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DETERMINATION OF WITHAFERIN - A IN ASWAGANDHA MARKETED PRODUCTS BY HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHY

Mogtaba Mutasim Alawad Mohammed*, M. Senthil Kumar, R. Sathish

Annai Veilankanni's Pharmacy College, Chennai, Tamil Nadu, India.

The Tamilnadu Dr.M.G.R.Medocal University, Chennai, Tamil Nadu, India.

***Corresponding Author: Email:** mogtabam77@gmail.com*

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ABSTRACT

Purpose: To develop a high performance thin-layer chromatography (HPTLC) procedure for quantitation of withaferin A in ethanol extract of Withania somnifera (L.) roots and in Aswagandha marketed products

Methods: Quantification of withaferin A was carried out using a CAMAG TLC system. A combination of toluene, ethyl acetate and formic acid (5.5: 4.3: 0.2 v/v) was used as mobile phase, with densitometry detection at 323 nm. The HPTLC procedure was subjected to validation as per ICH guidelines.

Results: A sharp withaferin A band at Rf of 0.28 was obtained. The detection limit (LOD) and quantification limit (LOQ) were 0.004 and 0.13 ng/band, respectively.

Conclusion: The developed HPTLC method is linear, precise, accurate and specific for the determination of withaferin A.

Keywords *– Withania somnifera, HPTLC, Withaferin A.*

1. INTRODUCTION

Traditional medicine plays a pivotal role in the treatment of various diseases. *Withania somnifera* (Ashwagandha), of Family Solanaceae root is widely used for therapeutic purposes which are mainly attributed to the active constituents, withanolides. Various clinical and preclinical trials exhibited the plant's potential in curing hepatotoxicity, neurological disorders, anxiety, Parkinson's disease, and hyperlipidemia. The roots and leaves of *W. somnifera* consist of 35 chemical phytoconstituents[1, 2]. In the last two decades, withanolide D and withaferin A are the active components of *W.somnifera* which have been shown tremendous cytotoxic activity suggesting its potential as an anti-carcinogenic agent in treatment of several cancers. The ability of withanolides to inhibit heat protein which in turn causes the death of breast cancer cell is also described [3]. Lalitha et al 2022 has been reported the *in vitro* MTT assay on MCF-7 breast cancer cell lines [4]. *W. somnifera* extract loaded phytosomes exhibit enhanced cytotoxic effect at IC50 values of 45 µg/mL compared to the extract of *W. somnifera* 74µg/mL.

Standardization of herbal drugs is important for validation of biological effects, safety and quality in relation to the chemical constituents of herbal drugs [5]. High performance thin layer chromatography (HPTLC)is the most preferred analytical tool. It is

a fast and inexpensive method of analysis used to separate, qualify and quantify a mixture of phytocomponents. The present study was focused on quantitative estimation of the biomarker withaferin A (Figure 1) using HPTLC in a pharmacologically significant herbal species *W. somnifera,* and validation of the proposed method as per ICH norms [6, 7].

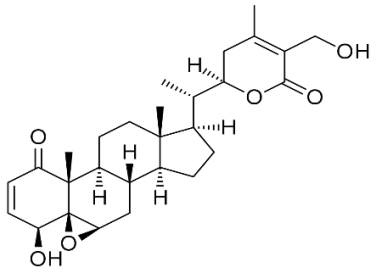

Figure 1: Chemical structure of Withaferin A

2. MATERIALS AND METHODS

2.1 Materials and reagents

A CAMAG TLC system containing a Linomat V applicator and CAMAG TLC III scanner was utilized for analysis of withaferin A. Pre-coated aluminum sheets (silica gel 60F $_{254}$ 20 × 10 cm with stationary phase thickness of 250 µm), analytical grade chemicals and solvents were procured from E. Merck, Germany. Standard withaferin A was procured from Sigma Aldrich, Mumbai.

2.2 Plant material

The roots of *W.somnifera* of Family Solanaceae were collected from Tambaram, Chennai, Tamil Nadu and collected plant was identified and authenticated by Dr. J.Sriram B.S.M.S., M.D., Siddha Lecturer, Government Siddha Medical College, Tirunelveli 627 002 and the Voucher number 1 was deposited in College Herbarium for future reference.

2.3 Extraction

The roots (200 g) were collected, dried in shade and powdered. Three different marketed herbal formulations Ashwagantha chooranam were purchased from different traders, India Market, India. The extraction of each sample using ethanol (80% v/v) was carried out separately by Soxhlet apparatus for 72 h. The ethanol extracts of *W. somnifera* WSR and marketed herbal formulation Ashwagantha chooranam (WSR1, WSR 2 and WSR 3) were concentrated *under vacua* using rota-evaporator.

