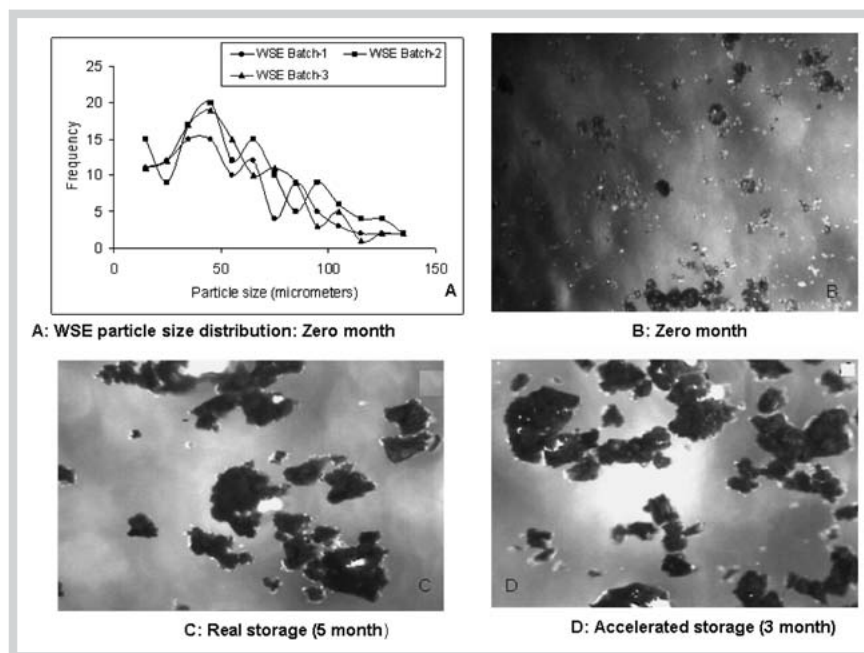


Fig. 1 WSE was studied for particle size distribution. Panel **A** represents the multimodal particle size distribution of three batches of WSE at zero month of storage. Panels **B**, **C**, and **D** represent optical microscope images of WSE on months 0, 5, and 3 of real and accelerated storage, respectively.

in Q3 scan mode. The data acquisition was done using Analyst 1.4.2 software (MDS SCIEX).

Validation

Withaferin A and withanolide A were used as external reference standards. The method was validated for linearity, limit of detection (LOD) and quantitation (LOQ), and precision. The calibration curves of withaferin A and withanolide A covered seven different concentrations ranging from 1–100 µg/mL. WSE samples were assayed for withaferin A and withanolide A content (µg/mL) for intra- ($n = 15$) and inter-day ($n = 5$) precision, respectively. The % RSDs were considered as a measure of precision.

Validation was carried out using reported methodology [17]. The fingerprint was validated on the basis of intermediate precision and repeatability of relative peak areas (RPA) and relative retention times (RRT) of selected peaks in the chromatograms. The similarity of month wise fingerprints was compared using Pearson sample correlation coefficients [18].

Biological activity

Balb/C mice of either sex, 10–12 weeks old were randomly distributed in groups as per experimental protocols ($n = 6$). Animals were housed and maintained following standard guidance as found in the Government of India guidelines. The study protocol was approved by the Institutional Animal Ethics Committee of the Indian Drugs Research Association and Laboratory, Pune, India. Each mouse received 1×10^9 sheep red blood cells (SRBC) in a volume of 0.2 mL i.p. for sensitization (day 0) and booster (day 6) at the required time schedule.

Immunomodulatory activity was assessed at the months 0, 3 and 6 of the study. Levamisole was used as a reference drug. The following basal group configurations were maintained during the study: vehicle control (PBS); positive control, levamisole at 2.5 mg/kg/b.w/p.o.; and test group WSE samples at 100 mg/kg/b.w/p.o. at the respective time point. The study was carried out using the previously reported protocol. Each animal received 1×10^9 SRBC cells/0.2 mL/i.p. for sensitization (day 0) and booster (day 6) at the required time schedule. Animals in the respective

groups received saline, levamisole, or WSE from 0–6 days, respectively. On day 8, sera were collected from all groups for the estimation of SRBC specific antibody titers using the Nelson method [19]. The highest serum dilution causing visible hemagglutination was shown as a titer. Bovine serum albumin in saline served as a control. The moisture content of each sample during the respective time point was taken into consideration before preparing the test doses.

