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Development of validated HPTLC and UHPLC methods for quantification of Withanolide in extract and formulation

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Abstract: High performance thin layer chromatography (HPTLC) and Ultra-High-Performance Liquid Chromatography (UHPLC) techniques were developed and validated to quantify Withanolide in extract and formulation. On Al-backed silica gel 60 F254 TLC plates (10 cm × 10 cm, layer thickness 0.2 mm), which had been prewashed with methanol, HPTLC separation was carried out. Dichloromethane: Methanol: Toluene: Acetone in various ratios produced good separation in mobile phase (5:1:1:0.5 v/v). Camag TLC scanner densitometric scanning at 365 nm determined and quantified. This approach produced compact Withanolide spots at Rf 0.48. ICH guidelines verified HPTLC's precision, reproducibility, and accuracy. Withanolide linearity was 500-3000 ng/spot with R^2= 0.9994. LOD & LOQ were found to be 9.48 & 28.73 ng respectively. For UHPLC, Cosmosil C18 was used with acetonitrile: water (0.2 % OPA) (70:30, v/v) mobile phase. Flow rate was 1.5 mL/min. Under optimal chromatographic conditions, Withanolide was retained for 5.9 min and detected at 254 nm. ICH guidelines verified UHPLC's precision, repeatability, and accuracy. Withanolide linearity was 10-60 µg/mL with R^2= 0.9994. LOD along with LOQ were 0.411 and 1.245 µg. HPTLC and UHPLC procedures utilized for regular quality control and quick screening of active components from plant extracts.

Introduction

Now more than ever, scientists are interested in plant-based bioactives for the health benefits they deliver from high-value secondary metabolites. Phytomedicines treat roughly eighty percent of the worldwide population, particularly in developing nations (Ashraf et al., 2016), given the current state of healthcare in the world. Phytomedicines have few adverse effects but are affordable, which contributes to the continued success of traditional therapy systems (Anwar et al., 2016; Jana et al., 2018; Nazar et al., 2020).

In the medical community, plants from the genus Withania in the family Solanaceae are held in high regard for their significant therapeutic and nutritional value. W. somnifera (Figure 1) was one of the plants in this genus that had a wide variety of therapeutic and

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pharmacological purposes. The xerophytic plant *Withania somnifera* (Syn: winter cherry, ashwagandha, suranjan, Indian ginseng) thrives on rich soils over the African continent, the Mediterranean region (Kalra et al., 2017; Verma et al., 2011; Singh et al., 1998; Hepper, 1991; Saleem et al., 2020). Ayurvedic medical tradition viewed the plant's roots and leaves as phytotherapeutic substances that capacity to treat extensive range of conditions. The plant has shown promise in treating hepatotoxicity (Bhattacharya et al., 2000), neurological (Tomoharu et al., 2005), anxiety (Sairam et al., 2000), Parkinson's (Ahmad et al., 2005), and hyperlipidemia (Visavadiya et al., 2007) in clinical and preclinical research. The leaves were effective at warding off insects, and the fruits had high concentrations of saponins (Bala et al., 2004).

Withania somnifera contains the scientifically active steroidal lactones Withanolides (Afewerky et al., 2021; Orabi et al., 2023), as shown by a phytochemical study. The group of alkaloids known as withanine was discovered in the plant's roots and accounts for 38 percent of the alkaloid weight (Hussain et al., 2023). Antitumor and cytotoxic activities (Sivasankarapillai et al., 2020) were found in the primary Withanolides isolated from W. somnifera: Withanolide D and withaferin A. Saponins, steroids, phenolics, phytophenols, flavonoids, and glycosides were also present in the plant (Polumackanycz et al., 2023; Alanazi et al., 2023; Indhira et al., 2023; Abdelwahed et al., 2023), in addition to the alkaloids. It is also utilised as a fever reducer, pain reliever, adaptogen, and anti-inflammatory in many herbal remedies (Kalra et al., 2017; Rao, 2023).

Withania somnifera (withania) is a vital component in Ayurvedic medicine because it contains the bioactive constituents Withanolide and withaferin-A. According to reports, *Withania somnifera* contains the therapeutically active compounds withaferin-A and Withanolide. The anti-inflammatory, anticonvulsive, anticancer, and antioxidant properties of Withaferin-A have been demonstrated. Ayurvedic formulations have seen increased demand in latest years appropriate to their low cost, efficiency, and low risk profile. A standardization protocol for the safety, efficacy, and quality control of Ayurvedic medications is required due to their rising popularity and demand. Consumers can feel safer using Ayurvedic products because of the methodology for standardization that has been put in place. Due to the polyherbal formulations' complex nature and intrinsic unpredictability of their bioactive chemical ingredients, it is difficult to develop quality control measures. The standardization of herbal medication quality control measures is crucial. Creating quality assurance tools that are dependable, targeted, and responsive is now crucial.

