Stability indicating studies on NMITLI 118RT+ (standardized extract of *withania somnifera* dunal)

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ABSTRACT

Background: *Withania somnifera* Dunal *(Ashwagandha*) is an Indian medicinal plant of great medicinal value; used in many clinically proven conditions. NMITLI-118RT+ is a candidate drug under a Council of Scientific and Industrial Research (CSIR) networking project. It is a chemotype of *W. somnifera*'s root extract, which has been used for the present study. **Objectives:** The present investigation aims to develop and validate a simple isocratic reverse phase-high performance liquid chromatography (RP-HPLC) system for the detection and estimation of Withanolide A (marker compound) and its analytical application for stability indicating studies on NMITLI-118RT+. **Material and Methods:** A validated RP-HPLC method for Withanolide A was established on a Waters HPLC system and the same was used on NMITLI-118RT+ for quantification and fingerprinting purposes, and for establishing forced degradation, isothermal stress tests, and drug-excipient testing protocols as per International Conference on Harmonization (ICH) guidelines. **Results:** A validated method was established, which could detect the marker at a retention time of around 6.3 minutes, with a linearity range of 2–100 µg/mL, by varying the amounts of the said marker, which were estimated in four different batches of NMITLI-118RT+. Photostability as per ICH guidelines suggested a slight loss of the active constituent and maximum degradation was afforded with alkali followed by acid, and then peroxide, in the forced degradation studies. In the drug-excipient studies, the maximum amount of active constituent could be detected in the samples with ethyl cellulose and the least with hydroxy propyl cellulose. **Conclusion:** The method developed here was simple and rapid. The various stability indicating studies carried out in the present investigation would be useful for formulation development and were suggestive of deciding the recommended storage conditions for NMITLI-118RT+.

Key words: *Ashwagandha*, excipients, forced degradation, photostability, Withanolide A

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INTRODUCTION

Out of the many species of genus *Withania* (Solanaceae), *Withania somnifera* Dunal also known as *Ashwagandha*, Indian ginseng or winder cherry, is the most important plant of medicinal significance in the traditional Indian systems of medicine such as Ayurveda, Unani, and Siddha for over 3000 years. Some important classical uses of the plant include its use as an aphrodisiac, in liver tonics, as an anti-inflammatory, and for bronchitis, asthma, ulcers, emaciation, insomnia, and senile dementia. The significant medicinal potential of *W. somnifera* is mainly associated with the presence of unique classes of steroidal lactones called Withanolides. These are ergostane-based moieties; triterpenoidal in origin, and include pharmaceutically active molecules like Withanolide A [Figure 1], Withaferin A, Withanolide D, Withanone, and so on.[1-3]

Some of the clinically proven properties of *W. somnifera,* in recent times, happen to be cell cycle disruption and an anti-angiogenic potential.[4] It has also been demonstrated to possess adaptogenic, anti-inflammatory, antioxidant, anti-platelet, antihypertensive, hypoglycemic, and hypolipidemic effects, which may contribute to its cardioprotective properties.[5] Its anti-stress and anti-hypertensive effects have also been validated by Kushwaha *et al.,* 2012.[6] *Ashwagandha* is also said to possess antitumor, anti-stress, immunomodulatory, hemopoetic, and rejuvenating properties.[7] Its anxiolytic and antidepressant potential has also been reported by Bhattacharya *et al.*[8]

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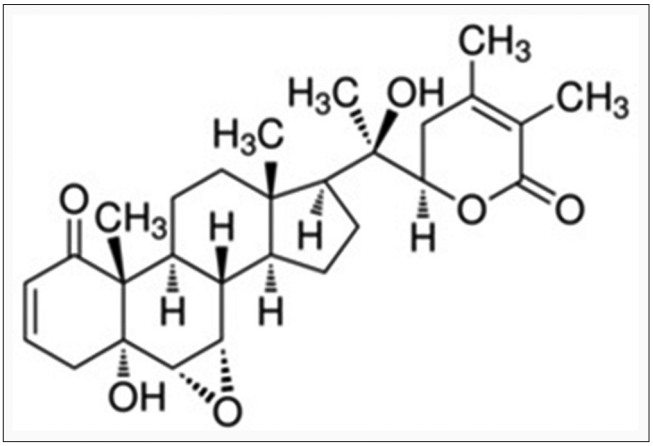

