

A BIOASSAY APPROACH TO COMPLEMENT CHEMICAL STANDARDIZATION OF ASHWAGANDHA ROOT EXTRACTS

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ABSTRACT

Withania somnifera (L.) Dunal, popularly known as ashwagandha, is an Indian plant that has long been utilized in Ayurveda for its adaptogenic effects, as evidenced by several clinical and preclinical research publications. Using an *in-vitro* acetylcholinesterase inhibition assay (AChE), we performed bioassay-guided fractionation of the ethanolic extract of ashwagandha roots. The inhibitory activity was found to be concentrated in the fractions containing withanolides, and the active fractions were purified to yield nine distinct withanolides. Five of these had a considerable inhibitory effect against acetylcholinesterase ($IC_{50} < 10 \mu\text{g/ml}$), with 12-deoxywithastramonolide being the most active ($IC_{50} = 1.54 \mu\text{g/ml}$). Ashwagandha extracts are typically standardized to the content of withanolides by chromatographic methods like HPLC. We evaluated if acetylcholinesterase inhibition assay can be utilized for biological standardization of commercial batches that can complement the current chromatographic methods. Nine different commercially produced batches of Ashwagandha extracts were evaluated with acetylcholinesterase inhibition *in-vitro*, HPLC profiling, and quantitative determination of nine withanolides. The IC_{50} values ranged from $15 \mu\text{g/ml}$ to $95 \mu\text{g/ml}$, while the total content of nine withanolides varied between 2.78%w/w to 3.58%w/w. Given the inherent variability in withanolide content, an acetylcholinesterase inhibition assay can be utilized to enhance quality control of commercially available ashwagandha extracts.

Keywords: Bioassay Guided Fractionation, Acetylcholinesterase Enzyme Inhibition, 12-Deoxywithastramonolide, Chromatographic Fingerprint, Similarity Analysis, Ashwagandha.

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INTRODUCTION

Withania somnifera (L.) Dunal (WS) native to the family Solanaceae, is a shrub that grows as a stout, evergreen, and tomentose (perennial) dispersed in India's drier regions, such as Madhya Pradesh, Uttar Pradesh, the Punjab plains, Gujarat, and Rajasthan serving as main producers¹⁻³, and is commonly known as ashwagandha, winter cherry, and "Indian ginseng". It is one of the most important herbs in Ayurveda, used as "Rasayana" and an adaptogen, which covers a wide range of health benefits by improving learning and memory, longevity, immunity, and preventing ageing.¹ It is used in various formulations in Ayurveda, Siddha, and Unani medicines because of its medicinal potential. Alkaloids, tannins, phenolics, carbohydrates, starch, terpenoids, and steroids have been found in its roots, with alkaloids and steroidal lactones being the predominant class of compounds.⁴⁻⁶ Withanolides are a group of naturally occurring steroids that have a C28 steroidal nucleus and a lactone with a nine-carbon side chain connected to the C-17 position and are hypothesized to contribute to the plant's bioactivity.⁷ Adaptogenic, anti-cancer, anti-convulsant, immunomodulatory, antioxidative, and neurological activities have been observed in WS extracts and extracted bioactive components. The herb has also been shown to help with arthritis, geriatric, behavioral, and stress-related issues.⁸⁻¹² The active compounds of ashwagandha such as sitoindosides VII-X, acylsterylglucosides, and withaferin-A have been demonstrated to exhibit considerable anti-stress efficacy in acute stress models.¹³ Many of its components have immunomodulatory properties.¹⁴ The adaptogenic, anxiolytic, and anti-depressive properties of WS methanolic extracts show therapeutic potential in stress mice.¹⁵ By suppressing the neuroadaptive processes associated with chronic morphine