2.4 Preparation of standard solution

10 mg of Withaferin-A was accurately weighed and dissolved in 10 mL methanol (1000 µg/mL) by sonication for 10 min. This solution was further diluted with methanol to attain the concentration of 50ng/µL by sonication for 10 min and used as standard solution for HPTLC analysis.

2.5 Preparation of sample solution

10 mg of WSR, WSR1, WSR 2 and WSR 3 were accurately weighed and was separately dissolved in 10 mL methanol (1000 µg/mL) by sonication for 10 min. This solution was further diluted with methanol to attain the concentration of 50 µg/µL.

2.6 Optimization of mobile phase

During the optimization of chromatographic conditions, the separation studies were performed using different solvent systems to obtain excellent quality and symmetrical shape of peaks. Trial separations were performed using several solvents of varying polarities i.e. hexane, toluene, chloroform, di-chloromethane and ethyl acetate. Based on the outcome of these trials, binary and tertiary solvent mixtures were tried. The other chromatographic parameters *viz.,*chamber saturation time, height of

solvent run, sample loading volume, sample loading rate, space between spots, and detection wavelength were optimized to obtain consistent R_f and toresolve phyto-constituents in an improved manner.

2.7 Chromatographic conditions

A continuous loading rate of 100 nL/sec and 18.8 mm of band separation for sample were maintained. The scanning was carried out at 6.00 × 0.45 mm slit dimension and speed of 10 mm/sec. The monochromator was maintained at a bandwidth of 20 nm. The scanning of each track was performed in triplicate with baseline correction. The combination of toluene, ethyl acetate and formic acid (5.5:4.3:0.2 v/v) was allowed to rise to 80 mm after 20 min of optimized chamber saturation time maintained at room temperature (25 ± 2 °C) and relative humidity of 55 ± 5 %. The average time taken for the development was 15 min. The validation was carried out as per ICH guidelines.

2.8 Quantification of withaferin A in WSR, WSR1, WSR2, WSR3

The WS and standard solutions in linear range were loaded and studied. The experiment was performed in six replicates. The amount of withaferin A present in WSR, WSR1, WSR2, WSR3 was computed as jn Eq 1.

$$Withaferin\ A\ content = [(TA \times SD)/(SA \times TD)] \times 100 \ldots (1)$$

where *TA* is the test area, *SD* is the standard dilution, *SA* is the standard area and *TD* is the test dilution.

2.9 Validation of the proposed method

2.9.1 Linearity

Standard withaferin A solution of volumes 2, 4, 6, 8 and 10 µL were loaded in HPTLC plates (100 – 500 ng/band) and studied. A linearity curve was constructed through a plot of peak area againstwithaferin A content.

This was evaluated in terms of LOQ. The lowest concentration detected under the chromatographic conditions was the LOD. Values of LOD and LOQ were determined using Eqs 2 and 3.

$$LOD = 3.3 \times N/B \ldots\ldots\ldots\ldots\ldots (2)$$

$$LOQ = 10 \times N/B \ldots\ldots\ldots\ldots\ldots (3)$$

where N refers to standard deviation of the peak areas of the drug sample taken as the measure of the noise, while B represents slope of the standard curve.

2.9.2 Precision

Six replicate loadings of freshly prepared standard at concentrations of 100, 200 and 300 ng/band were analysed on the same day for intra-day precision, and on three different days for inter-day precision.

2.9.3 Specificity

The specificity of method was determined by overlaying withaferin A in WSR, WSR1, WSR 2 and WSR 3 and Withaferin-A in standard spectra.

2.9.4 System suitability

The resolution and repeatability of the developedmethod were determined by analysing six repeated loads of fresh standard (200 ng/band). The mean peak area, SD and % RSD were determined.

2.9.5 Robustness

Tiny fluctuations in parameters like volume of solvent phase, chamber saturation time and duration of pre-conditioning of plate were introduced in the developed method, and the results were analyzed in triplicates of 200ng/band. The percentage RSD of peak area was also determined.

2.9.6 Accuracy

To determine how accurate the proposed methodwas, three-level recovery studies were carriedout using standard addition procedure. Knownquantities of standard withaferin A was added to the WSR (50, 100 and 150 ng) were added to the WSR (1 mg) and the resultant solutions were analysed.