Figure 1: Chemical structure of Withanolide A

The potential immunoprophylactic efficacies of several root extracts of *W. somnifera* chemotypes, NMITLI-101, NMITLI-118, and NMITLI-128, against an infective filarial pathogen were also studied.[9]

Many approaches for a broad-based chemical analysis have been used to identify targeted and non-targeted metabolites in the roots and leaves of *W. somnifera* and to quantify them. Sixty-two major and minor primary and secondary metabolites from the leaves, and 48 from the roots were unambiguously identified.[1] Phytochemical analysis and its correlation to genetic factors has been dealt with by Dhar *et al.*[2] Quantitative HPLC methods for Withanolide D and Withaferin A have also been reported.[10] However, assuming the generalization, most reported methods[11] on Withanolides are gradient-based methods, which increase the time of analysis. The authors report a simple, rapid, isocratic-validated reversed-phase-high performance liquid chromatography (RP-HPLC) method for the detection and quantification of Withanolide A (an active marker compound) and its utilization in a different stability, indicating analytical studies on a *Withania somnifera* extract (NMITLI-118RT+), a chemotype drug candidate, under preclinical development. The authors aim to generate data useful for further studies and formulation development on the said candidate drug.

MATERIALS AND METHODS

Withanolide A and NMITLI-118RT+ extracts derived from *Withania somnifera* L. were obtained from CSIR-CIMAP (Central Institute of Medicinal and Aromatic Plants), Lucknow, India. All solvents employed in the study were of HPLC grade; supplied by Merck India Ltd. Milli-Q pure water was obtained from a Millipore Elix water purification system purchased from Millipore India Pvt. Ltd., (New Delhi, India). Other reagents used were of analytical grade. Sodium hydroxide pellets, sodium bicarbonate, sodium metabisulfite, hydrochloric acid and hydrogen peroxide, and acetic acid used for forced degradation studies were purchased from SD Fine-Chem Ltd., Mumbai, India. The excipients, namely, microcrystalline cellulose, starch, dextrose, lactose, talc, stearic acid, magnesium stearate, and zinc stearate were purchased from SD Fine-Chem Ltd., Mumbai, India, while ethyl cellulose, hydroxy propyl cellulose, Eudagrit L 100, and PEG 6000, were supplied by Sigma Aldrich.

Instrumentation

High performance liquid chromatography

chromatographic conditions: The Waters HPLC (Milford, MA, USA) system used was equipped with a binary gradient pump (model 515, Waters), an autosampler injector (Model 2707, Waters), and a diode array detector (Model 2998, Waters). The Waters HPLC interface and Empower 2 software were utilized for data acquisition. The HPLC resolution of Withanolide A was achieved on an RP-18e Lichrosphere® column (250 × 4 mm, 5 µm, Merck, Germany) at 30 ± 3ºC utilizing the mobile phase consisting of acetonitrile: Water (1:1); flow rate of 1.0 mL/minute, and injection volume that remained at 20 µL. The column effluent was monitored at 240 nm (PDA detection).

Photostability chamber

Photostability chambers (Thermolab, Mumbai, India) were equipped with ultraviolet (UV) and visual light tubes.

Sample preparation

Withanolide A

About 1 mg of Withanolide A was accurately weighed and dissolved in 100 µL dimethyl sulfoxide and subsequently the volume was made up to 10 ml with methanol, to achieve a concentration of 100 µg/mL. Further dilutions in the range 2 to 50 µg/mL were made from this stock by the serial dilution method.

NMITLI 118RT+

Test solutions of 1 mg/mL were prepared in methanol-water (1:1)

Validation of the method

Limit of detection and limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) values were estimated as being thrice and ten times the signal-to-noise ratio, respectively.

Linearity

The detector response linearity was assessed by preparing the calibration sample solutions of Withanolide A (2, 5, 10, 20, 35, 50 µg/ml) from a stock solution of 100 µg/ml. The regression curve was obtained by plotting the peak area versus concentration. The percentage relative standard deviation (%RSD) of the slope and Y-intercept of the calibration curve were calculated.