exposure, WS extract protects rats from structural alterations by preventing the loss of spine density in the nucleus accumbens shell in rats associated with morphine withdrawal.¹⁶ WS extracts are desensitizers of stress-induced biological reactions involving thermoregulatory pathways, and they have a therapeutic promise for the prevention and treatment of diabetes-related mental health issues.¹⁷ In several cell survival studies, treatment with WS extract significantly protected the human neuroblastoma cell line SK-N-SH from A β peptide and acrolein, by significantly lowering the formation of reactive oxygen species in SK-N-SH cells. The WS extract is a powerful inhibitor of acetylcholinesterase activity, may operate as an antioxidant and cholinergic modulator and may have beneficial results in the treatment of canine cognitive dysfunction and Alzheimer's disease.¹⁸ WS extract at modest daily dosages suppresses a variety of stress responses, and its centrally acting analgesic and anxiolytic as well as antidepressant-like efficacies, rise with the number of treatment days.¹⁹ Audiogenic stress-induced elevated NO $_x$ levels in the cortex, hypothalamus, and hippocampus are considerably reduced when WS is administered. As a result, it exhibits strong adaptogenic action, which is likely mediated through the nitergic system.²⁰ Multiple pharmacological actions of ashwagandha and withanolides have boosted the interest in the plant in recent years. However, hardly any studies are available to link the key molecules responsible for the plant's specific or overall biological effect. Hence, this attempt was made to identify the bio-active molecules through a bioassay-guided fractionation (BAGF) of ethanolic extract of WS roots using acetylcholinesterase inhibitory activity. Nine withanolides were isolated with the help of BAGF utilizing column chromatography over silica gel. The structures of the isolated compounds were determined with spectroscopic data viz., NMR, and mass. The quality control of the herbs and their extracts is a topic of continuing research around the world. Considering the complex nature of their phytochemical makeup, it is nearly impossible to attribute a plant's biological activities to one or a few of its constituents. Since the medicinal herbs produce their physiological effects through a synergistic action of numerous phytochemicals, a combination of phytochemicals and biological assays can improve the quality control to a next level. Although numerous published papers are describing its therapeutic uses, there are limited literature available in terms of a quality control assessment based on the identification of bioactive components through BAGF. In this study, we attempted to correlate the acetylcholinesterase inhibition properties of WS extract and the isolated withanolides. The possibility of using acetylcholinesterase inhibition *in-vitro* as an additional parameter to the HPLC method was explored towards further strengthening the quality control of WS extracts.

EXPERIMENTAL

Chemicals and Reagent

Dihydrogen orthophosphate, acetylcholinesterase enzyme (from Electric eel, type VI-S, C-3389), Eserine hemisulphate salt- Positive control (E8625), Trizma base (T-1503) were purchased from Sigma, USA. Acetylthiocholine iodide substrate (RM 770), Ellman's reagent: 5, 5, Dithiobis (2- nitro benzoic acid) [DTNB] (RM 1677), Sodium bicarbonate (RM849), Sodium dihydrogen orthophosphate dihydrate (RM1255), Di-sodium hydrogen phosphate dihydrate (RM257), carboxymethyl cellulose were procured from Himedia, India and hydrochloric acid (H0090), methanol, acetonitrile, and water (HPLC grade) were procured from Rankem, India.

Plant Material

WS roots were obtained from the Neemuch district in Madhya Pradesh, India, and identified by the National Institute of Science Communication and Information Resources (NISCAR), New Delhi. Our in-house herbarium received a voucher specimen (RD-21879). Ethanolic extract of the WS roots was used for bioassay-guided fractionation. In this study, nine different batches of a commercially manufactured WS extract (AshwapureTM) were used to evaluate batch-to-batch consistency utilizing HPLC and *in-vitro* bioassay techniques.

Instrumentation

A Shimadzu system IR Prestige-21 spectrometer and a Shimadzu-2401 PC spectrometer (Shimadzu Corporation, Kyoto, Japan) were used to perform Fourier-transform infrared (FTIR) spectroscopy and UV spectra in methanol, respectively. Thermonik melting point device, made by Campbell Electronics in Mumbai, India, was used to determine the melting point of the isolated phytocompounds. A Bruker

AVANCE II 400 spectrometer was used to capture 1D spectra such as ^1H and ^{13}C NMR (Bruker Corporation, Chennai, India). On the same equipment, 2D spectra such as ^1H - ^1H COSY, HMQC, HMBC, and C-90 DEPT NMR investigations were recorded. LCQ Fleet-Thermo Fisher Scientific apparatus was used to measure MS spectra (Thermo Fisher Scientific, Inc., Waltham, MA). The Shimadzu Prominence 20AD included a binary delivery system, an online degasser-DGU-20A3R, SIL-20AC auto-sampler with sample cooler, a column oven-CTO-10ASVP, CBM-20ALite System Controller, SPD-M20A PDA detector coupled with an analytical workstation and a reverse-phase column Hibar® Purospher® STAR C18 (4.6 x 100 mm, 3 μm particle size) was used for HPLC chromatographic separation. Silica gel (60–120 mesh, ASTM; Merck Co.) for column chromatography and silica gel 60 F254 plates for performing TLC were purchased from Merck (White House Station, NJ), respectively.

Extraction and Isolation

To obtain the ethanolic extract, dried roots of WS (5kg) were coarsely pulverized and extracted three times with ethanol. By suspending the ethanolic extract (500 gm) of the root in water (H_2O), it was partitioned with ethyl acetate (EtOAc) and n-butanol (n-BuOH). Column chromatography (CC) over silica gel was used to fractionate the EtOAc fraction (150 g), which was first eluted with petroleum ether: EtOAc stepwise gradient and then with EtOAc: methanol stepwise gradient to yield six fractions (Fr. C1F1-Fr. C1F6). All the fractions were examined for biological activity (acetylcholinesterase inhibition assay), and TLC and HPLC were employed to analyze the selected bioactive fractions that were subjected to subsequent rounds of Bioactivity-guided fractionation. Fraction C1F3 was chosen for further separation over a silica column and eluted with a stepwise gradient of petroleum ether: EtOAc and EtOAc: MeOH to provide eight fractions (Fr. C3F1-Fr. C3F8). Fr. C3F4 and Fr. C3F5 were used to isolate withasomniferolide B and withasomniferolide A, respectively. Fraction C1F4 was subjected to CC over silica column and eluted with petroleum ether: EtOAc and EtOAc: MeOH stepwise gradient to give seven fractions, where withanolide B, 27-deoxy-withaferin-A were isolated from Fr. C2F4; withanolide A, 12-deoxywithastramonolide, Withaferin A from Fr. C2F5; withanoside V from Fr. C2F6 and withanoside IV from Fr. C2F7, respectively. The identities of the isolated compounds were confirmed by comparing the spectroscopic data (NMR and Mass) with the available literature.