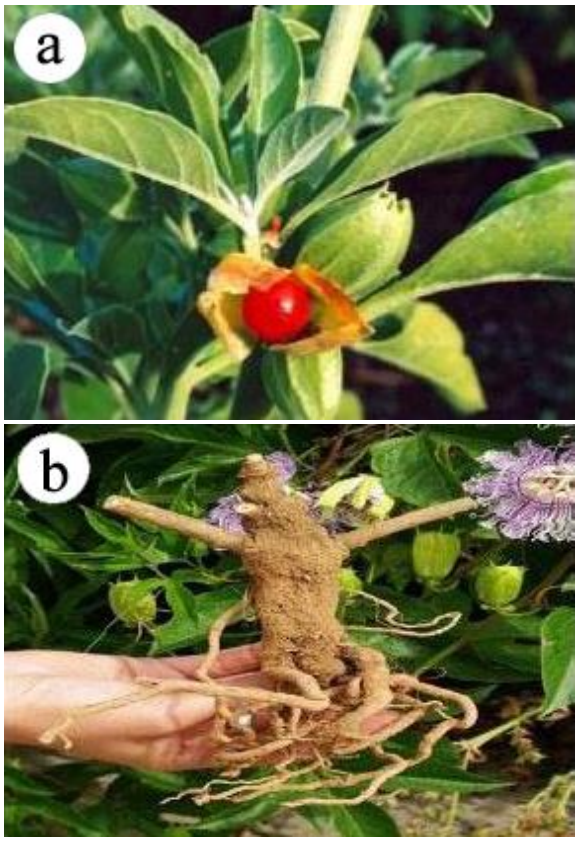

Figure 1.(a) Cherry fruit and leaves (b) *W. somnifera* root

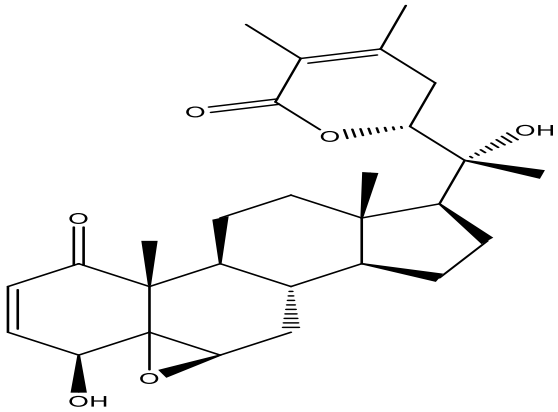

Figure 2. Structure of Withanolide

Materials and methods

Reagents

The entire chemicals were of HPLC grade (Finar Ltd., Mumbai) used without further purification.

Plant Material

Plant materials were procured from Konark Herbals & Health Care, Daman, India.

Selection of Solvents

The solubility of Withanolide in various solvents was tested, taking into account the drug's properties and the

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solvents. To dissolve the medication, methanol was ultimately used.

Method-I: HPTLC Method

Instrumentation and Chromatographic Conditions for HPTLC Method

Aluminium plate encrusted with 0.2 mm of silica gel 60 F254 was utilized for chromatography. Sample is spread in 6mm bands through CamagLinomat 5 applicator with 100 µl syringe (Hamilton, Switzerland). Space among 2 bands was 14 mm, and application rate was held constant at 150 nl sec^{-1}.

The ratio of dichloromethane to methanol to toluene to acetone in the mobile phase was (5:1:1:0.5). Plate was developed linearly upwards in a twin-trough glass chamber at 25ºC ±2 and relative humidity 60% ±5 for 15 minutes. About 80 millimetres was the length of the chromatogram run. After being removed from the developer, plate dried quickly. For densitometric scanning, we utilized a Camag TLC scanner 3 operating at 365 nm.

Preparation of standard stock solution for HPTLC Method

Weigh exactly 1mg of Withanolide and dissolve in methanol makeup volume 10ml. (obtain concentration 100 µg/ml)

Linearity Study of Withanolide for HPTLC Method

Withanolide at varying concentrations (500 ng/spot - 3000 ng/spot) was spotted onto a TLC plate using microlitre syringe with a Linomat 5 sample applicator. Under these well-established chromatographic conditions, the plate was developed then scanned. The maximum peak area for each drug concentration was measured.

Analysis of Extract for HPTLC Method

Accurately measured 1 gramme of the *Withania somnifera* extract was poured into a volumetric flask of 100 millilitre. The desired concentration was achieved by dilution with methanol. Then, 1 millilitre of this was transferred to a different volumetric flask and filled to the mark with the same solvent, diluting it to a concentration of 100 micrograms per millilitre. Spotted was the solution (10µL, 1000 ng). Concentration calculated by Regression equation.

Analysis of formulation for HPTLC Method

To determine the concentration of withanolide present twenty Ashwagandha capsules (Patanjali Ashwagandha Capsule; Manufactured by Patanjali Ayurveda, India) were finely powdered and weighed. Ten milligrammes of Withanolide powder was measured. The powder was processed using methanol extraction to obtain the Withanolide. After being 30 minutes sonication and having volume brought up to 100 mL, Withanolide was successfully extracted. A 0.45 µm filter (Milli filter, Milford, MA) was used to purify the final solution. After applying the aforementioned solution (10µL, 1000 ng each spot) to a TLC plate and scanned. Three consequent reading was taken and concentration determined by regression equation.

UHPLC Method

Instrumentation and Chromatographic Conditions for UHPLC Method

Thermo scientific, vanquish UHPLC system with quaternary pump, UV Visible detection system and chromeleon 7.2 data processor was used. The Cosmosil C_{18}, (250 mm × 4.6 mm i.d., 5 µm) was engaged in separation of Withanolide, which gives satisfactory resolution and run time. Mobile phase was optimized with a view to obtained Withanolide. Acetonitrile: Water (0.2% OPA) (70:30, v/v) was settled on as optimal mobile phase. Mobile phase flow rate was 1.5 mL/min, wavelength 254 nm & retention period of 5.9 minutes under ideal chromatographic conditions. The size of the sample used was 20 µL.

Preparation of standard stock solutions for UHPLC Method

By adding 7 milliliters of a solvent mixture to a 10-milliliter volumetric flask, a solution containing 10 milligrams of withanolide was prepared. After sonicating the solution for 15 minutes, the flask was filled to the mark with the solvent mixture. The resultant solution contained 1000 g/mL of withanolide and was well mixed by swirling.