Method precision and accuracy

The precision and accuracy of the method was checked by replicate injections ($n = 3$) on the same day and repeated on three different days using three different concentrations of Withanolide A. The system precision and method precision were performed for six successive injections. The inter- and intraday precision of the method was measured in terms of percentage coefficient of variation (%CV).[12] The accuracy was determined based on percent bias according to the following formula

$$\% \text{ bias} = \frac{[\text{Mean conc. found - conc. spiked}]}{\text{conc. spiked}} \times 100$$

Robustness

In order to determine the robustness of the method, the experimental conditions were purposely altered and its response to the chromatographic resolution of Withanolide A was evaluated. Robustness was defined as the ability of the method to remain unaffected by small changes in parameters, such as, the flow rate, mobile phase composition, and column temperature.

Recovery

Recovery assays were carried out by spiking the solutions with known amounts of Withanolide A. Recovery was calculated by the following formula:

Recovery% = (Amount detected) ×100/(Amount spiked)%

Solution stability and mobile phase stability

Stability of Withanolide A in a solution at analyte concentration was studied.

Fingerprint profile of NMITLI 118RT+

Four batches of NMITLI-118RT+ (maintained under refrigeration) were analyzed for determination of their Withanolide A content and fingerprint pattern. Test samples (1 mg/ml) along with standard Withanolide A calibration points were run and analyzed further.

Photostability studies

The studies were conducted in accordance with the concerned International Conference on Harmonization (ICH) guidelines.[13] The photostability chamber was calibrated initially, before initiating the studies. The calibration results revealed that light exposure for visual light was 318 hours and for UV light it was 75.57 hours, to achieve 1.2×10^6 Lux. On this basis the study was designed for a period of three days for UV light (withdrawal on third day) and 14 days for visual light (withdrawals were divided into three time points on day 4, day 8, and day 14), the initial values were taken as control (i.e., day 0 samples). A parallel study was also undertaken on samples exposed to UV light, which were continually extrapolated for visual light (i.e., UV + visual light treatment). Withanolide A content was estimated for all samples.

Forced degradation studies

Sample extracts (NMITLI-118RT+) and the standard Withanolide A underwent heat treatment (refluxed), at 40°C, with three different reagents, alkali (0.1 N NaOH), acid (0.1 N HCl), and peroxide (3% H_2O_2), for one hour, followed by neutralization with specific chemical agents namely acetic acid, sodium bicarbonate, and sodium metabisulfite, for alkali, acid, and peroxide, respectively, making up the volume to 10 ml with methanol–water. These prepared solutions were analyzed for their Withanolide A content by HPLC. An untreated comparator was also analyzed by HPLC, as also the standard Withanolide A, to facilitate calculations of % degradation in each of the test samples.[14]

Drug excipient studies

Interaction between the active constituent and excipients can alter the stability and bioavailability of drugs, thereby, affecting their safety and/or efficacy. Hence a careful choice of excipients is imperative for the formulation of an effective dosage form, and the pharmaceutical development of solid dosage forms should imply a previous preformulation study of the drug and the excipients. A number of experimental techniques (i.e., differential scanning calorimetry (DSC), Fourier-transform infrared (FT-IR) spectroscopy, x-ray powder diffraction, scanning electron microscopy (SEM), high performance liquid chromatography (HPLC), etc.) have been used to investigate the interaction between drug and excipients.[15,16] As the concerned drug here is an herbal extract, an isothermal stress testing (IST) methodology (binary compatibility study) has been designed, which has been analyzed by a stability indicating procedure - HPLC.

Equimolar mixtures (100 mg each) of the NMITLI-118RT+ extract and the chosen excipients were maintained at 40 ± 2ºC for a period of seven days in glass vials. A control sample containing only the extract was also kept under similar conditions. After seven days all the samples were withdrawn and 5 ml methanol–water (1:1) solution was added to each vial. These samples were then individually vortexed to ensure mixing and then filtered. Subsequently, the achieved filtrate, of concentration 20 mg/ml, was further used to achieve the final test samples of 1 mg/ml each. These were analyzed by HPLC for the estimation of Withanolide A content and to observe any changes in fingerprint pattern or additional peaks.