3. RESULTS AND DISCUSSION

The roots collected from Thambaram, Chennai, Tamil Nadu and the collected plant was identified and authenticated by Dr. J.Sriram B.S.M.S., M.D., Siddha Lecturer, Government Siddha Medical College, Tirunelveli 627 002 as *Withania somnifera* (Ashwagandha) of Family Solanaceae. Previous reports suggest that there are possibilities of adulteration of Aswaghantha roots with other roots which have close physical similarity with *W. somnifera*. Therefore, it is necessary to employ a suitable analytical technique for the rapid quality control of Aswaghantha roots and formulations. HPTLC is the most preferred analytical tool, which is a fast, inexpensive method of analysis and is used to separate, qualify and quantify mixed phytocomponents.

Very few reported analytical methods were available for the quantification of withanolides in Aswaghantha. An HPTLC method for the quantification of simultaneous determination of withaferin-A and withanolide-A [8], withaferin A and beta-sitosterol-D-glucoside [9], three withanolides (withaferin A, withanone and withanolide A) has been reported [10] . A HPLC method has been reported for the quantification of Withaferin-A in Aswaghantha [11] . Since Withaferin-A was found to present in ethanolic extracts, we decided to prepare the ethanolic extract of WSR, WSR1, WSR 2 and WSR 3 and to quantify Withaferin-A in it.

The WSR, WSR1, WSR 2 and WSR 3 yielded 0.81, 0.78, 0.82 and 0.75g residue. For quantification of withaferin A using HPTLC method, the developed HPTLC method gave a withaferin A peak with R_f 0.28 with optimized by using Toluene: Ethyl acetate: formic acid (5.5:4.3:0.2 v/v). Chromatograms of standard withaferin A and WSR, WSR1, WSR 2 and WSR 3 are shown in Fig 2, 3, 4, 5 and 6 respectively. The amount of withaferin A in WSR was 1.08, 0.92, 0.99 and 0.90 % (w/w) respectively at detection wavelength of 323 nm (Fig. 7).The linearity curve in the range 100 to500 ng/band of withaferin A is shown in Fig.8. The values of intercept, correlation coefficient, and slope were 845.6, 0.9991, and 265, respectively. The LOD and LOQ values were 0.004 and 0.013 ng, respectively, which indicated adequate sensitivity of the method. The withaferin A peak of the extract was identified through comparison of its R_f and absorbance spectrum with those of the standard Fig 9. Peak areas at 3 different withaferin A concentrations showed low % RSD from 0.31 to 0.63 (< 2 %) with respect to inter- and intra-day fluctuations, suggesting excellent precision (Table 1).