HPLC Analysis of Isolated Compounds

In methanol, a standard stock solution of withanoside IV, withanoside V, withaferin A, 12-deoxywithastramonolide, withanolide A, and withanolide B at a concentration of 1 mg/mL was prepared. For the calibration curves, a series of concentrations ranging from 1.0 to 100 ppm were obtained by diluting the standard solution with methanol and stored at -4°C until use. For the sample preparation, 250 mg of finely milled dried extract sample was taken in a 50 mL volumetric flask; methanol was added to it and heated gently on a water bath, and then allowed to cool at room temperature mixed well, and filtered through a 0.45 μm filter membrane prior to use. The analytical HPLC method uses a buffer as the mobile phase (A) and acetonitrile (B). A 1000 mL buffer was prepared by dissolving 0.136 g of anhydrous potassium dihydrogen orthophosphate and 0.5 mL of orthophosphoric acid in 900 mL HPLC grade water. The prepared buffer was filtered and degassed before use with a 0.45 μm membrane filter. The analytical gradient used for HPLC elution was: in 0 min, 5% B; in next 20 min to 45% B; in next 10 min to 50% B; in next 5 min to 80% B and maintained at 80% B for next 5 min, decreased the solvent B to 5% in the next 5 min, followed by the equilibration time of 5 min with the flow rate of 0.4 mL/min having injection volume of 5 μL . The wavelength selected for the detection was 227 nm. The column temperature was maintained at 30°C . The purities of the isolated withanolides were >95% by HPLC analysis, coupled with photodiode array detection.

Acetylcholinesterase Inhibition *in-vitro*

The acetylcholinesterase (AChE) inhibitory assay was carried out using Ellman *et al.*²¹ colorimetric methods with slight modifications. In a pre-incubation volume of 250 μL in phosphate buffer (200 mM; pH 7.7), 160 μL of the reference standard (Eserine)/test compounds at varying concentrations (0.25–10 $\mu\text{g/mL}$), 80 μL of DTNB (1 mM DTNB and 1.79 mM sodium bicarbonate dissolved in phosphate buffer), and 10 μL of the enzyme (U/mL) were added. At 25°C , the mixture was incubated for 5 min. After the pre-incubation period, 15 μL of the substrate (7.5 mM acetylthiocholine iodide dissolved in phosphate buffer) was added

to the mixture and incubated for another 5 min. The enzyme hydrolyzes the substrate to produce thiocholine, which then combines with Ellman's reagent (DTNB) to produce the yellow-colored molecule that was detected at 412 nm in a microwell plate reader (Versamax, Molecular Devices, USA). Log-probit analysis was used to calculate the IC₅₀. The enzyme's percent inhibition was computed as follows:

$$\text{Percentage Inhibition} = \frac{\text{Absorbance (control)} - \text{Absorbance (test)}}{\text{Absorbance (control)}} \times 100$$