RESULTS AND DISCUSSION

Method development

The present study aimed to produce an improved, rapid, validated RP-HPLC method for the resolution of Withanolide A. Out of the many permutations and combinations employed, the most suitable and optimized solvent system was chosen, which could provide the desired results on a C18 column. A mobile phase comprising of acetonitrile and water (30:70) was successful in tracing the eluted Withanolide A, however, at a retention time of 18.21 minutes, which was further reduced to 12.87 minutes by the use of acetonitrile: water (40:60). The final separation was achieved in the mobile phase comprising of acetonitrile: Water (50:50), which afforded Withanolide A at a retention time of about 6.3 minutes [Figure 2].

Validation of method

The LOD and LOQ was found to be 0.5 µg/mL and 2 µg/mL, when a signal-to-noise ratio of 3 and 10 were used as the criteria. Withanolide A exhibited good linearity over the concentration range of 2 to 100 µg/mL (y = 27933x - 21605, R^2 = 0.999), as was evident from its calibration curve. The various validation parameters have been precisely presented in Table 1. Intra-assay and inter-assay validations were carried out and the results are presented in Table 2. Percentage CV and bias were found to be within defined limits. The experimental conditions altered for the purpose of studying system robustness did not bring about any significant changes in the resolution of Withanolide A, however, slight changes in its retention time were observed at altered flow rates [Table 3]. The stability of Withanolide A in the solution at the analyte concentration was studied and it was found to be stable for more than 10 days. The percentage recoveries of both standards were calculated and were found to be in the range 97.50-101.75%.

Fingerprint profile

A typical chromatogram for NMITLI-118RT+ is shown in Figure 2. Four batches of NMITLI-118RT+ were analyzed, their fingerprint profiles recorded [Figure 2], and the Withanolide A content [Table 4] estimated. The fingerprint pattern remained largely identical in all four

Table 1: Assay of Withanolide A (validation parameters)

Validation parameters	Values
LOD	0.5 µg/mL
LOQ	2.0 µg/mL
Linearity range	2 µg/mL -100 µg/mL
Regression equation	y=27933x–21605
R^2	R^2=0.999
Recovery range	97.50%-101.75%

LOD: Limit of detection; LOQ: Limit of quantification

Table 2: Intra-assay and inter-assay validation of Withanolide A

Conc. taken (µg/ml)	Intra-assay validation			Inter-assay validation		
	Conc. found	% CV	% Bias	Conc. found	% CV	% Bias
5	5.28±0.08	1.55	5.60	5.25±0.07	1.27	5.13
45	45.22±0.16	0.35	0.62	45.23±0.16	0.35	0.51
90	90.31±0.02	0.02	0.35	90.30±0.02	0.02	0.34

CV: Coefficient of variance

Table 3: Method robustness parameters

Validation parameters	Effect on resolution time in minutes
Flow rate (ml/minute)	
0.8	7.8
1.0	6.1 to 6.3
1.2	5.43
Column temperature (ºC)	
30	6.12
25	6.34