Linearity studies for UHPLC Method

Aliquots from the stock standard solution were taken, and they were diluted with the mobile phase to a total volume of 10 milliliters in a succession of volumetric flasks to achieve a final concentration between 10-60 g/mL. The same injection volume was used for each sample.All concentrations were measured five times to construct a calibration curve, and the peak area was compared to the concentration of Withanolide.

Analysis of *Withania somnifera* extract for UHPLC Method

A 10 mg aliquot of *Withania somnifera* extract was precisely weighed and then was put into a 100 mL volumetric flask. After additional dilution, the volume was adjusted using mobile phase to provide a final extract concentration of 10.0 g/mL. The column was injected with a constant volume of 20 µL, and the resulting peak area was calculated. The linearity curve was used to

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compute the concentration. This process was repeated six times.

Analysis of formulation for UHPLC Method

20 capsules with a label claim of 500 mg were accurately weighed and powdered to ascertain the amount of withanolide present in Patanjali Ayurveda's Ashwagandha Capsule formulation. The resultant powder was weighed out and added to a volumetric flask filled with about 100 mL of a solvent combination along with 100 mg of the powder. A 0.45-micron membrane filter was used to purify the solution. The volume of the sample, which was filtered to yield 1 mL, was adjusted with diluent and thoroughly mixed before being placed in the volumetric flask. Six times the column was injected with the sample solutions.

The linearity curve was employed to ascertain the concentrations.

Method validation for HPTLC and UHPLC Method

Accuracy, specificity, precision, linearity, repeatability, LOD, and LOQ were all verified during validation per ICH guidelines (ICH Guidelines, 1995).

Accuracy

There were three stages of recovery tested: 80%, 100%, and 120%. Adding a standard drug solution of Withanolide in a known quantity to the previously analyzed sample solutions, and then putting them through the suggested HPTLC method and the UHPLC method, respectively and % recovery was calculated.

Linearity and range

Appropriate standard solutions were derived from the stock solutions. Concentration range over which Withanolide was found to be linear was determined separately for both HPTLC method & UHPLC method separately. The assay technique was applied to the solutions for analysis. Peak area versus standard solution concentration was used to generate the calibration curve. Intercept and slope were determined using the calibration curve.

Precision

Both intra-day & inter-day precision of HPTLC and UPLC were calculated. Standard solutions of Withanolide were analyzed 3 times within same day to identify within-day changes. % RSD was computed after analyzing a standard solution of Withanolide at varying concentrations for 3 consecutive days over course of a week.

Repeatability

Standard Withanolide was applied six times to evaluate sample application repeatability, and the % RSD was computed.

Sensitivity LOD and LOQ

The suggested approach's LOD and LOQ for quantifying Withanolides were determined. These values were obtained by using the equations LOD = 3.3 × SD/S and LOQ = 10 × SD/S, where 'SD' represents the standard deviation of pharmaceutical peak areas (n = 3), which is regarded a measure of noise, and 'S' represents the slope of the related calibration curve. In this study, we established both the LOD and LOQ for Withanolide.

Ruggedness

Two analysts used exact same exploratory and environmental settings to study how tough proposed method was. This method was recurring in triplicates and the Percentage RSD was calculated for both HPTLC and UHPLC method separately.

Robustness for HPTLC Method

The study aimed to assess the robustness of the method by deliberately altering a few factors. For example, stock solution's durability was evaluated by altering the mobile phase's chemical make-up. The impacts of various variables on the results were studied. 1 factor was altered at a time to determine effect, and % RSD was calculated separately for both the HPTLC and UHPLC methods.

Results and Discussion

The amount of Withanolide present was quantified using HPTLC and UHPLC. These methods can be used to check the quality of medicinal dosage forms on a regular basis.

Analysis of Extract by HPTLC Method

Percentage of drug within extract, mean and relative standard deviation was premeditated. Regression equation was used to figure out the concentration, and it was found that RSD was less than 2%. Table 1 show results (Koshy et al., 2016).

Table 1. Analysis of extract by HPTLC

Drug	Amount Taken (ng/band)	Amount Found (ng)	Amount Found %
***Withania somnifera* extract**	1000	995.65	99.565
	1000	999.64	99.964
	1000	997.69	99.769
	1000	999.64	99.964
	1000	998.87	99.887
	1000	998.36	99.836
	Mean ± SD	998.30 ± 1.504	99.83 ± 0.1504
	%RSD	1.3731	0.1373

Analysis of formulation by HPTLC method

They figured out the amount of drug in the formulation, as well as mean and relative standard deviation. Results of research indicated that amount of