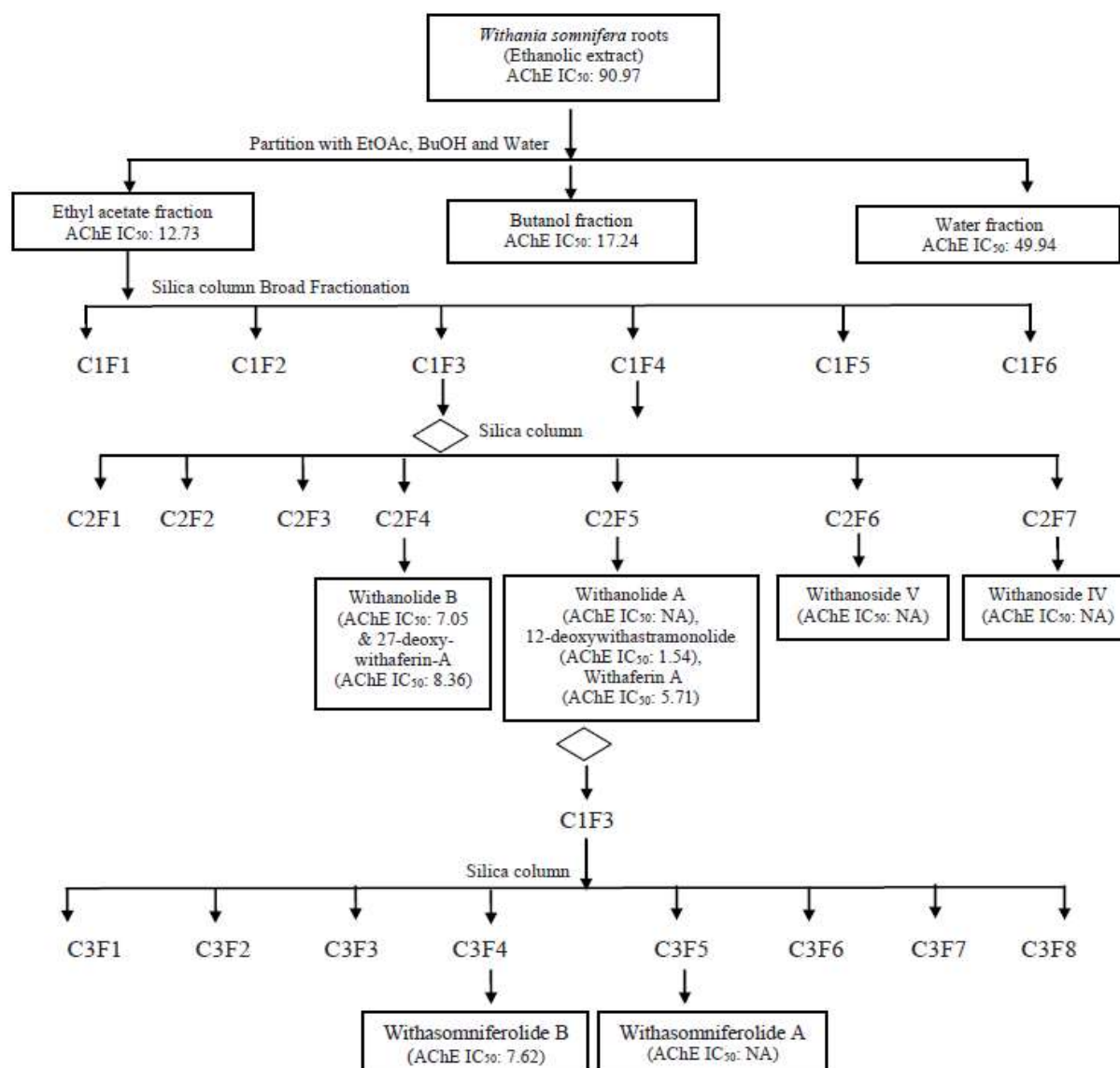
RESULTS AND DISCUSSION

It is well known that medicinal plants have a complex chemical composition with a multitude of phytochemical constituents comprising of both primary and secondary metabolites. Unlike the case of chemical drugs, the biological activities of the plant extracts are believed to be contributed by multiple constituents acting on multiple biological targets *in-vivo*. This situation makes it complex not only to understand the biological activities of the medicinal plants but also to control the quality of the extracts derived from these plants. Adding to the complexity is the fact that the chemical composition of the plants is prone to natural variations arising out of geographical location, age of the plant, the season of collection, harvesting conditions, etc.²² Another important point is that a majority of the chemical constituents of the plant is unknown, and therefore monitoring the quality of plant extracts based on 100% chemical characterization is virtually impossible. Therefore, an emphasis is often laid on gaining control from seed to shelf, involving good practices related to cultivation, harvesting, transport, storage, and manufacturing aspects.²³ The quality control parameters for botanical preparations often involve identity tests based on microscopic techniques, macroscopic techniques, chromatographic analysis, safety-related tests based on microbiological and residual analysis, and assay tests typically involving a determination of 'bioactive marker constituents'. Analytical monographs based on these tests are now available for many medicinal plants and their extracts in Pharmacopoeia of different countries like India, China, USA, and Europe.²⁴ In the context described above, the limitations of current analytical approaches are quite visible, and the scope for further research towards understanding the bioactive chemistry of medicinal plants is enormous. One such new approach is the use of bioassays as a quality control parameter, in addition to the current phytochemical approaches on the assay front. The major advantage of the bioassays when applied to the whole extract is that it captures the overall activity contributed by both the known and unknown constituents of the extract. The quantification of the strength of the activity are expressed with IC₅₀ and EC₅₀ values and defining a range for these values has been found to be a useful additional parameter for the quality control of botanical extracts.²⁵ *Withania somnifera*, popularly known as ashwagandha, is a medicinal plant of Ayurvedic origin that has gained tremendous traction in the dietary supplement and complementary medicine market across the globe.²⁶ Its use as an adaptogen and anti-stress agent has quadrupled in the COVID-19 context as more people suffer from uncertainties of life. The extracts derived from ashwagandha are typically 'standardized' to the content of withanolides, the steroidal lactones present in the plant. Withanolides have been studied for multiple biological activities in the scientific literature and are often considered the bioactive constituents of the plant. However, there is a lack of studies linking the content of withanolides to specific bioactivities exhibited by the extracts of the plant. Also, there are reports to indicate that withanolide-free fractions of ashwagandha have adaptogenic properties, clearly indicating that there is more to this plant than just withanolides.²⁷ Keeping these factors in mind, we have attempted this work to perform a bioassay-guided fractionation of the extract derived from the roots of the plant and isolate bioactive constituents. Acetylcholinesterase inhibition *in-vitro* was chosen, as the extract showed promising bioactivity in the preliminary studies. Furthermore, we evaluated the possibility of using inhibition of acetylcholinesterase *in-vitro* as a biological quality control parameter to complement the regular methods given in Pharmacopoeial monographs on ashwagandha.

