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Standardization of *Ashwagandhadi lehya*—An important *Ayurvedic* formulation of *Withania somnifera*

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Ashwagandhadi lehya is an important Ayurvedic formulation containing *Withania somnifera* as the main ingredient. The present study was undertaken to develop standardization parameters for *Ashwagandhadi lehya*. Samples of the formulation were prepared in the lab and those marketed by different manufacturers were procured, and all the samples were evaluated for various physicochemical parameters and botanical characterization. Different extracts of *Ashwagandhadi lehya* were prepared and subjected to HPTLC fingerprinting. Withaferin A, the main constituent present in *W. somnifera,* was used as the marker compound. Its content was determined in the different samples of *Ashwagandhadi lehya* for standardization.

Keywords: *Ashwagandhadi Lehya*, Standardization, *Withania somnifera*, Withaferin A

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Ayurveda is an ancient Indian medical system dating back to the Vedic period about 3000–1500 BC[1]. It is considered to be one of the oldest of healthcare medical systems, and is based on sound scientific principles. The word Ayurveda (Sanskrit) is derived from the words *Ayur* and *Veda*. *Ayur* means life, while *Veda* means Science. Therefore, Ayurveda literally means the Science of Life. It is not just a medicinal system, but also a way of life. Ayurveda deals with the physical, as well as spiritual health.

Ashwagandhadi lehya is a classical Ayurvedic polyherbal formulation included in Ayurvedic formulary of India. *Lehya* or *Avaleh* is one of the several groups of Ayurvedic formulations. It arises from Sanskrit root word *Lih Aswadane*, the form of medicine which can be tasted with help of tongue. It is a semi-solid preparation of drugs, prepared with the addition of jaggery, sugar or sugar candy and boiled with prescribed drug juice or decoction. The major ingredient of *Ashwagandhadi lehya* is *Ashwagandha* (*Withania somnifera*) which is the most important medicinal plant mentioned in various Indian Systems of Medicine (Ayurveda, Siddha and Unani). It has been described in the *Nighantus* as tonic, alterative, pungent, astringent, hot and aphrodisiac and recommended for rheumatism, cough, dropsy, and senile debility. Besides *Withania somnifera*, the other ingredients present are *Elettaria cardamonum*, *Myristica fragrans*, *Glycyrrhiza glabra*, *Hemidesmus indicus* and *Cuminum cyminum*. *Ashwagandhadi lehya* is therapeutically used for *Raktavikāra* (disorder of blood), *Krśatva* (Cachexia), *Arśa* (Piles), *Unamada* (Psychosis) and is used as *Balya Rasāyana* (Rejuvenating agent) and Vājikara (Aphrodisiac)[2].

Although the herbal medicines have been enjoying renaissance among the customers throughout the world, one of the impediments in the acceptance of these formulations is the lack of standardization and quality control profiles. Due to the complex nature and inherent variability of the chemical constituents of plant based drugs, it is difficult to establish quality control parameters[3]. However, with the increasing demands for these phyto-pharmaceuticals, their standardization is also becoming mandatory. Standardized products provide more security and increase the level of trust that people have in herbal drugs[4]. It is, therefore, essential that definite and accurate analytical protocols be available to ascertain consistency and quality of herbal formulations, and more so these should be adhered to so that these

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products exhibit the desired medicinal effects. The Pharmacopoeial Standards for Ayurvedic Formulations published by the Government of India, Ministry of Health and Family Welfare give certain physical parameters as standards for *Ashwagandhadi lehya*[2]. However, these are not sufficient and there is a need to develop other additional methods for quality control based on modern analytical methods. Efforts are being made by our group to develop analytical protocols both for single herbal drugs[5-8] as well as for compound herbal formulations[9-13] that can be used as valuable analytical tools in the routine standardization of Ayurvedic drugs and formulations.

The present study was, therefore, undertaken to develop standardization parameters for *Ashwagandhadi lehya*. Samples of the formulation, marketed by different manufacturers, were procured and evaluated for various physicochemical parameters and botanical characterization. Besides, *Ashwagandhadi lehya* was also prepared in our lab according to the AFI method and studied so as to define the differences amongst the various commercial samples. Thus, the present study aims to develop standardization methods for *Ashwagandhadi lehya* and to identify analytical marker/s in the formulation for maintaining product consistency.

Methodology

All solvents used for extraction and chromatography were of analytical grade from Merck. Whatman No.1 filter paper was used for filtration of the samples. Withaferin A was obtained from IIIM, Jammu, India. Four commercially available samples of *Ashwagandhadi lehya* (AL-1, AL-2, AL-3 and AL-4) prepared and marketed by different pharmaceutical companies were procured from local market. Besides, *Ashwagandhadi lehya* was also prepared in our lab (AL-5) as per the AFI. All the 6 samples of *Ashwagandhadi lehya* were analysed for various physicochemical parameters such as *p*H, total ash, acid insoluble ash, ethanol soluble extractives and water soluble extractives according to the methods given in the AFI.