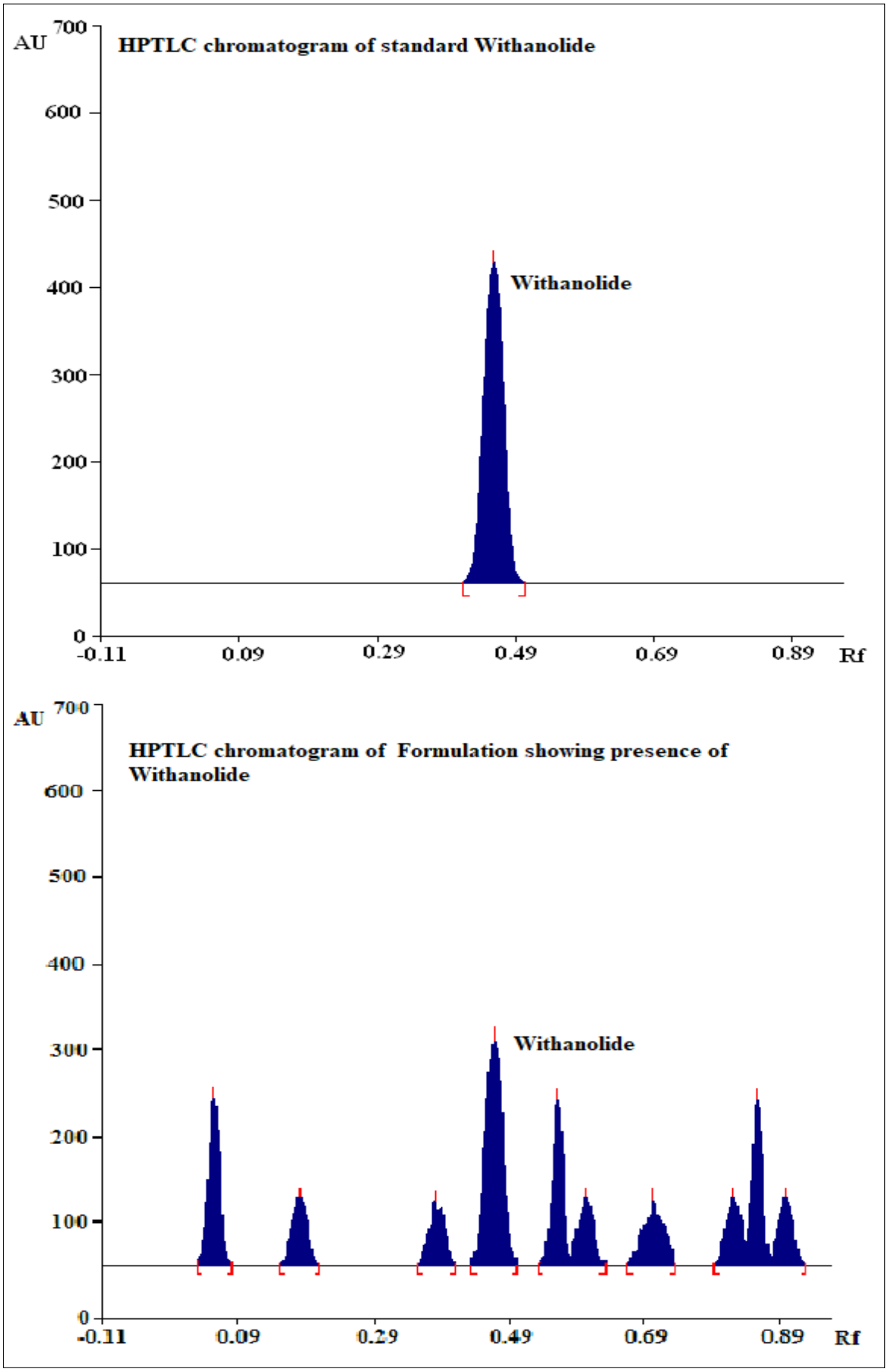

Figure 3. HPTLC chromatogram of standard Withanolide and formulation

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drug in formulation is close to what it says on the label. Proportional standard deviation was found to be less than 2% when regression equation was applied to calculate the concentration. Table 2 shows how things turned out. Figure 3 shows the HPTLC chromatogram of standard Withanolide and mixture (Koshy et al., 2016).

Table 2. Analysis of Formulation by HPTLC

Drug	Amount Taken (ng/band)	Amount Found (ng)	Amount Found %
Formulation: Patanjali Ashwagandha Capsule	1000	998.6	99.86
	1000	999.4	99.94
	1000	1001.3	100.13
	1000	995.9	99.59
	1000	994.8	99.48
	1000	997.9	99.79
	Mean ± SD	997.98 ± 2.360	99.79 ± 0.236
	%RSD	2.155162	0.215516

Analysis of *Withania somnifera* extract for UHPLC Method

Approximately ten milligrams of *Withania somnifera* extract were accurately measured, and the makeup volume by mobile phase was 100mL. It was reduced even more until there was 10.0 µg/mL of extract. The peak area was determined by injecting 20 µL of a known concentration into column. From linearity plot, the concentration was found. Six times, the same steps were done, and it was found that the relative standard variation was less than 2%. Table 3 shows the results (Ganzera et al., 2003).

Table 3. UHPLC Analysis of Withanolide extract

Drug	Amount Taken (µg/mL)	Amount Found (µg/mL)	Amount Found (%)
***Withania somnifera*extract**	30	29.8	99.3333
	30	29.98	99.9333
	30	29.87	99.5667
	30	29.94	99.8000
	30	29.98	99.9333
	30	29.99	99.9667
	Mean ± SD	29.92 ± 0.0763	99.75 ± 0.2544
	%RSD	0.06968	0.23227

Analysis of formulation for UHPLC Method

Twenty pills with a label claim of 500 mg were carefully weighed and ground into a powder to find out how much Withanolide they contained. A 100 mg amount of formulation powder was measured and makeup volume 100ml by solvent. The solution was separated using a 0.45-micron membrane filter paper. Following filtration, a 1 mL sample was transferred to a 10 mL volumetric flask, which was then filled to volume with diluent and properly mixed. The sample solutions were loaded six times into the column, and the linearity graph was used to calculate the concentrations. It was found that the relative standard variation was less than 2.0% (Table 4). A UHPLC chromatogram of standard Withanolide and formulation is shown in figures 4, 5 and 6 (Ganzera et al., 2003).

Table 4: Formulation analysis by UHPLC

Drug	Label Claim (mg)	Amount Found mg ± SD [n = 6]	% Amount found	% RSD
Formulation Patanjali Ashwagandha Capsule; Manufactured by Patanjali Ayurveda	500 mg capsule	499.4 ± 0.3829	99.88	0.382971

Method validation

By ICH guideline method is validated.

Linearity Study of Withanolide by HPTLC Method

Between 500 and 3000 ng/spot, Withanolide was found to be continuous. Withanolide was found to have a correlation value of 0.9994. Table 5 showed the uniformity range of Withanolide. Figure 7 shows calibration graph.