Data analysis

Marker content in terms of mg/100 mg of WSE was calculated using calibration curves. These values were further converted into residual percentages where zero-month values were considered as 100%. Antibody titers in control, reference, and test groups were compared using Dunnett's test for multiple comparisons. The level of significance was considered as $p < 0.05$. The percent immunomodulatory activity was derived using the method of Kaul et al. and the formula (Test group – sensitized control group/sensitized control group) $\times 100$ [20].

Supporting information

Total ion chromatograms (TIC) of marker compounds and WSE are provided in **Fig. 15** as Supporting Information.

Results

Three batches of WSE were analyzed for particle size distribution studies using optical microscopy at month zero. WSE showed a multimodal distribution with particle size range from 20–140 µm (◉ **Fig. 1**). The angle of repose $< 30^\circ$ indicates free flowing material and $> 40^\circ$, poor flow properties [21]. The results suggest values for angle of repose at 30.14° , 39° , and 38.63° for WSE (month 0), AL, and MCC, respectively. The Carr compressibility index (%) < 10 indicates excellent and > 38 poor flow properties. Compressibility index (%) for WSE (29.78), AL (16.69), and MCC (8.09) suggested good flow properties at month zero. A significant physical change from powder form to clump was observed

Table 1 Effect of real-time and accelerated storage condition on physical and pharmaceutical properties of WSE.

Month	Physical form	Bulk density (gm/cm ³)	Tapped density (gm/cm ³)	Carr's index (%)	Angle of repose (θ)	Moisture content (w/w)
Real storage						
0	free	0.3322 ± 0.0021	0.4721 ± 0.0053	29.78	30.1432 ± 0.5023	4.5723 ± 0.0522
1	free	0.3513 ± 0.0032	0.4843 ± 0.0072	29.05	29.5443 ± 0.5542	6.4923 ± 0.0673
2	free	0.3644 ± 0.0024	0.4932 ± 0.0062	28.31	29.2534 ± 0.7532	6.5342 ± 0.0741
3	free	0.3746 ± 0.0043	0.5034 ± 0.0073	27.25	29.0321 ± 0.6024	7.3342 ± 0.0641
4	free	0.3837 ± 0.0024	0.5134 ± 0.0062	26.81	28.5432 ± 0.6531	7.7324 ± 0.0773
5	clumps	NA	NA	NA	NA	8.5342 ± 0.0664
6	clumps	NA	NA	NA	NA	9.5433 ± 0.0852
Accelerated storage						
0	free	0.3322 ± 0.0021	0.4721 ± 0.0053	29.78	30.1432 ± 0.5253	4.5723 ± 0.0522
1	free	0.3542 ± 0.0033	0.4843 ± 0.0074	29.05	28.5642 ± 0.5532	7.0635 ± 0.064
2	free	0.3732 ± 0.0042	0.5043 ± 0.0073	27.25	29.0234 ± 0.6331	7.3343 ± 0.0634
3	clumps	NA	NA	NA	NA	9.5106 ± 0.0573
4	clumps	NA	NA	NA	NA	10.7423 ± 0.0854
5	cake	NA	NA	NA	NA	11.9026 ± 0.0864
6	cake	NA	NA	NA	NA	12.1425 ± 0.0943

WSE 0–6 represents the real and accelerated storage samples tested at monthly intervals from 0 to 6 months. Carr index, angle of repose and densities were calculated using standard formulae mentioned in *Material and Methods*. All values are mean ± S.D. NA: not applicable due to change in physical form