For TLC each sample of *Ashwagandhadi lehya* (5 gm) was extracted successively with n-hexane (50 ml x 3) and acetone (50 mL × 3) by continuous stirring with a magnetic stirrer for 30 min at 40°C. The extracts were pooled, filtered through Whatman No. 1 filter paper, concentrated at reduced temperature (below 50°C) by rotary evaporation (Büchi, USA) to yield the respective samples. Each sample (20 mg) was re-dissolved in 1mL solvent to give test solutions (20 mg mL^{-1}) for TLC analysis. TLC fingerprinting of all the extracts were carried out using pre-coated silica gel 60 F_{254} plates (E. Merck) as stationary phase and toluene : ethyl acetate (9:1) and chloroform : methanol (9.5:0.5) as mobile phase. Spots were observed under UV and visible light after spraying with anisaldehyde sulphuric acid followed by heating at 105°C for 5-10 min. Precoated silica gel 60 F_{254} plates procured from E. Merck, Mumbai were used for HPTLC analysis. The acetone soluble extracts (20 mg each) of the different samples of the formulation were dissolved in 1.0 mL methanol and 15 µL of each of the extracts was used for quantitative estimation of Withaferin A in *Ashwagandhadi lehya*. A stock solution (0.5 mg/mL) of standard was prepared by dissolving 5.0 mg Withaferin A, accurately weighed, in 10 ml methanol. Aliquots 20, 40, 60, 80, 100 and 120 µL) of the stock solution were transferred to Eppendorff tubes and the volume of each was adjusted to 2.0 mL with methanol to furnish standard solutions containing 5, 10, 15, 20, 25 and 30 µg/ mL Withaferin A, respectively. Twenty µL each of the standard solutions of withaferin A was applied onto silica gel 60 F_{254} HPTLC plates using a Linomat 5 Automatic Sample Applicator. The plates were developed in a CAMAG glass twin trough chamber up to a distance of 9.0 cm. After development, the plates were dried in air. They were sprayed with anisaldehyde sulphuric acid reagent followed by heating at 110°C for 10 min. The plates were scanned at 600 nm and the peak areas were recorded. Calibration curve was prepared by plotting peak areas vs concentrations and the regression equation was computed.

For microscopical examination, about 2 gm of each sample was washed thoroughly with hexane and then with water repeatedly till sugar and honey were removed. Then a small portion of the washed material was taken, stained with iodine and mounted in 50% glycerin. Another small quantity was clarified with chloral hydrate and mounted in 50% glycerin. The different mounts were examined for the characteristics of the main ingredients and compared with that reported in the API.

Results and discussion

Standardization of *Ashwagandhadi lehya* was carried out based on the physicochemical parameters[2]. Three commercial preparations of *Ashwagandhadi lehya*, AL-1, AL-3, AL-4, were found to pass most of

the physicochemical tests as per AFI (Table 1). However, some of the physicochemical parameters of AL-2 were found to be quite different.

Ashwagandhadi lehya constitutes of 6 ingredients namely *Withania somnifera*, *Elettaria cardamonum*, *Myristica fragrans*, *Glycyrrhiza glabra*, *Hemidesmus indicus* and *Cuminum cyminum*. Botanical characterization was thus carried out as specified in the AFI to see whether the characteristics of all the ingredients were observed in the different samples or not. The results of the microscopical examination indicated that the characteristics of the main ingredient, i.e. *W. somnifera* were present in all the samples. AL-1 and AL-5 exhibited the characteristics of all the ingredients while AL-3 showed the presence of only *W. somnifera*. The results have been tabulated in Table 2.

Ashwagandhadi lehya samples were successively extracted with hexane and acetone and TLC studies of all the extracts were carried out. The TLC chromatograms of the n-hexane and acetone extracts were recorded for fingerprinting. Table 3 gives a comparative profile of the bands observed in the two extracts of the different samples of the formulation. Six bands at R_f 0.17, 0.24, 0.31, 0.62, 0.69 and 0.77 were observed in the hexane extracts of all the samples. Besides, an extra light blue band at R_f 0.10 and a brown band at R_f 0.55 were seen in the n-hexane fractions of AL-3 and AL-4, respectively. All the acetone extracts showed the presence of four bands at R_{fs} 0.12, 0.26, 0.52 and 0.75. Two more bands at Rf 0.40 and 0.46 were present in all other samples except in AL-2 wherein instead a band at 0.43 was observed. One additional brownish yellow band at R_f 0.65, present in case of AL-3, was however absent in all other samples.