Table 5. Linearity study of Withanolide by HPTLC Method.

Sr. No.	Conc. of Withanolide in (ng/spot)	Peak area(means ± S.D., n = 3)	% RSD
1	500	698 ± 3	2.4499
2	1000	1387.66 ± 2.51	2.0548
3	1500	2025.33 ± 4.50	3.6817
4	2000	2646.33 ± 7.09	5.7927
5	2500	3395 ± 3	2.4499
6	3000	4004.33 ± 2.30	1.8856
*- each value is the mean of three observations			

Linearity studies by UHPLC Method

The concentration of Withanolide was discovered to be constant between 10 and 60 µg/mL. Generation of calibration curves were made by graphing peak area versus Withanolide concentration and testing each

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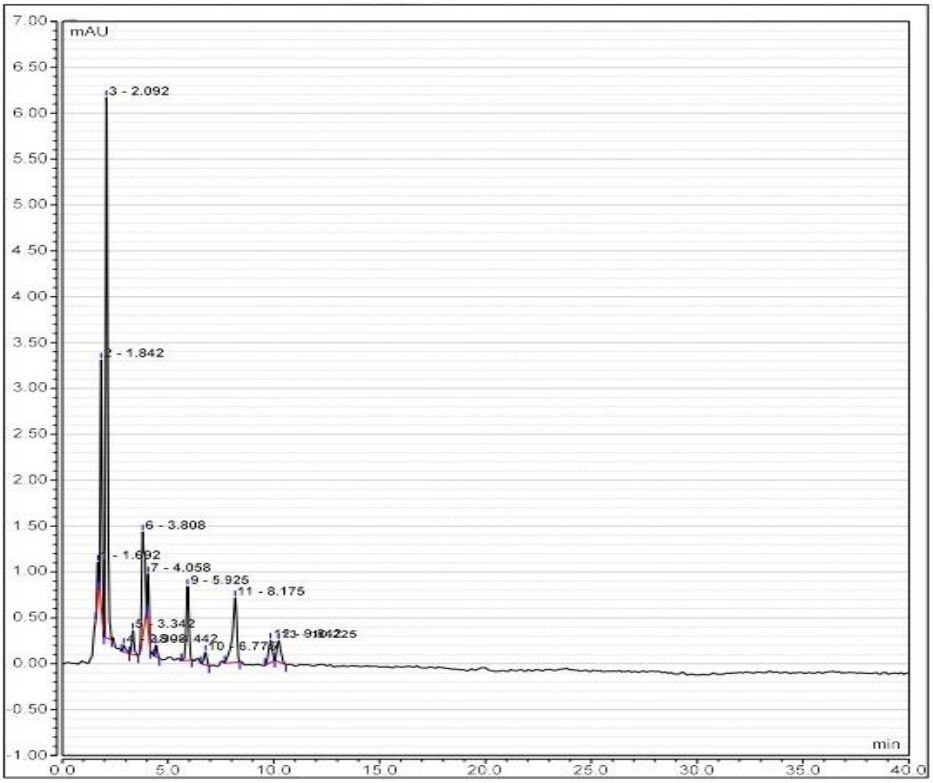