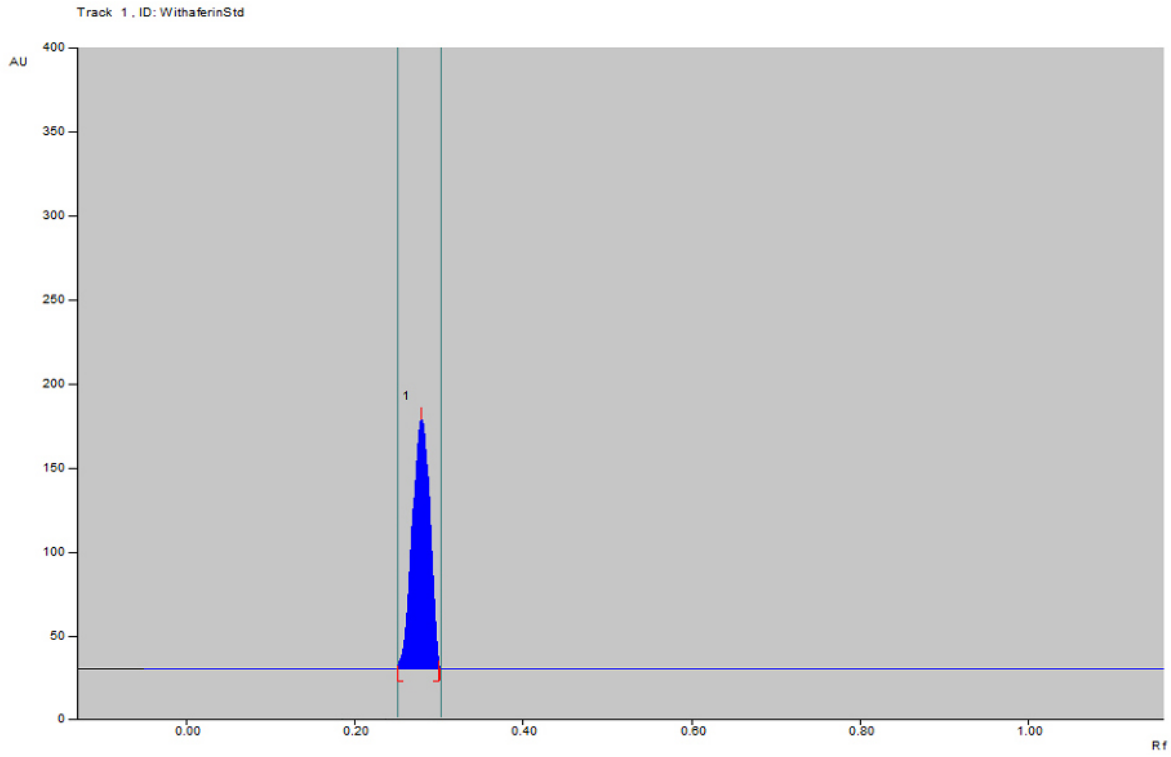

Fig. 2 Standard Chromatogram of Withaferin-A

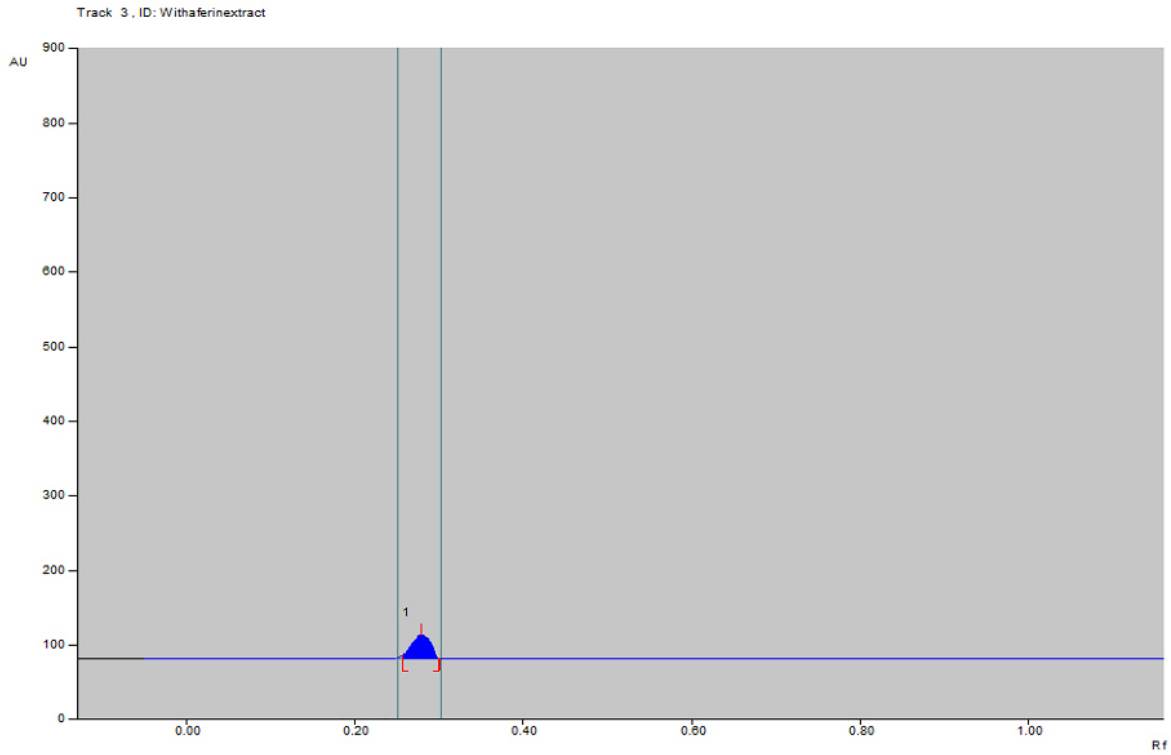

Fig. 3 Chromatogram of Withaferin-A in collected *W. somnifera* sample WSR

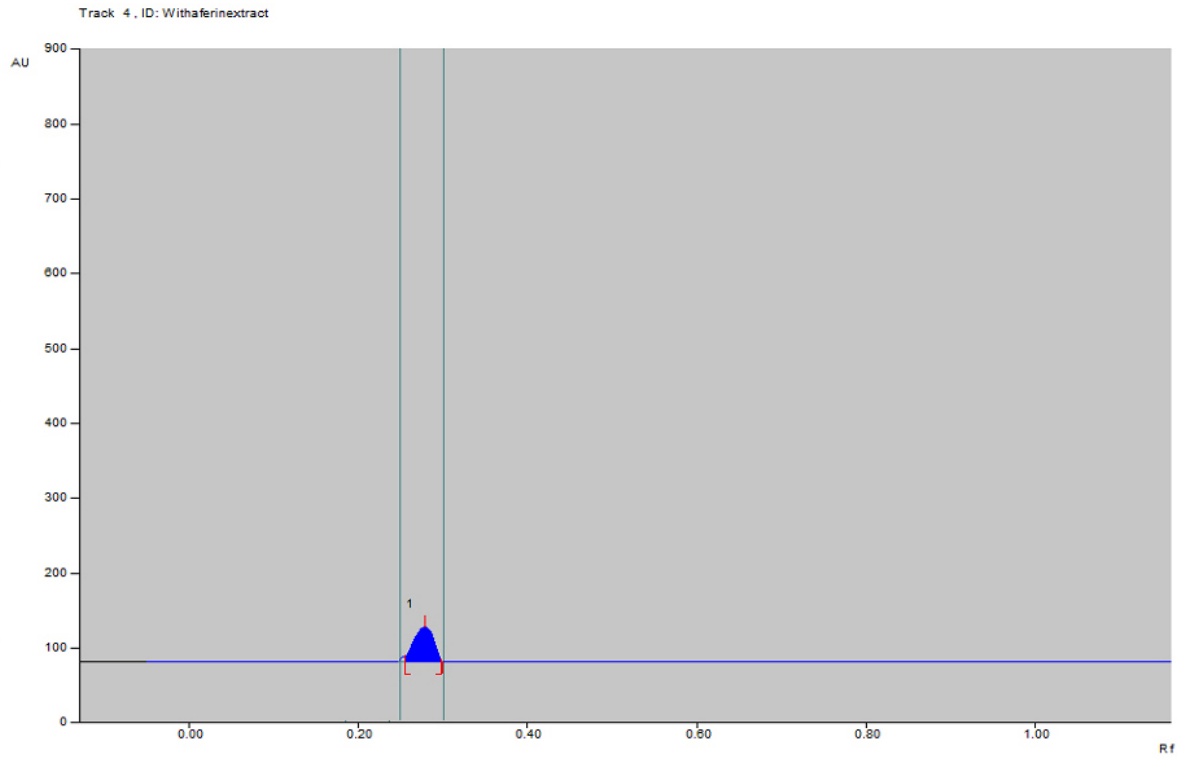

Fig.4 Chromatogram of Withaferin-A in marketed herbal formulation Ashwagantha chooranam Trader 1 sample WSR1