After preliminary studies of the extracts, it was decided to focus attention on the estimation of Withaferin A in the different formulation samples as it is best known biomarker reported for *Withania somnifera*. Different mobile phases were tested for HPTLC analysis of *Ashwagandhadi lehya* samples and Withaferin A to obtain high resolution and reproducible results. The desired objective was achieved by use of Chloroform : Methanol (9.5:0.5) as mobile phase, which gave a peak at R_f 0.26 for Withaferin A. Extraction with different solvents was carried out and it was found that acetone is the solvent of choice as it led to complete extraction of the marker compound. Fig. 1 shows the HPTLC analysis of the acetone fractions of different samples. Different dilutions of the standard Withaferin A were applied

Table 2—Microscopical characterization of *Ashwagandhadi Lehya* samples

Ingredient	Sample codes				
	AL-1	AL-2	AL-3	AL-4	AL-5
Withania somnifera Dunal	✓	✓	✓	✓	✓
Elettaria cardamonum Maton	✓	-	-	✓	✓
Myristica fragrans	✓	✓	-	✓	✓
Glycyrrhiza glabra Linn.	✓	-	-	✓	✓
Hemidesmus indicus R. Br.	✓	-	-	-	✓
Cuminum cyminum Linn.	✓	-	-	-	✓

✓ Present; - not observed

Table 1—Physicochemical parameters of *Ashwagandhadi Lehya* samples

Physico-chemical Parameter	Standard values	Sample Codes				
		AL-1	AL-2	AL-3	AL-4	AL-5
Description	A blackish brown, semisolid paste with spicy pleasant odour and bitter astringent taste	A blackish brown, semisolid paste with spicy pleasant odour and bitter astringent taste	A blackish brown, granular product with spicy pleasant odour and bitter astringent taste	A blackish brown, semisolid paste with spicy pleasant odour and bitter astringent taste	A blackish brown, semisolid paste with spicy pleasant odour and bitter astringent taste	A blackish brown, semisolid paste with spicy pleasant odour and bitter astringent taste
Loss on drying	Not more than 28%	22.41	3.33	22.30	13.61	25.69
Total ash	Not more than 2%	1.40	2.08	2.34	1.04	1.60
Acid-insoluble ash	Not more than 1%	0.41	0.58	0.27	0.25	0.22
Alcohol soluble extractive	Not less than 19%	53.38	12.40	39.63	32.52	22.25
Water-soluble extractive	Not less than 46%	63.75	80.03	56.25	70.25	53.57
*p*H (1%)	Between 4.70–5.00	4.98	6.0	5.1	5.5	4.93

*values mean of three observations each

Table 3—TLC fingerprinting of n-hexane and acetone extracts of *Ashwagandhadi Lehya* samples after spraying with Anisaldehyde sulphuric acid

Extract	TLC bands		Sample Codes				
	Rf	Colour	AL-1	AL-2	AL-3	AL-4	AL-5
Hexane	0.10	light blue	-	-	✓	-	-
	0.17	bluish grey	✓	✓	✓	✓	✓
	0.24	bluish grey	✓	✓	✓	✓	✓
	0.31	bluish grey	✓	✓	✓	✓	✓
	0.55	brown	-	-	-	✓	-
	0.62	bluish grey	✓	✓	✓	✓	✓
	0.69	bluish grey	✓	✓	✓	✓	✓
	0.77	dark bluish grey	✓	✓	✓	✓	✓
Acetone	0.12	bluish grey	✓	✓	✓	✓	✓
	0.26	violet	✓	✓	✓	✓	✓
	0.40	violet	✓	-	✓	✓	✓
	0.43	violet	-	✓	-	-	-
	0.46	violet	✓	-	✓	✓	✓
	0.52	violet	✓	✓	✓	✓	✓
	0.65	brownish	-	-	✓	-	-
	0.75	yellow violet	✓	✓	✓	✓	✓

✓Present; - not observed

and the calibration plot was prepared by plotting peak area against amount of Withaferin A applied and the linear range was determined. The linear range for was found to be 100-600 ng per spot. The linear regression equation obtained was y=205.212+5.728 x with a correlation coefficient (r) of 0.9945. This correlation coefficient indicated good linearity between the concentration and peak area in the applied concentration range. The presence of Withaferin A was confirmed by comparing the R_f values and the spectra of the standard with corresponding spots in the extracts. The overlay spectra of identified peaks in the standards as well as the corresponding peak in the extracted samples showed total superimposition at peak start, peak maximum and peak end, thus confirming the purity of the peaks in the extracted solutions. Withaferin A content was determined quantitatively in the different samples and was found that in different market samples of *Ashwagandhadi Lehya* the amount of Withaferin A ranged from 0.1 mg to 5.2 mg per 100 g of the formulation (Table 4). AL-2 and AL-4 contained the lowest amounts of Withaferin A while AL-3 contained the highest amount of it. The content of Withaferin A was somewhat in the same range in case of AL-1 and AL-5. These results indicate that Withaferin A can be used as the biomarker for standardization of *Ashwagandhadi lehya* however the limits need to be defined.