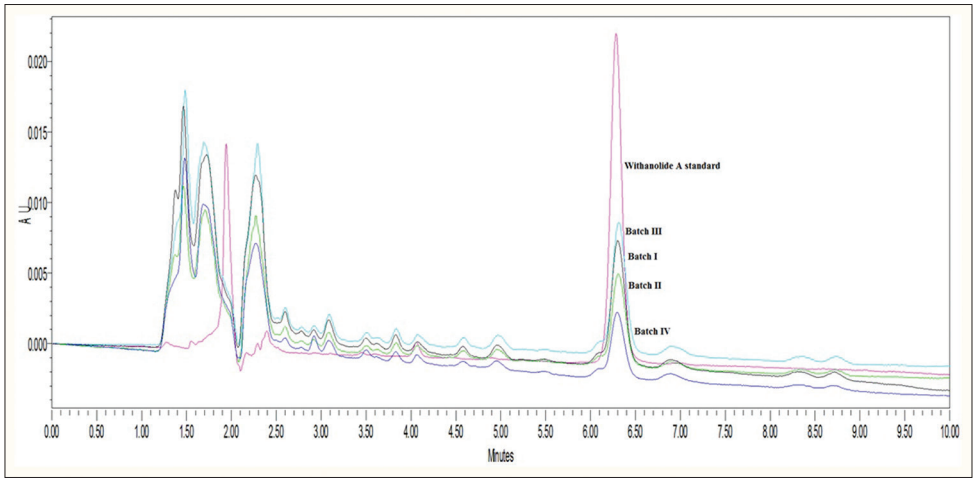

Figure 2: Fingerprint pattern of NMITLI-118RT+ (Overlay comparison of all four batches with standard Withanolide A)

batches, however, variation in the Withanolide A content was observed.

Photostability studies

Photostability studies were carried out for the time period, as obtained by the calibration of the photostability chamber. The samples were analyzed for Withanolide A content [Table 5]. In general, a slight loss in Withanolide A content was observed in all three treatments, with a higher decrease observed in the treatment by UV + visual light, as compared to the control samples.

Forced degradation studies

Stress conditions utilizing alkali medium afforded maximum degradation, followed by acidic, and consecutively peroxide; and the trend was obvious in all sample sets. Another practical observation during the studies was change in color of the plant extracts from yellowish-brown to colorless on treatment with H_2O_2, owing to its bleaching effect.

Order of degradation: Alkali > Acid > Peroxide

Percent degradation and % drug remaining was calculated and the results are presented in Table 6. RP-HPLC chromatograms for stress-induced Withanolide A standard and NMITLI-118RT+ are shown in Figure 3a-c and Figures 4a-c for alkali, acid, and peroxide stress, respectively.

Drug-excipient studies

All samples containing the chosen excipients and the control were analyzed; their Withanolide A content was estimated [Table 7], and all observations recorded. The fingerprint profile remained largely similar in all samples; as many as 17 peaks could be detected in the control sample. An additional peak at a retention time of 6.9 minutes was observed in the control and samples with ethyl cellulose, dextrose, and polyethylene glycols (PEG), whereas, it remained absent in samples containing hydroxy propyl cellulose, lactose, talc, stearic acid, zinc stearate, and Eudagrit. The lowest number of peaks[6] were seen in the

Table 4: Estimation of Withanolide A content in different batches of NMITLI-118RT+

Sample batch no.	Withanolide A Conc. µg/mg
Batch-I	4.25
Batch-II	2.21
Batch-III	4.66
Batch-IV	1.22

Table 5: Estimation of Withanolide A content in photostability samples

Sample specifications	Conc. of withanolide A (µg/mg)
Control under refrigerated conditions	4.53
UV	4.15
Visual light	4.48
UV+visual light	3.89

UV: Ultraviolet

Table 6: % Withanolide A content remaining/ degraded (forced degradation studies)

Sample specification	% Withanolide A (mean values)	
	Content remaining	Content degraded
Withanolide A in 0.1N NaOH	11.75	88.25
NMITLI-118RT+in 0.1N NaOH	19.05	80.50
Withanolide A in 0.1N HCl	29.22	70.78
NMITLI-118RT+in 0.1N HCl	26.39	73.61
Withanolide A in 3% H_2O_2	45.46	54.54
NMITLI-118RT+in 3% H_2O_2	51.68	48.32

