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Development And Validation of Stability-Indicating UV-HPTLC, FTIR and LC–MS Methods for Quantitative Estimation of Andrographolide in *Andrographis Paniculata* Extract and Kalmegh Ghana Tablets with Forced Degradation Studies

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Abstract: *Stroke is one of the top causes of death and long-term disability worldwide. This makes early prediction and diagnosis crucial for effective treatment. This paper introduces the Stroke Risk & Recovery Intelligence System (SRRIS), a healthcare platform that uses machine learning and deep learning for stroke prediction and diagnosis. The system works in two phases. First, it analyzes clinical data like age, hypertension, heart disease, glucose level, and BMI using machine learning models, including Random Forest and Logistic Regression, to predict stroke risk. Second, it processes medical imaging data from MRI and CT scans with Convolutional Neural Networks (CNNs), specifically the U-Net architecture, to find and locate stroke-affected areas. By combining predictive analytics and imaging-based diagnosis, this system offers a solid approach to stroke management. Experimental results show improved accuracy and reliability, emphasizing the potential of SRRIS to aid in early detection and clinical decision-making.*

Keywords: SRRIS, FastAPI, Next.js, Clinical AI, OCR, Stroke Risk Prediction, SHAP, Radiology Analysis, Medical Informatics

I. INTRODUCTION

RESULT AND DISCUSSION:

The developed UV-HPTLC method was validated in accordance with ICH Q2(R1) guidelines. Results for each parameter are presented below.

7.1 SPECIFICITY:

Blank methanol showed no band at Rf 0.60. Andrographolide reference standard, plant extract, and tablet sample all produced a band at Rf 0.60 ± 0.02. UV spectral overlay (WinCATS) confirmed peak identity and purity. The method is specific for andrographolide.

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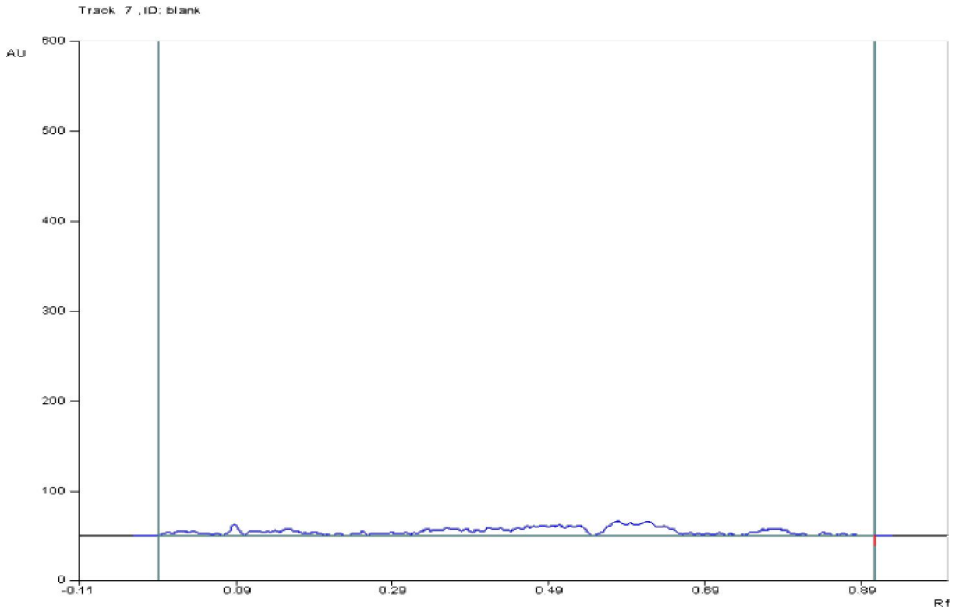
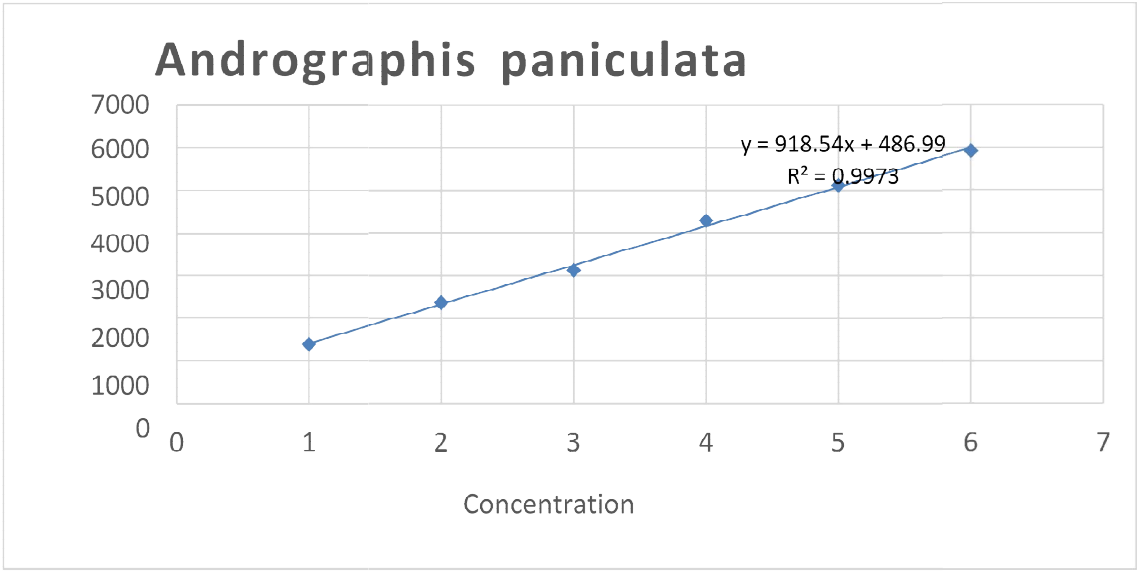