Figure 4.RP-HPLC graph of Standard Withanolide Extract

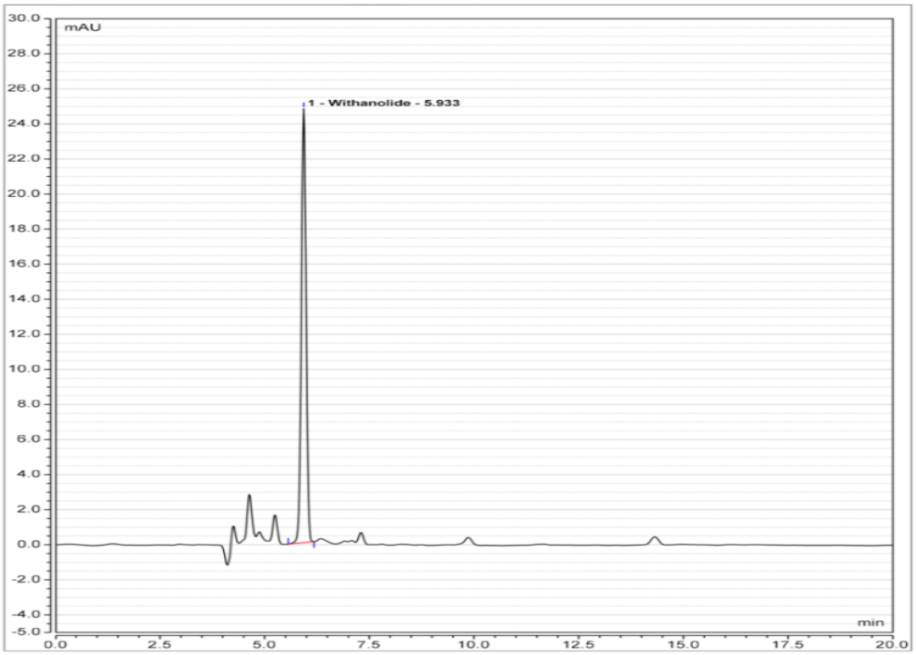

Figure 5. RP-HPLC graph of Standard Withanolide

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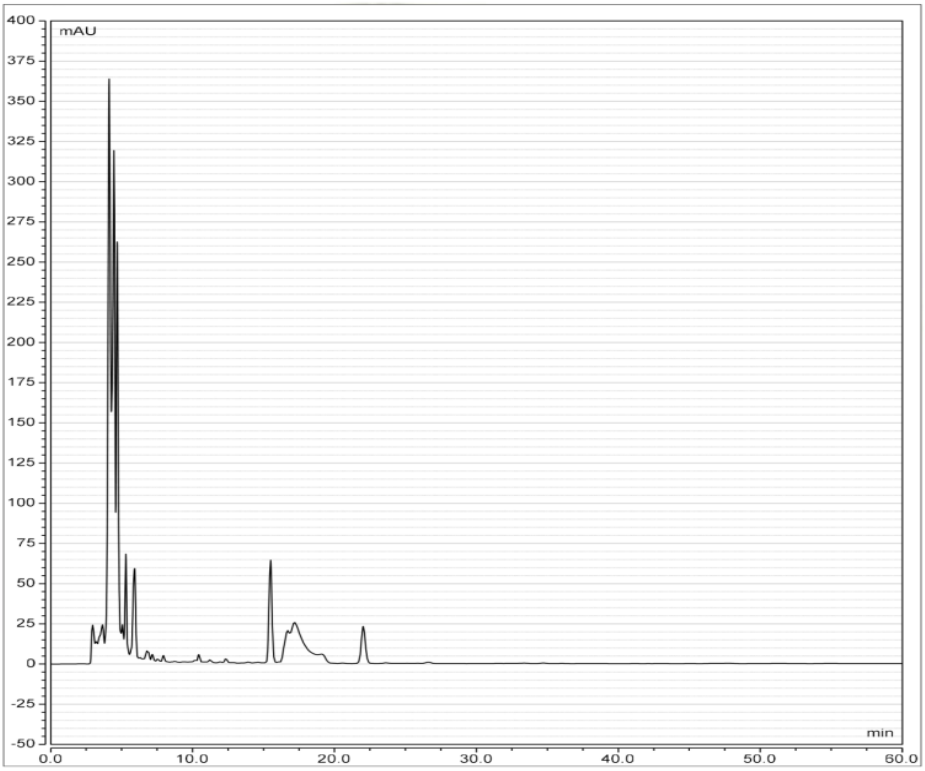

Figure 6. RP-HPLC graph of Formulation

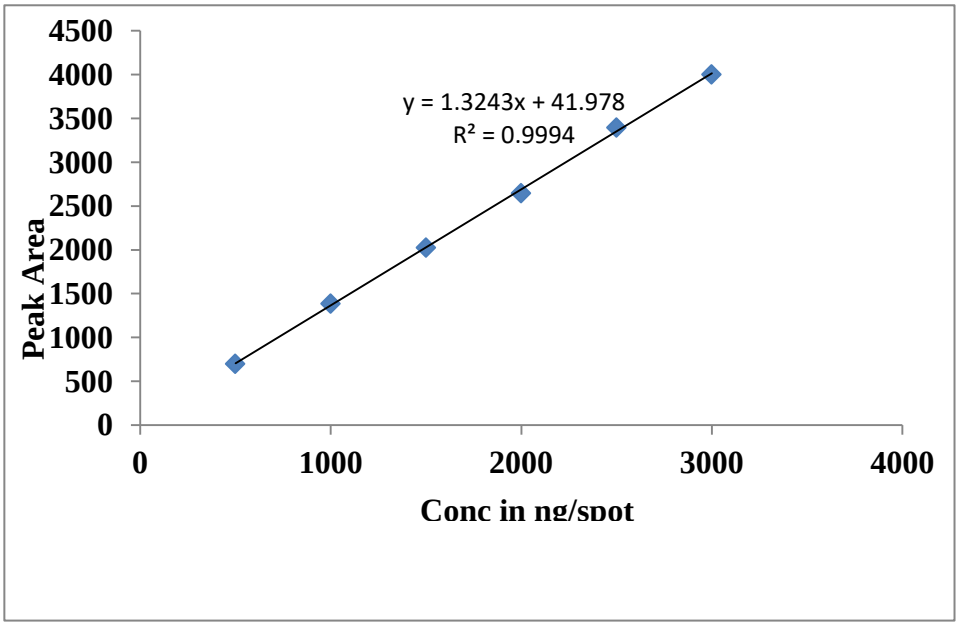

Figure 7. Calibration Curve for Withanolide

Y= 1.3243X + 41.978; Coefficient of correlation = 0.9994; Intercept = 41.978, Slope = 1.3243. The calibration curve shows that more entire range of concentrations used in assay method, a linear response was found. The range shows that the method works in a straight way.

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concentration five times. Table 6 shows the data, and Figure 8 shows the calibration curves.

Table 6. Linearity results of Withanolide (UHPLC)

Sr. No.	Concentration [µg/mL]	Peak area (mAU*min) [Mean ± SD; n = 3]	% RSD
1	10.00	2.548 ± 0.0623	00.0508
2	20.00	5.361 ± 0.0151	00.0123
3	30.00	8.105 ± 0.002	00.0017
4	40.00	10.666 ± 0.0276	0.0225
5	50.00	13.273 ± 0.0223	00.0182
6	60.00	15.715 ± 0.0671	00.0548

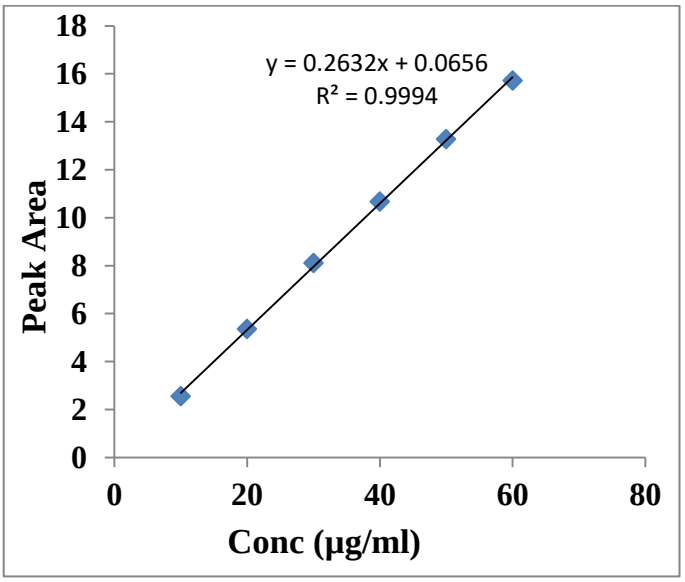

Figure 8.Calibration Curve of Withanolide

Y = 0.2632X + 0.0656;

Slope = 0.2632; Intercept = 0.0656; Correlation = 0.9994

As a result, linearity characterizes the UHPLC technique for Withanolide.