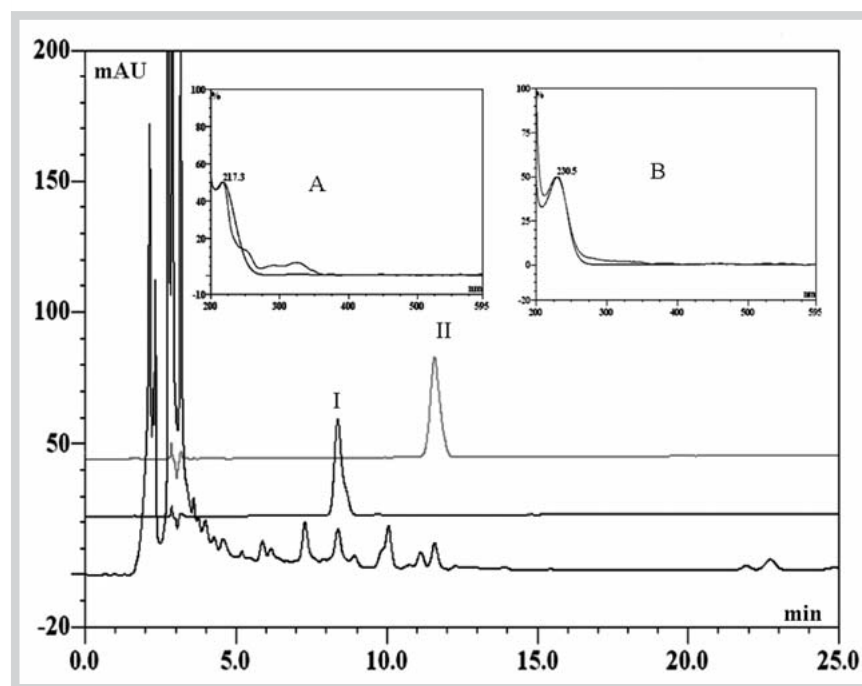


Fig. 2 HPLC chromatogram of WSE showing the presence of marker compounds withaferin A (I; retention time, t_R 8.25 mins) and withanolide A (II; t_R 11.59 mins) as confirmed by retention time and spectral overlays. Windows A and B show spectral overlays of markers I (λ max 217.3 nm) and II (λ max 230.5 nm) with corresponding peaks in WSE, respectively.

at month five of real-time and month three of accelerated storage. Additionally, the moisture content was increased during real and accelerated storage, respectively (► **Table 1**).

Chemical stability of WSE was examined using a previously reported modified HPLC method [16]. The solvent gradient was modified as 60A/40B to 55A/45B during a run time of 25 min to improve resolution of peaks in our experimental conditions. Withaferin A and withanolide A were identified using spectral overlays of reference standards and WSE samples at retention times (t_R) of 8.25 and 11.59 minutes, respectively (► **Fig. 2**). The presence of these markers was further confirmed by LC-MS analysis. Total ion chromatograms of WSE and reference standards as shown as Supporting Information confirmed the presence of withaferin A and withanolide A (**Fig. 1S**). The mass spectrum

showed signals at m/z of 471.2 [M+ H]⁺ and 493.2 [M+ Na]⁺ and signal at m/z of 488.2 [M+ NH₄]⁺, 493.2 [M+ Na]⁺, and 413.2 for withaferin A and withanolide A, respectively (**Fig. 1S**). The modified assay showed excellent linearity for withaferin A ($r^2 = 0.9991$) and withanolide A ($r^2 = 0.9992$) over the concentration range of 10 to 100 $\mu\text{g/mL}$. Other validation parameters for the marker content are shown in ► **Table 2**. The method was also validated for fingerprint analysis following Xie et al. [22]. The fingerprint region from t_R 5 to 24 min was selected due to peaks with good resolution and reproducibility as seen in ► **Fig. 3**. The selected region and eight selected peaks as 1–8 in the chromatogram are also shown in this figure. Withanolide-A peak was chosen as a reference because of its high and stable content. This method conforms to other validation parameters and supports

Table 2 Method validation: marker content.