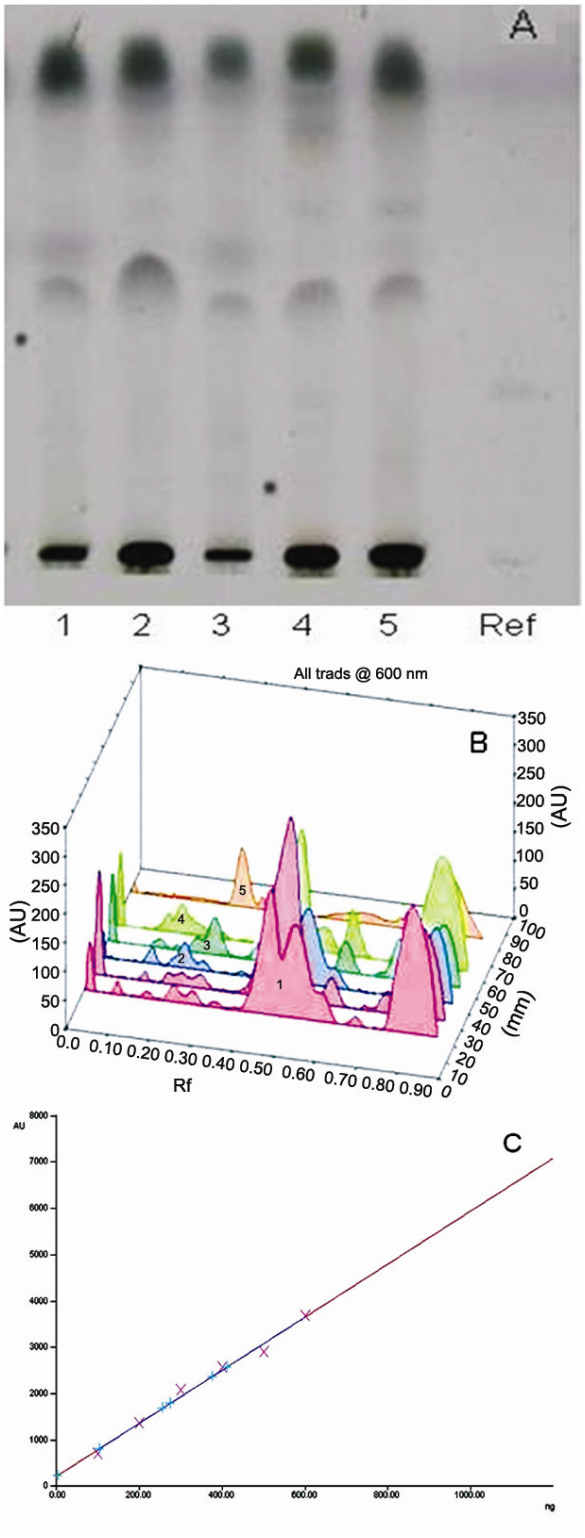

Fig. 1—(A, B) HPTLC fingerprint profiles and densitograms of acetone extracts of different *Ashwagandhadi Lehya* samples. 1: AL-1, 2: AL-2, 3: AL-4, 4: AL-3, 5: AL-5, Ref: Withaferin A. (C) Calibration graph for Withaferin A standard solutions

Table 4—Withaferin A content in different *Ashwagandhadi Lehya* samples

Amount of Withaferin A (mg/100g)				
AL-1	AL-2	AL-3	AL-4	AL-5
1.9	0.1	5.2	0.1	1.1

*values mean of three observations each

Conclusion

Although Ayurveda advocates the use of quality control tests to make sure that the prepared medicines adhere to the standards mentioned in Ayurveda, most of the tests described appear to be based on observation and seem subjective without valid scientific backing. Hence, standardization and development of reliable quality protocols for Ayurvedic formulations using modern techniques of analysis is extremely important[9]. Since standardization and development of reliable protocols for quality control of Ayurvedic formulations using modern techniques of analysis are extremely important, the generated TLC fingerprints of the n-hexane and the acetone extracts of *Ashwagandhadi lehya* could be used as a valuable analytical tool in the routine standardization of *Ashwagandhadi lehya* to check the batch to batch variations. Also Withaferin A can be used as an appropriate bio-marker for standardization of *Ashwagandhadi lehya*.

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