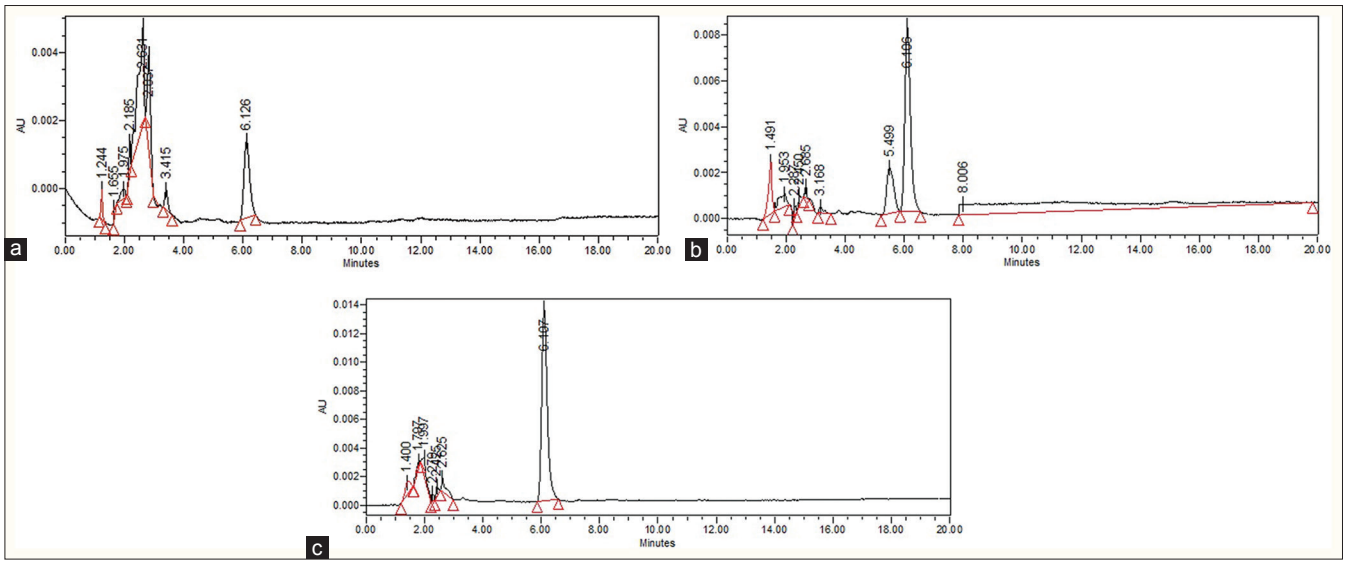

Figure 3: RP-HPLC chromatogram for stress-induced Withanolide A standard (a) alkali treatment (b) acid treatment (c) peroxide treatment

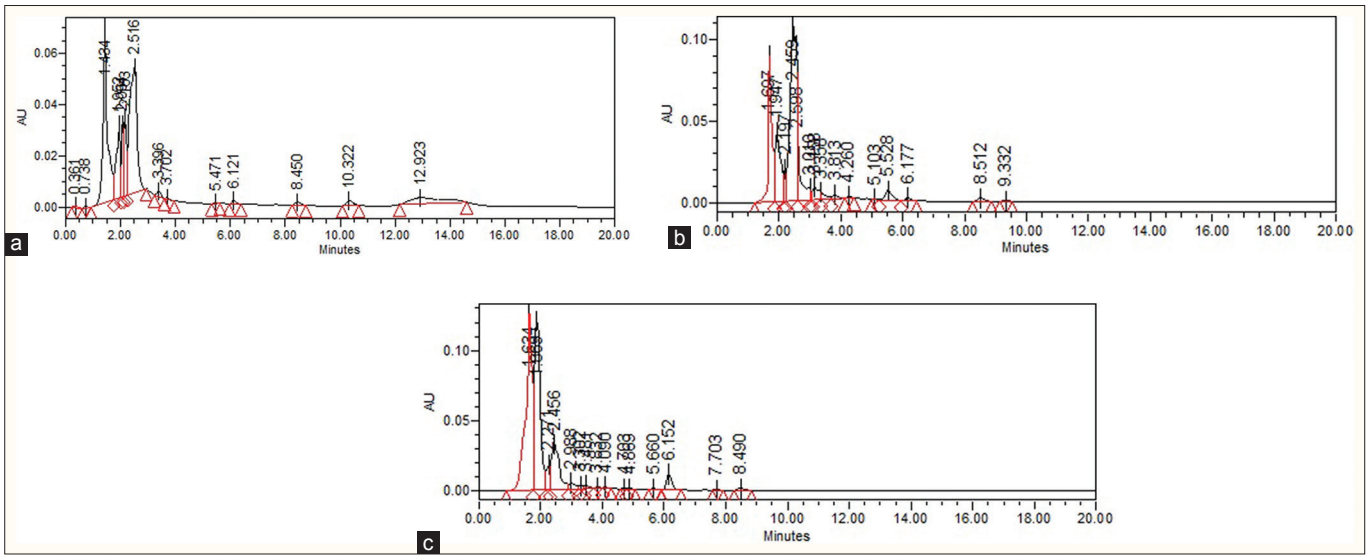

Figure 4: RP-HPLC chromatogram for stress-induced NMITLI-118R T+ (a) alkali treatment (b) acid treatment (c) peroxide treatment