Parameter	r^2	Linearity equation	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)	Precision			
					Intraday	Inter-day		
Withaferin A	0.9991	$y = 0.222x - 0.487$	1.0	3	458.28 (1.08)	457.18 (1.19) Day 1	458.62 (2.38) Day 2	453.41 (4.07) Day 3
Withanolide A	0.9992	$y = 0.185x - 1.388$	5.0	15.0	478.41 (1.91)	483.70 (1.13) Day 1	479.91 (2.28) Day 2	475.41 (3.98) Day 3

The adapted HPLC-DAD method was validated following ICH guidance. y : Peak area, x = concentration ($\mu\text{g/mL}$), r^2 : correlation coefficient, LOD: limit of detection, LOQ: limit of quantitation. Values in parentheses under precision indicate relative standard deviations (RSDs)

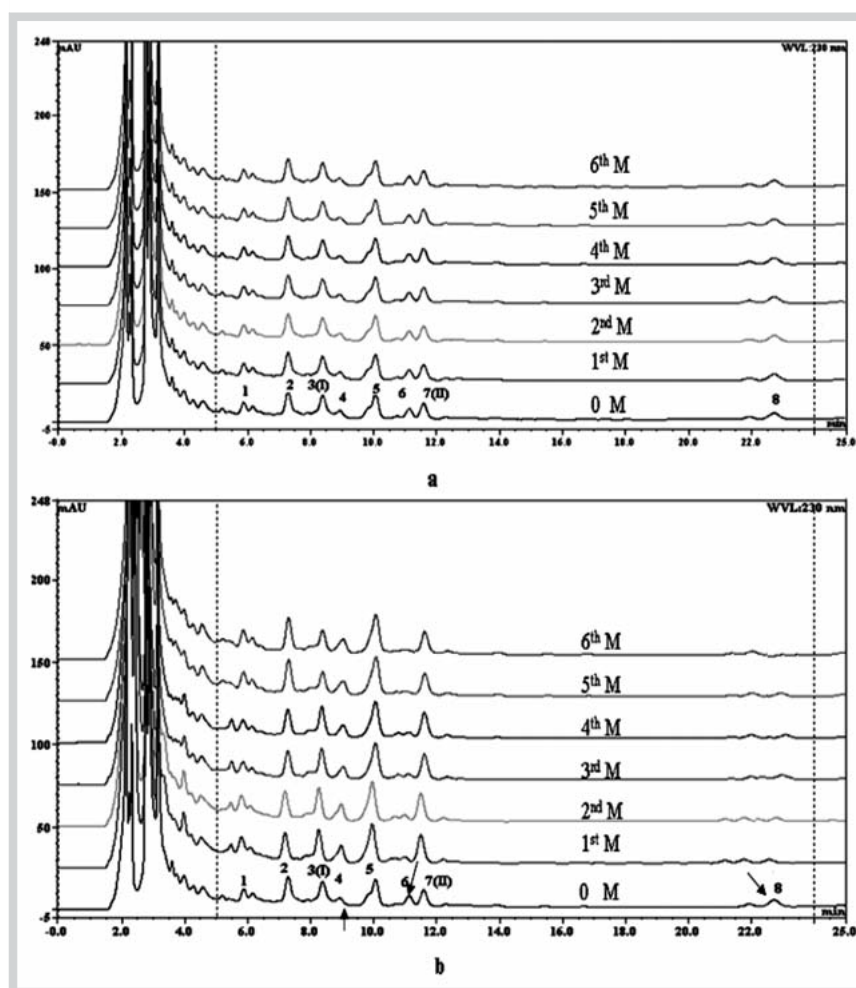


Fig. 3 Effect of real **a** and accelerated storage **b** conditions on WSE fingerprint. Month wise chromatograms under real and accelerated storage are shown in overlay plots. Dotted lines represent fingerprint region (t_R 5 to 24 mins) showing selected eight peaks (1–8). M represents month. Refer to **Table 5** for results.

marker suitability and fingerprint analysis (**Table 2** and **3**). WSE samples stored at real-time and accelerated storage conditions were analyzed for withaferin A and withanolide A at monthly intervals for six months (**Table 4**). Under real-time storage, withaferin A and withanolide A showed at the end of the six-month period residual percentages of 90.40 and 92.82, respectively, whereas the accelerated stability testing at the end of this period showed residual percentages of 63.60 and 88.86 for withaferin A and withanolide A, respectively.