Bioactivity-Guided Fractionation

Bioactivity-guided fractionation was performed on the ethyl acetate fraction of ethanolic extract of WS roots, which was found to inhibit the acetylcholinesterase with an IC₅₀ value of 12.73 µg/mL. Fractionations and purifications of the active fractions led to the isolation of compounds namely withanoside IV, withanoside V, withaferin A, 12-deoxywithastramonolide, withanolide A, withanolide B, 27-

deoxywithaferin A, withasomniferolide A and withasomniferolide B (Fig. 2). These isolated compounds were subjected to detailed 1D and 2D NMR investigation (^1H - ^1H COSY, HMBC and HSQC, DEPT 135 spectrum data) for their identity confirmation, which was matched with previously published literature.²⁸⁻³³ Out of these compounds, 12-deoxywithastramonolide showed the most potent inhibition activity against acetylcholinesterase (AChE) enzyme with an IC_{50} value of 1.54 $\mu\text{g}/\text{mL}$, whereas other molecules such as withaferin A, withanolide B, 27-deoxywithaferin A and withasomniferolide B showed moderate AChE inhibition activity with IC_{50} value of 5.71, 7.05, 8.36 and 7.62 $\mu\text{g}/\text{mL}$, respectively. Other compounds such as withanoside IV, withanoside V, withanolide A and withasomniferolide A were found to be inactive. The IC_{50} value details are mentioned in Table-3. The details for acetylcholinesterase assay inhibition for the different commercial batches of extracts are shown in Table-4, and the IC_{50} values of the extract were observed in the range of 15-91 $\mu\text{g}/\text{mL}$ when tested at concentrations between 5-1000 $\mu\text{g}/\text{mL}$.



*All IC_{50} values are expressed as $\mu\text{g}/\text{mL}$

NA- not active up to the tested concentration

Fig.-2: Bioactivity-guided fractionation of the ethanolic extract of *Withania somnifera* roots

Table 3: The inhibitory activity of BAGF fractions and isolated compounds from the roots of *Withania somnifera* against acetylcholinesterase inhibition assay

Fractions/ isolated compounds	AChE inhibition assay, IC ₅₀ (µg/mL)
Ethanol extract	90.97
Ethyl acetate fraction	12.73
Butanol fraction	17.24
Aqueous fraction	49.94
Withanoside IV	NA
Withanoside V	NA
Withaferin A	5.71
12-deoxywithastramonolide	1.54
Withanolide A	NA
27-deoxywithaferin A	8.36
Withanolide B	7.05
Withasomniferolide A	NA
Withasomniferolide B	7.62

NA - not active up to the tested concentration

Table 4: Acetylcholinesterase inhibition assay IC₅₀ values of *Withania somnifera* batches for consistency

Commercial batches	AChE inhibition, IC ₅₀ (µg/mL)
NR-WS-01	21.72
NR-WS-02	91.12
NR-WS-03	65.99
NR-WS-04	50.69
NR-WS-05	40.7
NR-WS-06	15.97
NR-WS-07	15.03
NR-WS-08	61.01
NR-WS-09	57.47