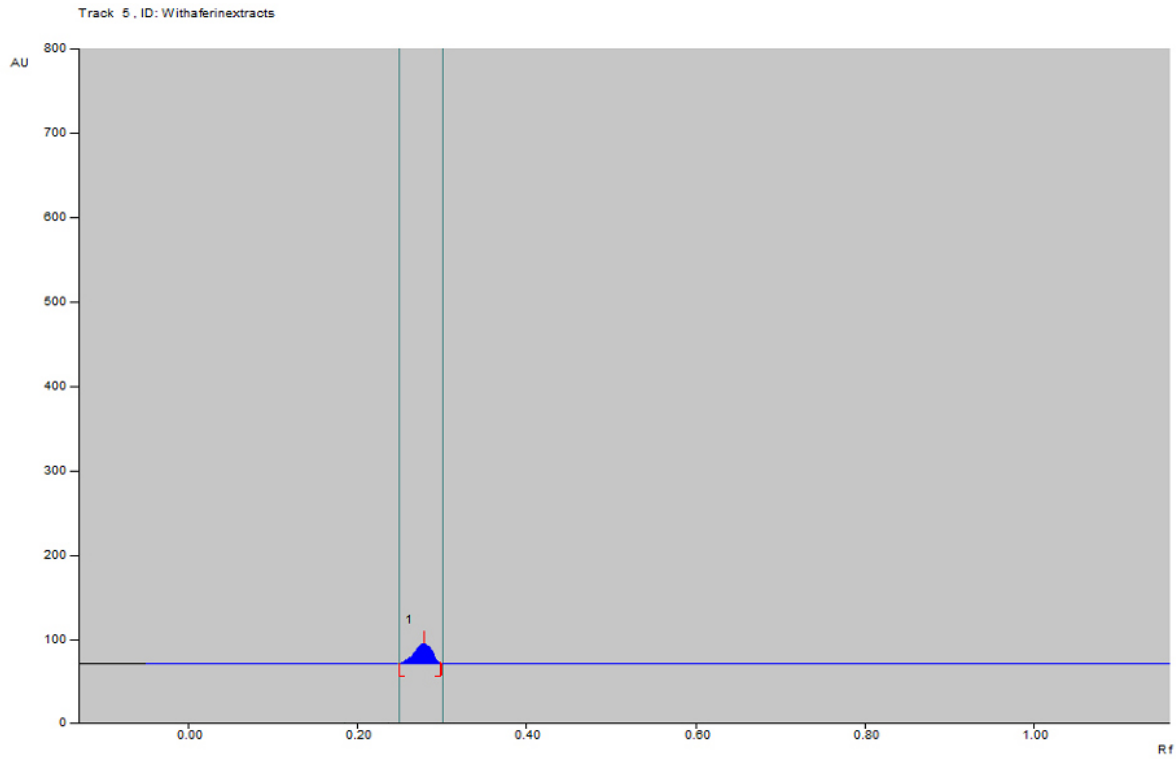

Fig. 5 Chromatogram of Withaferin-A in marketed herbal formulation Ashwagantha chooranam Trader 2 sample WSR2

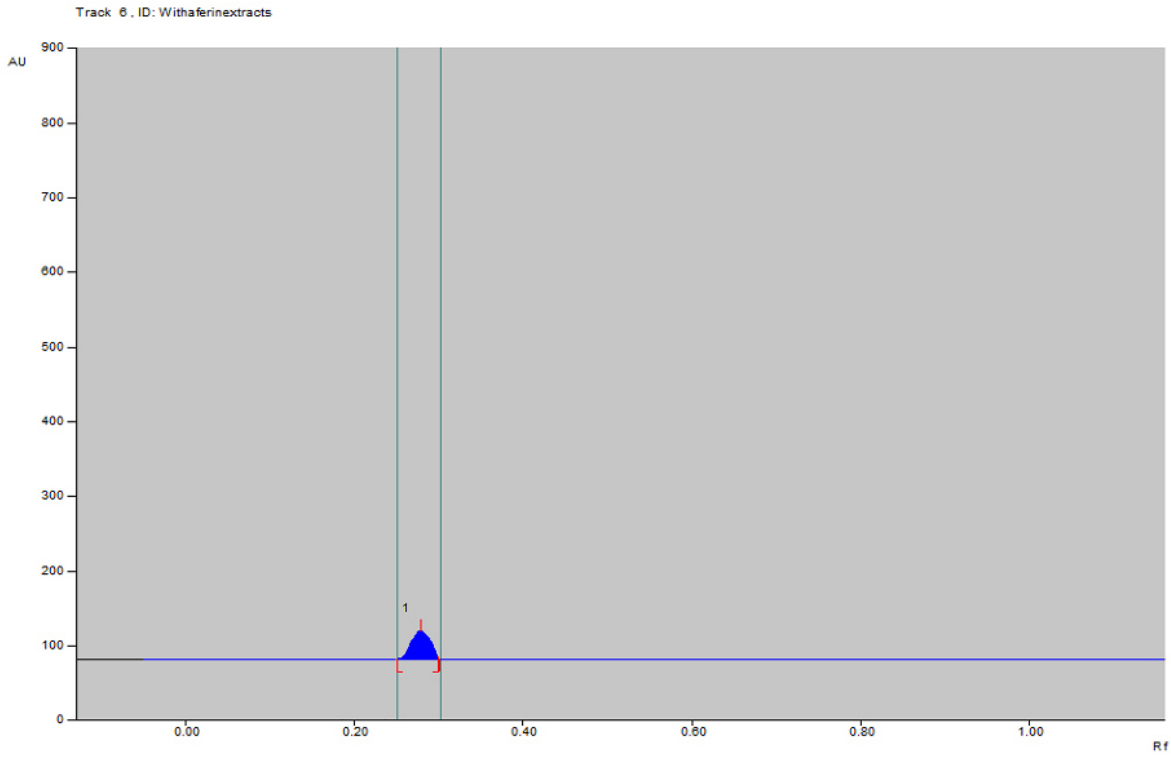

Fig.6 Chromatogram of Withaferin-A in marketed herbal formulation Ashwagantha chooranam Trader 3 sample WSR3