In fingerprint analysis, a limit of $\pm 20\%$ variation in RPA was defined as a significant change [23]. In real-time storage samples, peaks 2, 4, and 6 showed a significant change at month six, month three, and month one of storage. Other peaks were relatively stable as made evident by the similarity coefficients that ranged from 0.99 to 0.98 during six months (**Fig. 3 a** and **Table**

5). However, in the accelerated stability testing, significantly higher degradation was observed wherein peak 6 and 8 could not be detected from month one to month six, respectively. Further, peak 4 showed a significant change from the first month onwards. These changes reflected further on the correlation coefficients (r^2), wherein values dropped from 0.83 in the first month to 0.67 at the sixth month of accelerated storage (**Fig. 3 b** and **Table 5**).

Biological activity measured as the percent immunomodulatory activity of levamisole and WSE (month 0) was observed to be 35.21 and 23.45%, respectively. The results suggest activity decline however, a relatively higher decline was observed in samples under accelerated storage ($p < 0.01$; **Fig. 4**).

Table 3 Method validation towards development of fingerprint.

Peak no.	Repeatability		Intermediate precision						Intraday	
	RRT	RPA	Inter-day		Day 2		Day 3		RRT	RPA
			Day 1	Day 1	Day 2	Day 2	Day 3	Day 3		
1	0.504	0.381	0.507	0.384	0.501	0.388	0.501	0.384	0.506	0.383
	(0.30)	(0.58)	(0.38)	(0.77)	(0.32)	(0.88)	(0.45)	(0.95)	(0.32)	(0.58)
2	0.628	0.821	0.618	0.811	0.614	0.815	0.628	0.819	0.628	0.812
	(0.64)	(0.95)	(0.34)	(0.55)	(0.31)	(0.58)	(0.84)	(0.75)	(0.31)	(0.58)
3 Withaferin A	0.719	0.536	0.718	0.541	0.714	0.538	0.710	0.534	0.718	0.542
	(0.59)	(0.92)	(0.11)	(0.61)	(0.41)	(0.72)	(0.90)	(0.99)	(0.21)	(0.72)
4	0.768	0.385	0.774	0.378	0.761	0.372	0.770	0.371	0.771	0.376
	(0.65)	(1.32)	(0.25)	(1.02)	(0.25)	(1.13)	(0.85)	(1.63)	(0.25)	(1.13)
5	0.851	1.40	0.854	1.44	0.856	1.442	0.857	1.441	0.855	1.44
	(0.49)	(0.70)	(0.25)	(0.70)	(0.27)	(0.79)	(0.47)	(0.97)	(0.27)	(0.70)
6	0.981	0.30	0.971	0.299	0.965	0.297	0.963	0.296	0.971	0.298
	(0.96)	(0.97)	(0.92)	(0.97)	(0.95)	(0.99)	(0.97)	(1.17)	(0.92)	(0.97)
7 (Ref Peak) Withanolide A	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
8	1.962	0.403	1.964	0.407	1.965	0.405	1.966	0.403	1.964	0.407
	(0.16)	(0.92)	(0.06)	(0.62)	(0.06)	(0.71)	(0.16)	(0.91)	(0.06)	(0.71)

HPLC-DAD method was validated for repeatability and precision towards development of reproducible chromatographic fingerprints. A total of 8 peaks were selected for fingerprint analysis. Peaks 3 and 7 were identified as withaferin A and withanolide A. All other peaks were normalized with respect to peak area and retention time of withanolide A (reference peak no. 7) and are reported here as relative peak area (RPA) and relative retention time (RRT). RRT and RPA values were further used for determining the repeatability and precision of the method. Value in parentheses indicates RSD