HPLC Analysis

The isolated molecules were utilized to generate HPLC fingerprints with nine different commercial batches of WS, which were then analyzed using Pearson correlation coefficient with the software XLSTAT Base, version 18.06. The least similar value for these samples was 0.951, while the majority of the values were above >0.98 (Table-1), indicating that while WS extract commercial batches were made at different times, their contents were comparable, as determined by HPLC fingerprinting (Fig.-1). The nine withanolides isolated under the current research methodology were also considered for quantification, out of which content of withanoside IV (>0.7%), withaferin A (>0.5%), and withanoside V (>0.4%) was on the higher side, whereas withanolide B (>0.02%), withasomniferolide B (>0.01%) and 27-deoxywithaferin A (>0.01%) towards the minimal side. The sum of all nine withanolides leads to the total content ranging from 2.78% to 3.58% w/w in the different commercial batches of the extract analyzed, and the details are mentioned in Table-2. The present work indicates that many of the withanolides do possess strong acetylcholinesterase inhibitory properties, as revealed in the BAGF. However, when we tried to correlate the IC₅₀ values of the isolated withanolides to the IC₅₀ value of extract, we could not see a strong correlation (see Table-5). This indicated that there could be possibilities of synergistic actions at play in the extract, and also the possibilities of other constituents contributing to the AChE inhibitory properties of ashwagandha extract. Since the regular quality evaluations of ashwagandha may involve analysis of selected withanolides, we saw a possibility to expand the scope of quality control by incorporating specification limits for IC₅₀ values in an acetylcholinesterase inhibition assay. In this case, we propose an IC₅₀ value of less than 100 mcg/ml as an additional parameter to the regular specification of the content of withanolides. The combination of phytochemical and biological assay-based controls may contribute to further enhancing the quality control of ashwagandha extracts. The AChE inhibition assay studied here is just an example of the utilization of bioassays for quality control of ashwagandha extract, and there appears a clear scope for studying many other assays for the purpose.

Table-1: Similarity indexes of the nine batches of *Withania somnifera*

	NR-WS-01	NR-WS-02	NR-WS-03	NR-WS-04	NR-WS-05	NR-WS-06	NR-WS-07	NR-WS-08	NR-WS-09	Reference
NR-WS-01	1									
NR-WS-02	0.998	1								
NR-WS-03	0.977	0.979	1							
NR-WS-04	0.996	0.995	0.981	1						
NR-WS-05	0.971	0.967	0.988	0.971	1					
NR-WS-06	0.993	0.989	0.988	0.994	0.991	1				
NR-WS-07	0.997	0.998	0.984	0.999	0.972	0.993	1			
NR-WS-08	0.959	0.951	0.984	0.961	0.997	0.983	0.959	1		
NR-WS-09	0.982	0.983	0.999	0.984	0.989	0.991	0.986	0.985	1	
Reference	0.984	0.985	0.989	0.985	0.990	0.992	0.982	0.993	0.987	1

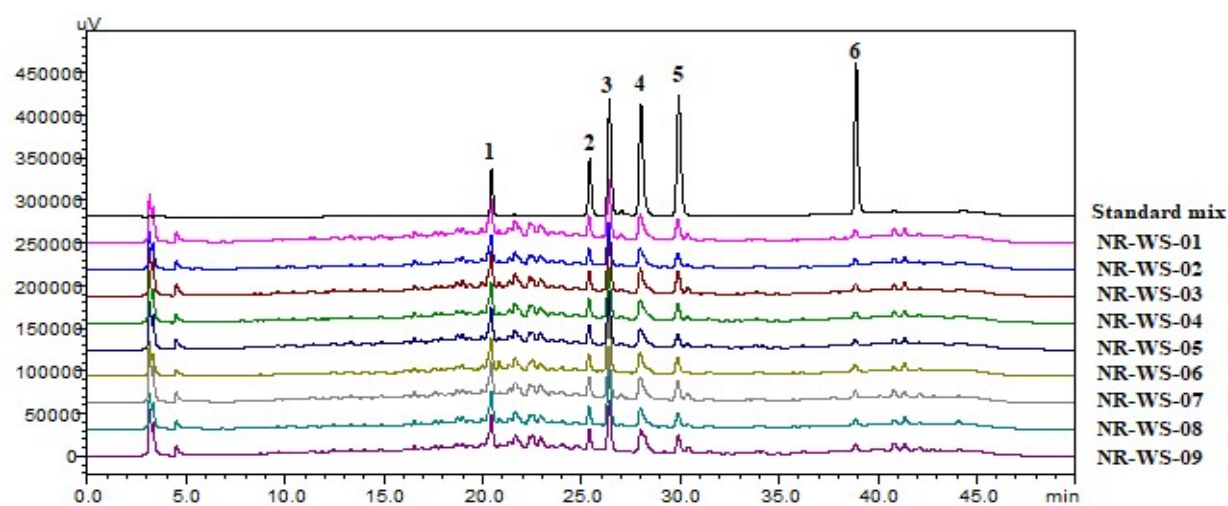
Fig.-1: HPLC fingerprint chromatograms of the nine batches of *Withania somnifera* obtained at 227 nm with standard mixture compounds including (1) withanoside IV, (2) withanoside V, (3) withaferin A, (4) 12-deoxywithastramonolide, (5) withanolide A and (6) withanolide B

Table 2: Quantification of isolated withanolides in the commercial extract batches of Ashwagandha.