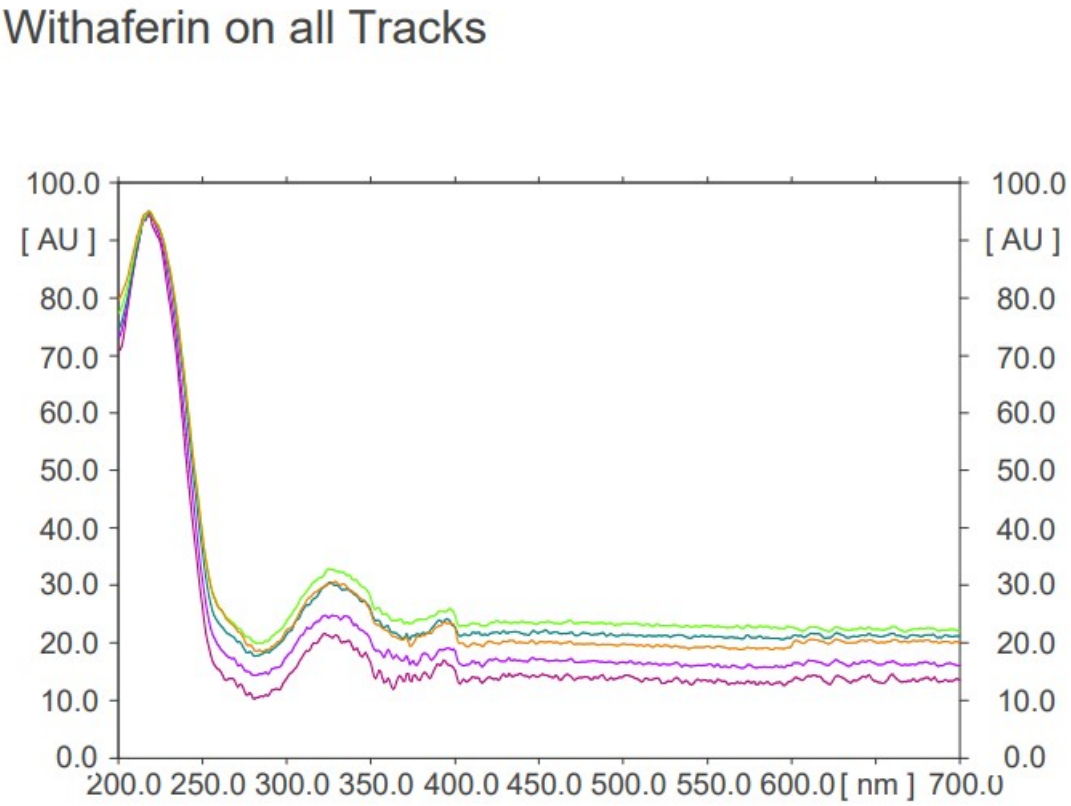

Fig. 7 Overlain spectra of Withaferin-A in standard and sample WSR, WSR1, WSR 2 and WSR 3