Month	Real conditions		Accelerated conditions	
	mg/100 mg ± S.D	Residual %	mg/100 mg ± S.D	Residual %
Withaferin A				
0	0.0458 ± 0.0005	100	0.0458 ± 0.0005	100
1	0.0448 ± 0.0006	97.92	0.0437 ± 0.0022	95.37
2	0.0442 ± 0.0005	96.42	0.0404 ± 0.0015	88.20
3	0.0437 ± 0.0007	95.39	0.0389 ± 0.0009	85.02
4	0.0432 ± 0.0004	94.22	0.0366 ± 0.0013	79.99
5	0.0426 ± 0.0004	93.09	0.0329 ± 0.0018	71.92
6	0.0414 ± 0.0005	90.40	0.0291 ± 0.0019	63.60
Withanolide A				
0	0.0478 ± 0.0001	100	0.0478 ± 0.0004	100
1	0.0470 ± 0.0003	98.22	0.0465 ± 0.0005	97.35
2	0.0466 ± 0.0002	97.38	0.0453 ± 0.0006	94.83
3	0.0460 ± 0.0002	96.22	0.0446 ± 0.0002	93.39
4	0.0457 ± 0.0002	95.57	0.0444 ± 0.0003	92.82
5	0.0453 ± 0.0001	94.77	0.0435 ± 0.0005	90.27
6	0.0444 ± 0.0002	92.82	0.0425 ± 0.0004	88.86

Month wise real and accelerated time WSE samples were analyzed for withaferin A content using HPLC-DAD analysis. The content was converted into mg/100 mg levels and further reported here as residual percentages considering 0 month values as 100%

Discussion

Stability testing at different stages of herbal drug development is currently under contest due to analytical complexities and likely additional costs during the manufacture process. Detailed stability profiling of extracts at preformulation stages may help the formulator to identify inherent weaknesses of the extract at an early stage and enable him to choose few but rational stability-indicating parameters for further stages of development. Botanical materials may have poor flow, variable particle size distributions, and inconsistent compression properties. Stability testing of botanicals, especially during preformulation stages, should include flowability, hygroscopicity, and compression properties [24,25].

However, such investigations on herbal drugs are rarely reported. The particle size distribution is one of the most important characteristics of a powder, affecting properties and performance of intermediate and final products. Our results suggest that WSE contains multiple fractions of different particle sizes in fine powder range as evident by the angle of repose and Carr indices at month zero of storage. However, there were incidences of clump formation during the real-time and accelerated storage probably due to moisture sensitivity which showed an increase from initial 4.5% to 9% and to 12% in real and accelerated humidity conditions, respectively as shown in **Table 1**. Further, high moisture content may adversely affect the stability of key phytochemicals in the extract. These trends suggest the need for extra care during formu-

Table 5 Effect of real-time and accelerated storage conditions on WSE fingerprint.

Peak	1	2	3	4	5	6	7	8	r ² #	
	Withaferin A						Withanolide A (Ref. peak)			
Month	(Relative peak areas calculated with ref to peak 7)									
Real-time storage										
0	0.38	0.81	0.54	0.37	1.44	0.29	1.00	0.40	NA	
1	0.38	0.81	0.53	0.41	1.51	0.22 [°]	1.00	0.40	0.997	
2	0.38	0.81	0.53	0.43	1.52	0.20 [°]	1.00	0.40	0.995	
3	0.37	0.81	0.53	0.48 [^]	1.56	0.18 [^]	1.00	0.39	0.989	
4	0.37	0.82	0.53	0.50	1.58	0.16 [^]	1.00	0.39	0.985	
5	0.37	0.84	0.53	0.52	1.59	0.27	1.00	0.39	0.988	
6	0.36	0.99 [^]	0.53	0.54	1.59	0.30	1.00	0.39	0.982	
Accelerated storage										
0	0.38	0.81	0.54	0.37	1.44	0.29	1.00	0.40	NA	
1	0.38	0.81	0.53	0.41	1.43	ND	1.00	0.40	0.831	
2	0.38	0.80	0.53	0.43	1.42	ND	1.00	0.40	0.825	
3	0.36	0.81	0.53	0.48 [^]	1.42	ND	1.00	0.40	0.817	
4	0.36	0.82	0.53	0.45	1.41	ND	1.00	0.40	0.824	
5	0.36	0.84	0.53	0.43	1.41	ND	1.00	0.38	0.820	
6	0.35	0.79	0.52	0.42	1.41	ND	1.00	ND	0.672	

Effect of real and accelerated storage on fingerprint is presented here. Relative peak areas of selected peaks were calculated with reference to withanolide A (peak no. 7). #Month wise Pearson correlation coefficients (r²) as compared to month zero values are presented here. ^ represents significant change, i.e., RPA > 80–120% of month zero values. NA: not applicable. ND: not detected

lation development when moisture sensitive botanical materials like WSE are used.