Accuracy by HPTLC Method

Experiments on recovery were done at three levels: 80%, 100%, and 120%. Three different amounts of a normal drug solution of Withanolide were added to the sample solutions that had already been tested. The chromatogram was made, and then it was scanned. Table 7 shows the data of % recovery.

Accuracy by UHPLC Method

It was accomplished using recovery research employing conventional method of addition at intensities of 80%, 100%, and 120%. A previously determined quantity of standard Withanolide was added to a sample that had already been tested (10.0 µg/mL of Withanolide) and then put through the suggested UHPLC method. Table 8 shows that the healing rate was between 98% and 102%.

Table 7. Recovery studies for Withanolide by HPTLC method

Drug	Initial Amount [ng]	Added amount [ng]	Recovered amount ± S.D.	% Recovered	% R.S.D.
Withanolide	1500	1200	2698.5 ± 0.8185	99.94	0.6683
	1500	1500	2996.8 ± 4.3485	99.89	3.5505
	1500	1800	3299.43 ± 0.5033	99.98	0.4109

Table 8. Recovery data for Withanolide by UHPLC method

Drug	Initial amount [µg/mL]	Excess drug added to analyte [%]	Amount recovered ± S.D. [µg/mL]	Recovery [%]	%RSD [n = 3]
Withanolide	10	80	17.97 ± 0.01	99.83	0.0081
	10	100	19.94 ± 0.0472	99.71	0.0385
	10	120	21.96 ± 0.0152	99.84	0.0124

Precision (Intra- day & Inter- day precision) by HPTLC method

Variations within and between days were used to figure out how accurate the method was. By analyzing 1500, 2000, and 2500 /spot of standard solution of Withanolide 3 times on the same day, intra-day differences were found. Inter-day accuracy was measured by looking at 1500, 2000, and 2500 ng/spot of standard solution of Withanolide for three days in a row over a week. Table 9 and Table 10 show the findings.

Table 9. Precision studies for Withanolide(Inter - day) by HPTLC method

Drug	Conc. [ng/spot]	Inter-day			
		Peak area Mean ± S.D.	% RSD [*n* = 3]	Amount found	% Amount Found
Withanolide	1500	2027.53 ±2.5006	2.0417	1499.2 ±0.3605	99.94
	2000	2651.03 ±1.9553	1.5965	1999.86 ±1.193	99.99
	2500	3401.26 ±1.5275	1.2472	2499.53 ±0.2081	99.98

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Table 10. Precision studies for Withanolide (Intra – day) by HPTLC Method

Drug	Conc. [ng/spot]	Intraday			
		Peak area Mean ± S.D.	% RSD [*n* = 3]	Amount found	% Amount Found
Withanolide	1500	2020.73 ±1.5502	1.2657	1499.63 ±0.1527	99.97
	2000	2651.83 ±2.1594	1.7632	1999.4 ±0.4358	99.97
	2500	3402.4 ±0.7549	0.6164	2498.87 ±0.2516	99.95

Precision by UHPLC Method

Repeatability and middle precision studies were done to make sure that the method was accurate. To study intra-day precision, Withanolide was measured at 10, 20, and 30 µg/mL 3 times on same day. The accuracy between days was tested by analyzing same quantity on 3 different days over a week. It was found that the RSD was less than 2%. Table 11 shows how things turned out.

Table 11. Results of Precision study for Withanolide by UHPLC method

Drug	Conc. [µg/mL]	Amount Found Intra –day [µg/mL] [n = 3]		Amount Found Inter- day [µg/mL] [n = 3]	
		Mean	% RSD	Mean	% RSD
Withanolide	10	9.97 ± 0.01	0.0124	9.99 ± 0.0152	0.0124
	20	19.96 ± 0.01	0.0081	19.95 ± 0.0251	0.0205
	30	29.93 ± 0.04	0.0326	29.94± 0.0230	0.0188

Repeatability by HPTLC method

Repeatability of sample application was tested by putting 15 µL containing 1500 ng/spot of standard Withanolide on a TLC plate 3 times, letting it develop, and scanning it. The results are shown in Table 12. The spot that was split was scanned six times without moving the plate. It was found that RSD was less than 2

Table 12. Repeatability data of Withanolide by HPTLC Method

Sr. no.	Application volume [µL]	Area of Withanolide
1	15	2021.3
2	15	2020.6
3	15	2022.6
4	15	2021.9
5	15	2020.1
6	15	2019.4
Mean		**2020.98**
S.D.		**1.1822**
%R.S.D.		**1.0792**

Repeatability by UHPLC method

Analyzing 10 µg/mL of Withanolide six times was used to measure repeatability. Table 13 shows how things turned out. The spot that was split was scanned six times without moving the plate. It was found that the RSD was less than 2%.