Table 7: Estimation of Withanolide A content in NMITLI-118R-excipient samples

Sample Specification	Withanolide A content (µg/mL)
Control under refrigerated conditions	4.530
NMITLI-118RT++Ethyl cellulose	3.793
NMITLI-118R T++Starch	3.112
NMITLI-118R T++PEG 6000	2.854
NMITLI-118R T++Dextrose	2.549
NMITLI-118R T++Zinc stearate	2.542
NMITLI-118R T++Eudagrit L 100	2.375
NMITLI-118R T++Talc	2.322
NMITLI-118R T++Stearic acid	2.145
NMITLI-118R T++Magnesium stearate	2.092
NMITLI-118R T++Lactose	1.979
NMITLI-118R T++Hydroxypropyl cellulose	0.940

PEG: Polyethylene glycols

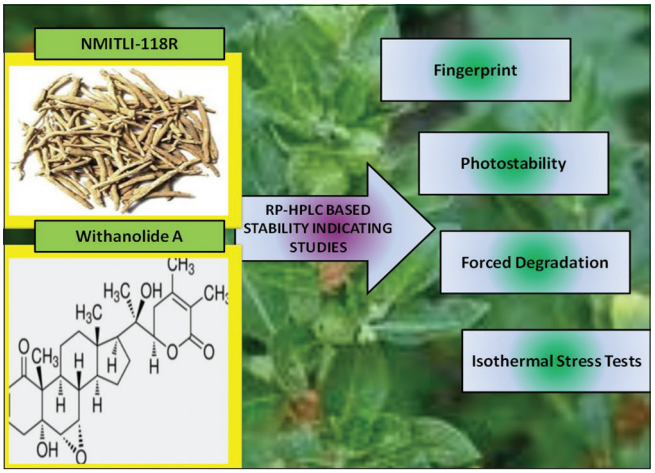

Figure 5: Stability indicating studies: An Overview

chromatogram of the sample containing hydroxypropyl cellulose (HPC) and the lowest amount of Withanolide A content was recorded for the same.

Order of Withanolide A content among different samples: Control > Ethyl cellulose > Starch > PEG 6000 > Dextrose > Zinc stearate > Eudagrit L 100 > Talc > Stearic acid > Magnesium stearate > Lactose > Hydroxypropyl cellulose.

CONCLUSION

The present studies resulted in the successful development and validation of a rapid isocratic method for the detection and estimation of Withanolide A. Method validation was dealt with exhaustively and it could be deduced that the said method was an improved one, with rapid elution of the marker, with no interference on the solvent front, over other reported methods, which employed gradient systems and increased the time of resolution. Analytical studies were attempted on NMITLI-118RT+ (a candidate drug under a CSIR networking project) to generate data beneficial for formulation development, with further studies and investigational new drug (IND) application on the same. The fingerprinting pattern was recorded and Withanolide A contents were estimated in different batches. On the basis of the results of stability studies and behavior of NMITLI-118RT+ under stress conditions, the authors recommended the following storage conditions: It should be protected from heat, moisture, aerial oxidation, and acidic or alkaline pH. For formulation development pH 7 was the most suitable pH. Excipient studies were undertaken as a preliminary forerunner for formulation development and a range of excipients including lubricants, glidants, binders, polymers, and the like, were chosen under the study. Maximum drug content and minimum interaction were achieved with ethyl cellulose, while minimum drug

content and maximum interaction were achieved with hydroxypropyl cellulose [Figure 5].

The data generated here gives useful leads for further studies on NMITLI-118RT+ and the authors plan to undertake formulation studies on the same in the near future.

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