S. No.	Batch No.	Withanoside-IV	Withanoside-V	Withaferin-A	12-deoxywithastramonolide	Withanolide-A	27-deoxywithaferin A	Withanolide B	Withasomniferolide-A	Withasomniferolide-B	Sum of all markers
1	NR-WS-01	0.93	0.53	0.74	0.32	0.21	0.02	0.05	0.45	0.02	3.27
2	NR-WS-02	1.02	0.62	0.8	0.3	0.26	0.02	0.06	0.47	0.03	3.58
3	NR-WS-03	0.89	0.59	0.69	0.22	0.23	0.02	0.05	0.6	0.03	3.32
4	NR-WS-04	0.77	0.49	0.67	0.29	0.24	0.02	0.06	0.43	0.03	3.00
5	NR-WS-05	0.83	0.56	0.58	0.31	0.16	0.02	0.05	0.58	0.03	3.12
6	NR-WS-06	0.74	0.46	0.58	0.29	0.18	0.02	0.04	0.45	0.02	2.78
7	NR-WS-07	0.88	0.56	0.72	0.29	0.26	0.02	0.06	0.46	0.03	3.28
8	NR-WS-08	0.81	0.51	0.58	0.3	0.16	0.02	0.04	0.62	0.03	3.07
9	NR-WS-09	0.88	0.54	0.65	0.22	0.22	0.02	0.06	0.56	0.03	3.18

Table 5: Regression coefficient of Withanolides content vs Acetylcholinesterase inhibition (IC_{50})

Withanolides content	Regression coefficient of Withanolides content vs Acetylcholinesterase inhibition (IC_{50})
Total Withanolides (sum of 9 markers)	0.3009
Withaferin A	0.08209

12-deoxywithastramonolide	0.1005
27-deoxywithaferin A	0.0
Withanolide B	0.06607
Withasomniferolide B	0.3754

CONCLUSION

It was observed that ashwagandha extracts derived from roots possess significant AChE inhibitory properties *in-vitro*. The BAGF using AChE inhibition assay led to the isolation of many withanolides, out of which 12-deoxywithastramonolide was found to be the most active. However, the AChE inhibitory activity of the extract could not be fully correlated to the enzyme inhibitory activity of the isolated withanolides, indicating that there could be possibilities of synergistic actions at play in the extract and also the possibilities of many other constituents contributing to the AChE inhibitory properties of ashwagandha extract. Therefore, utilizing a bioassay for the whole extract as an additional quality control parameter may add further value to the existing phytochemical approaches based on the analysis of withanolides in ashwagandha extracts.

REFERENCES

1. A. Girme, G. Saste, S. Pawar, A. K. Balasubramaniam, K. Musande, B. Darji, N. K. Satti, M. K. Verma, R. Anand, R. Singh and R. A. Vishwakarma, *ACS omega*, **5**(43), 27933(2020), <https://doi.org/10.1021/acsomega.0c03266>
2. J. John, *International Journal of Pharmaceutical Sciences and Research*, **5**(6), 2131(2014), [http://dx.doi.org/10.13040/IJPSR.0975-8232.5\(6\).2131-48](http://dx.doi.org/10.13040/IJPSR.0975-8232.5(6).2131-48)
3. M. H. Mirjalili, E. Moyano, M. Bonfill, R. M. Cusido and J. Palazon, *Molecules*, **14**(7), 2373(2009), <https://doi.org/10.3390/molecules14072373>
4. S. D. Desai, G. A. Hadimani, I. B. Bagoji, N. HM and S. Hugar, *Journal of Advanced Scientific Research*, **6**(1), 27(2015)
5. B. Ganguly, N Kumar, A. H. Ahmad and S. K. Rastogi, *Journal of Ginseng Research*, **42**(4), 463(2018), <https://doi.org/10.1016/j.jgr.2017.05.002>
6. K. Narinderpal, N. Junaid and B. Raman, *Research and Reviews: Journal of Botanical Sciences*, **2**(4), 6(2013)
7. S. G. Musharraf, A. Ali, R. A. Ali, S. Yousuf, A. U. Rahman and M. I. Choudhary. *Rapid Communications in Mass Spectrometry*, **25**(1), 104(2011), <https://doi.org/10.1002/rcm.4835>
8. J.N. Dhuley, *Phytotherapy Research*, **15**(6), 311(2001), <https://doi.org/10.1002/ptr.874>
9. P. Kaur, S. Mathur, M. Sharma, M. Tiwari, K. K. Srivasatava and R. Chandra, *Indian Journal of Biochemistry*, **16**(2), 195(2001), <https://doi.org/10.1007/BF02864860>
10. L. C. Mishra, B. B. Singh and S. Dagenais, *Alternative Medicine Review*, **5**(4), 334(2000).
11. R. Schliebs, A. Liebmman, S. K. Bhattacharya, A. Kumar, S. Ghosal and V. Bigl, *Neurochemistry International*, **30**(2), 181(1973), [https://doi.org/10.1016/S0197-0186\(96\)00025-3](https://doi.org/10.1016/S0197-0186(96)00025-3)
12. P. D. Sethi and A. R. Thiagrajan, Studies on the anti-inflammatory activity of withaferin-A, *Indian Journal of Pharmacology*, **2**(4), 165(1970)
13. S. K. Bhattacharya, R. K. Goel, R. Kaur and S. Ghosal, *Phytotherapy Research*, **1**(1), 32(1987), <https://doi.org/10.1002/ptr.2650010108>
14. S. Ghosal, J. Lal, R. Srivastava, S. K. Bhattacharya, S. N. Upadhyay, A. K. Jaiswal and U. Chattopadhyay, *Phytotherapy Research*, **3**(5), 201(1989), <https://doi.org/10.1002/ptr.2650030510>
15. A. Dey A, S. S. Chatterjee and V. Kumar, *Journal of intercultural ethnopharmacology*, **5**(3), 274(2016), <https://doi.org/10.5455/jice.20160414104917>
16. S. Kasture, S. Vinci, F. Ibba, A. Puddu, M. Marongiu, B. Murali, A. Pisanu, D. Lecca, G. Zernig and E. Acquas, *Neurotoxicity research*, **16**(4), 343(2009), <https://doi.org/10.1007/s12640-009-9069-2>
17. A. K. Thakur, A. Dey, S. S. Chatterjee and V. Kumar, *Current Traditional Medicine*, **1**(1), 51(2015). <https://doi.org/10.2174/2215083801999150527115205>
18. M. Singh and C. Ramassamy, *Journal of Nutritional Science*, **6**, 2017, <https://doi.org/10.1017/jns.2017.48>