Table 13. Repeatability study for Withanolide by UHPLC Method

Sr. No.	Conc. (10 µg/mL)	Amount Found
1	10	9.99
2	10	9.97
3	10	9.96
4	10	9.99
5	10	9.97
6	10	9.98
Mean		9.97666667
S.D.		0.0121
%R.S.D.		0.0110

Sensitivity by HPTLC Method

The sensitivity of the suggested method for measuring Withanolide was assessed using the limit of detection (LOD) and limit of quantification (LOQ). The formulas used to determine the LOD and LOQ were LOD = 3.3 × N/B and LOQ = 10 × N/B, respectively, where 'N' stands for the standard deviation of peak areas of drugs (n = 3), which is an indicator of noise, and 'B' stands for the slope of the related calibration curve. Y = 1.3011X + 92.24 was discovered to be linearity equation. The LOD and LOQ for Withanolide were found to be 9.48 ng and 28.73 ng, respectively.

Table 14. Results of sensitivity studies of Withanolide

Sr. no.	Application volume [ng/spot]	Area of Withanolide Mean ± SD	% RSD
1	500	698 ± 3	2.4499
2	1000	1387.66 ± 2.51	2.0548
3	1500	2025.33 ± 4.50	3.6817
4	2000	2646.33 ± 7.09	5.7927
5	2500	3395 ± 3	2.4499
LOD = (3.3* Avg SD) / Slope		9.48	
LOQ = (10* Avg SD) / Slope		28.73	

Sensitivity by UHPLC Method

Quantitation limit is the minimum detectable concentration of a substance in sample matrix that can be detected using a quantitative test. Finding contaminants and/or degradation products is where it really shines. The formulas LOD = 3.3 (SD)/S and LOQ = 10 (SD)/S, where SD stands for the standard deviation of the response and S for the slope of the calibration curve, were used to compute the limits of detection (LOD) and

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quantification (LOQ). Withanolide's LOD and LOQ were established to be 0.411 µg and 1.245 µg, respectively.

Ruggedness by HPTLC Method

Two different analysts looked at how hardy the planned method was using the exact same experimental and environmental conditions. On HPTLC plates, 1500 ng/band of Withanolide was put on each band. Bands were made and scanned in the same way that was explained above. This process was done three times, and the percent RSD was less than 2%. Table 15 shows how things turned out.

Table 15. Results of ruggedness of Withanolide by HPTLC Method

Analyst	Amount found of Withanolide (%)	% RSD [n=3]
I	099.98	0.0489
II	099.67	0.2309

Ruggedness by UHPLC Method

From stock solutions, a sample solution of Withanolide at a concentration of 10 µg/mL was prepared and analyzed using identical procedures and equipment by two different analysts. The peak area of the solution was measured six times at the same concentration. It was found that the RSD was less than 2%. Table 16 shows how things turned out.

Table 16. Ruggedness study Withanolide by UHPLC Method

Drug	Amount in µg/mL	Amount Found [n = 6] Mean ± SD		% Amount Found [n = 6]		% RSD	
		Analyst I	Analyst II	Analyst I	Analyst II	Analyst I	Analyst II
Withanolide	10	9.96 ± 0.0147	9.94 ± 0.0499	99.68 ± 0.1471	99.41 ± 0.4996	0.013437	0.045613

Robustness by HPTLC Method

The robustness of the method was evaluated by systematically altering several variables, including composition at the mobile phase and the stock solution's stability. The impact of each variable was investigated by varying one factor at a time by maintaining a constant amount of Withanolideat 1000.0 ng/band. Outcome is revealed in table 17.

Table 17.Robustness Withanolide by HPTLC Method.

Parameters	Withanolide	
	SD of peak area	% RSD
Mobile phase composition		
a Dichloromethane: Methanol: Toluene: Acetone (5:1:1:1)	22.98	0.21
b Dichloromethane: Methanol: Toluene: Acetone (5:1:1.5:0.5)	13.43	0.13
Mobile phase volume (mL)		
6	13.16	0.12
10	09.35	0.08
Development distance (mm)		
70	16.75	0.34
75	06.05	0.11
80	11.89	0.24
Relative humidity (%)		
55	14.72	0.154
65	12.85	0.11
Duration of saturation (min)		
20	20.21	0.15
25	16.75	0.11
30	10.29	0.09
Activation of prewashed TLC Plates (min)		
08	10.56	0.16
10	4.29	0.04
12	6.49	0.06
Lag time between detection and chromatography	5.22	0.04
Time from chromatography to scanning	13.18	0.11

Robustness by UHPLC Method

The method's robustness was investigated by systematically altering a few of operating conditions. Withanolide injections at 10 µg/mL were used to examine the effects of varying just one variable at a time, with the results summarized in table 18.

Table 18. Results of Robustness Studies Withanolide by UHPLC Method.

Parameters	Withanolide R_t
Acid Concentration change for pH adjustment	
00.15%	6.5
00.2%	5.9
00.3%	5.1
Flow Rate change (ml/min)	
00.8	10.4
01.2	7.8
01.5	5.9
Mobile phase composition change	
(60 : 40 *v/v*)	12.6
(65 : 35 *v/v*)	10.9
(70 : 30 *v/v*)	5.9

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Conclusion

Simple, accurate, reproducible, robust and cost effective HPTLC and& UHPLC chromatographic methods requiring simple reagents were developed and reproducibility and selectivity in quantifiable assessment of Withanolide in formulation were demonstrated statistically. It was determined that the created procedures provided various benefits, including rapidity, cost-effectiveness, a simple mobile phase, and good concordance with the drug's label claim. Withanolide was successfully determined despite the presence of additives in the pharmaceutical formulation of the sample used in the experiment. Withanolide in its dose form can be routinely analyzed using HPTLC and UHPLC techniques, as they are not affected by the presence of excipients.

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Conflict of Interest

None

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