Regulatory agencies such as the US FDA and EMEA have made fingerprinting analysis mandatory for quality evaluation of herbal medicinal products. Moreover, various chemometric approaches are being suggested for assessment of similarity in fingerprints. *Withania somnifera* has been studied extensively for its biologically active entities especially the steroidal lactones known as withanolides. Several analytical approaches have been suggested for the identification and quantification of withanolides in WS samples [26,27]. We used the HPLC-DAD method reported by Ganzera et al. [16] and modified it for analysis of withanolide A, which is reported to be responsible for immunomodulatory activity. This method was modified in gradient conditions to improve resolution of peaks and was found to be precise and reproducible for the identification of withaferin A, withanolide A, and the fingerprint analysis (● Tables 2 and 3). Our results indicate that WSE showed signs of chemical instability during real-time and accelerated storage (● Tables 4 and 5). Interestingly, the withaferin A content showed a relatively higher decline as compared to withanolide A.

This preliminary finding will be important as withaferin A and withanolide A are reported to modulate distinct targets [28]. For instance, withaferin A is reported to modulate T cell apoptosis, inhibit angiogenesis, and quinone reductase activity [29], whereas withanolide A is described to have immunostimulatory effects through T-helper cell pathways. Previously, we have reported *in vivo* T cell modulatory activity of WSE in comparison to levamisole in an experimental murine model using SRBC as an antigenic stimulus [30]. This prompted us to explore whether the observed instability trends reflect on the immunomodulatory activity of WSE. A significant decline in immunomodulatory efficacy was observed during accelerated storage ($p < 0.01$). This may be due to the reduction of withanolide content; however, it warrants further investigations. Use of a combinative analysis approach was beneficial as fingerprint analysis (● Fig. 3, Table 5) identified

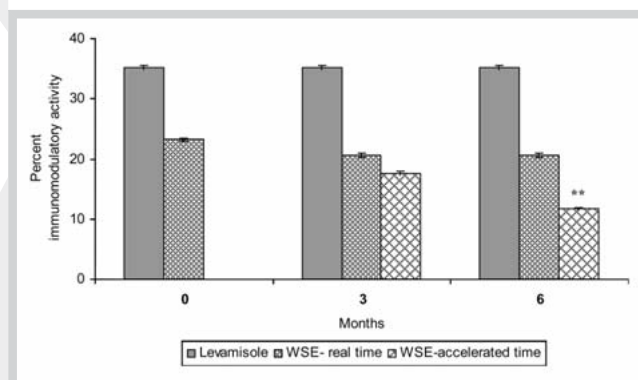


Fig. 4 Effect of real and accelerated storage on biological stability of WSE. Immunomodulatory activities of WSE and levamisole were compared with respect to humoral response (antibody titres) against sheep red blood cells (SRBC). Observations were made at month 0, 3, and 6 under real and accelerated storage. Bars in graph represent percent modulatory activity calculated according to formulae given in the data analysis. Error bars represent standard deviation of mean titers of the respective group ($n = 6$). ** $P < 0.01$ as calculated using Dunnett's Test.

three other peaks (no. 2, 4, and 6) that showed concurrent significant reduction in peak areas. It would be interesting to elucidate the identity and biological activity of peak numbers 2, 4, and 6, which will lead to a better understanding of the instability in WSE formulations. Further, our modified analytical method may qualify as a stability indicating method once appropriate stress studies are completed. The observed moisture sensitivity of WSE may be responsible for the instability trends; however, more focused studies are needed for conclusive correlations. Our study demonstrates that detailed stability profiling of herbal extracts with respect to pharmaceutical, biological, and chemical properties at preformulation stages can facilitate a more efficient botanical product development and pharmaceuticals.

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