19. A. Dey, S. S. Chatterjee and V. Kumar, *Oriental Pharmacy and Experimental Medicine*, **16(4)**, 295(2016), <https://doi.org/10.1007/s13596-016-0245-7>
20. K. U. Bansod and S. N. Umathe, *Journal of Pharmaceutical Biology*, **7(2)**, 69(2017), <http://dx.doi.org/10.21276/jpb.2017.7.2.4>
21. G. L. Ellman, K. D. Courtney, V. Andres and R. M. Featherstone, *Biochemical Pharmacology*, **7(2)**, 88(1961), [https://doi.org/10.1016/0006-2952\(61\)90145-9](https://doi.org/10.1016/0006-2952(61)90145-9)
22. K. D. Anna and P. M. Stephen, *The Medical Journal of Australia*, 166: 538(1997), <https://doi.org/10.5694/j.1326-5377.1997.tb123246.x>
23. V. M. Shinde, K. Dhalwal, M. Potdar and K. R. Mahadik, *International Journal of Phytomedicine*, **1**, 4(2009), <https://doi:10.5138/ijpm.2009.0975.0185.05786>
24. C. H. Yeh, H. E. Wang and J. T. Cheng, *Journal of Food and Drug Analysis*, **15(4)**, 2007. <https://doi.org/10.38212/2224-6614.2388>
25. D. Prashanth, A. Amit, S. Yogisha and R. Padmaja, *Journal of Natural Remedies*, **3(2)**, 166 (2003), <https://doi.org/10.18311/jnr/2003/158>
26. V. K. Singh, D. Mundkinajeddu, A. Agarwal, J. Nguyen, S. Sudberg, S. Gafner and M. Blumenthal. *Botanical Adulterants Bulletin*. Retrieved June. 2019, 10, 2020.
27. B. Singh, A. K. Saxena, B. K. Chandan, D. K. Gupta, K. K. Bhutani and K. K. Anand, *Phytotherapy Research*, **17(5)**, 531(2003), <https://doi.org/10.1002/ptr.858>
28. H. Matsuda, T. Murakami, A. Kishi and M. Yoshikawa, *Bioorganic & Medicinal Chemistry*, **9(6)**, 1499(2001), [https://doi.org/10.1016/S0968-0896\(01\)00024-4](https://doi.org/10.1016/S0968-0896(01)00024-4)
29. J. Deshbandhu and A. P. Dahake, *Der Pharma Chemica*, **1(2)**, 109(2009), <https://doi.org/10.4103/0250-474X.27824>
30. M. Gupta, A. Bagchi and A. B. Ray, *Journal of Natural Products*, **54(2)**, 599(1991), <https://doi.org/10.1021/np50074a042>
31. N. Widodo, K. Kaur, B. G. Shrestha, Y. Takagi, T. Ishii, R. Wadhwa and S. C. Kaul, *Clinical Cancer Research*, **13(7)**, 2298(2007).
32. P. Neogi, M. Kawai, Y. Butsugan, Y. Mori and M. Suzuki, *Bulletin of the Chemical Society of Japan*, **61(12)**, 4479(1988), <https://doi.org/10.1246/bcsj.61.4479>
33. V. P. Sonar, B. Fois, S. Distinto, E. Maccioni, R. Meleddu, F. Cottiglia, E. Acquas, S. Kasture, C. Floris, D. Colombo, C. Sissi, E. Sanna and G. Talani, *Journal of Natural Products*, **82(5)**, 1250(2019), <https://doi.org/10.1021/acs.jnatprod.8b01023>

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