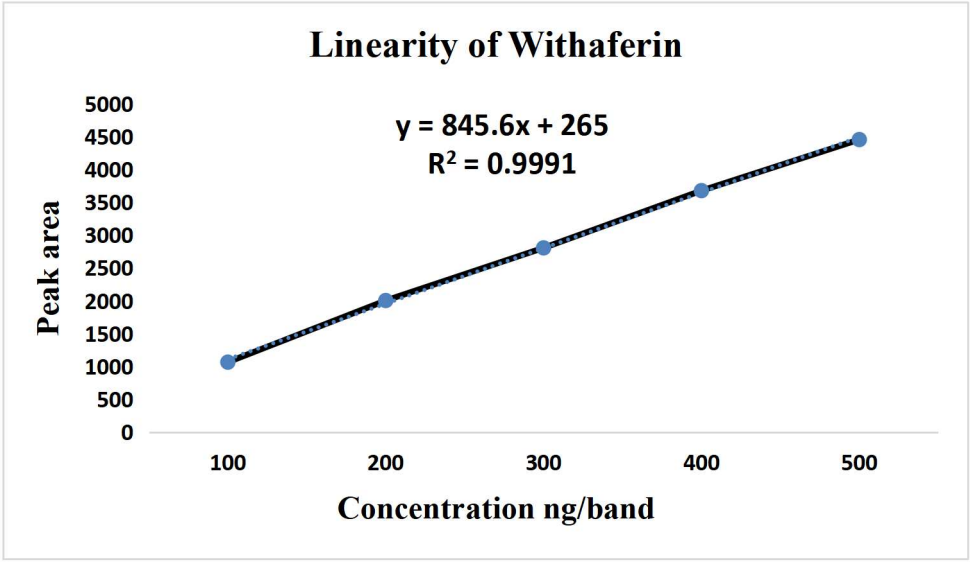

Fig. 8 Linearity plot of Withaferin-A

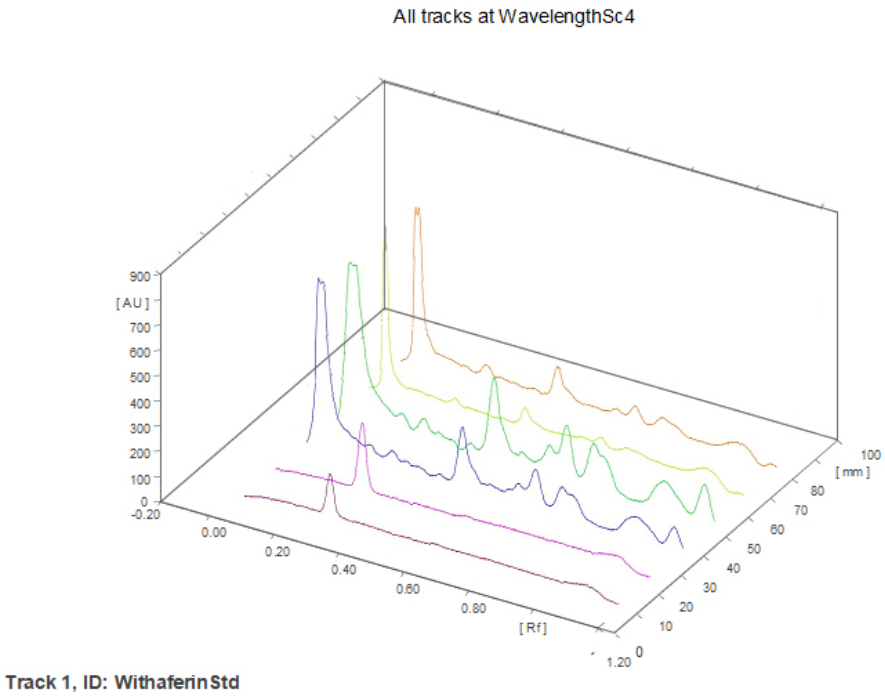

Fig. 9 3D spectra of Withaferin-A and WSR, WSR 1, WSR 2 and WSR 3

The accuracy of the method analysed through percentage recovery of withaferin A showed values of 98.08 to 100.97 % (Table 2), which were within the acceptable limits (98.0 to 102.0 %). The low values of SD and % RSD (Table 3) obtained after introducing small deliberate changes in the developed method indicated the robustness of the method.

With respect to suitability, the RSD of apigenin peak areas and R_f values as calculated were 1.29 and 0.11, respectively which were within theacceptable limits (< 2). This indicates that the developed method is suitable for the intended purpose.

The amount of Withaferin-A present in the WSR, WSR1, WSR 2 and WSR 3 was successfully quantified by the HPTLC method using toluene, ethyl acetate and formic acid in the ratio of 5.5:4.3:0.2 v/v/v as mobile phase. The content of withaferin A in *W.*

somnifera extract WSR was found to be 1.08%. Amount of Withaferin-A present in WSR1, WSR 2 and WSR 3 were 0.92, 0.99 and 0.91% w/w respectively.

Table 1: Intraday precision data of Withaferin-A

Withaferin-A concentration (ng/band)	Intra-day precision		Inter-day precision	
	Concentration found* (Mean ± SD)	%RSD	Concentration ound*(Mean ± SD)	%RSD
100	100.03 ± 0.06	0.60	100.48 ± 0.63	0.63
200	200.50 ± 1.20	0.60	200.65 ± 1.13	0.56
300	301.55 ± 1.11	0.37	300.37 ± 1.11	0.31

*Average of six determinations

Table:2 Accuracy data of Withaferin-A

% Standard Withaferin-A added to WSR 1mg	Theoretical calculation (Withaferin-A ng)	Amount found (Withaferin-A ng)	RSD (%)	Recovery (%)
50	15.7	15.4 ± 0.21	1.85	98.08
100	20.6	20.8 ± 0.19	1.29	100.97
150	25.8	25.9± 0.26	1.37	100.38

***Average of three determinations**

Table 3: Robustness studies of Withaferin-A

Optimization conditions	Withaferin-A	
	200 (ng/band)* ±SD	% RSD
Mobile phase volume		
18 mL	200.52 ± 0.59	0.29
20 mL	200.25 ± 0.34	0.18
22 mL	200.53 ± 0.82	0.29
Saturation time		
18 min	200.27 ± 0.75	0.37
20 min	200.19 ± 0.61	0.24
22 min	200.12 ± 0.53	0.19
Duration of pre conditioning of plate		
3min	200.25 ± 0.50	0.25
5 min	200.15 ± 0.23	0.31
7min	200.07 ± 0.38	0.33

**Average of three determinations*

4. CONCLUSION

A HPTLC method has successfully been developed and validated for the quantification of withaferin A in *Withania somnifera* (Ashwagandha). The method can also be applied as a quality control tool and thus can facilitate thestandardization of plant-based medicines for defined content of bio-actives to ensure their quality and safety.

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