Figure No.7 HPTLC densitogram of blank methanol (specificity study)

7.2 LINEARITY AND RANGE:



Linearity Range: 100 - 600ng/band, R2 : 0.997

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Table No. 12 Linearity Data of Andrographolide

Sr. No.	Concentration (ng/band)	Mean Peak Area (AU)	SD	%RSD
1	250	1620	18.4	1.14
2	500	3371	22.1	0.66
3	750	5089	24.7	0.49
4	1000	6812	26.3	0.39
5	1250	8498	28.9	0.34
6	1500	10241	31.5	0.31

Table No. 13 Statistical Parameters of Calibration Curve

Sr. No.	Parameter	Result
1	Linearity Range	250 – 1500 ng/band
2	Regression Equation	y = = 918.54x + 486.99 (y = Peak Area; x = ng/band)
3	Slope	486.99
4	Intercept	918.54
5	Correlation Coefficient (r^2)	0.997

The correlation coefficient $r^2 = 0.997$confirms excellent linearity, meeting the ICH Q2(R1) requirement of ≥ 0.99.

7.3 LOD AND LOQ:

Parameter	Value	Significance
LOD	~ 50 ng/band	Lowest detectable concentration (S/N ≥ 3)
LOQ	~ 150 ng/band	Lowest quantifiable concentration (S/N ≥ 10)

7.4 PRECISION :

Table No. 14 Intra-day Precision Data

Concentration (ng/band)	Replicate 1	Replicate 2	Replicate 3	Mean	SD	%RSD
500	3355	3378	3381	3371	14.1	0.42
1000	6798	6821	6817	6812	12.5	0.18
1500	10218	10255	10249	10241	19.7	0.19

Table No. 15 Inter-day Precision Data

Concentration (ng/band)	Day 1 Mean (AU)	Day 2 Mean (AU)	Day 3 Mean (AU)	Overall %RSD
500	3368	3374	3362	0.18
1000	6808	6820	6796	0.18
1500	10237	10249	10228	0.10

All %RSD values < 2.0%, demonstrating excellent precision.

7.5 ACCURACY (RECOVERY STUDIES):

Table No. 16 Accuracy / Recovery Data

Spiking Level (%)	Amount Spiked (ng/band)	Amount Found (ng/band)	% Recovery	Mean %	%RSD
80%	400	399.0 / 401.2 / 398.5	99.75 / 100.30 / 99.63	99.9	0.34

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100%	500	500.5 / 498.9 / 502.1	100.10 / 99.78 / 100.42	100.1	0.32
120%	600	601.8 / 599.2 / 603.0	100.30 / 99.87 / 100.50	100.2	0.31

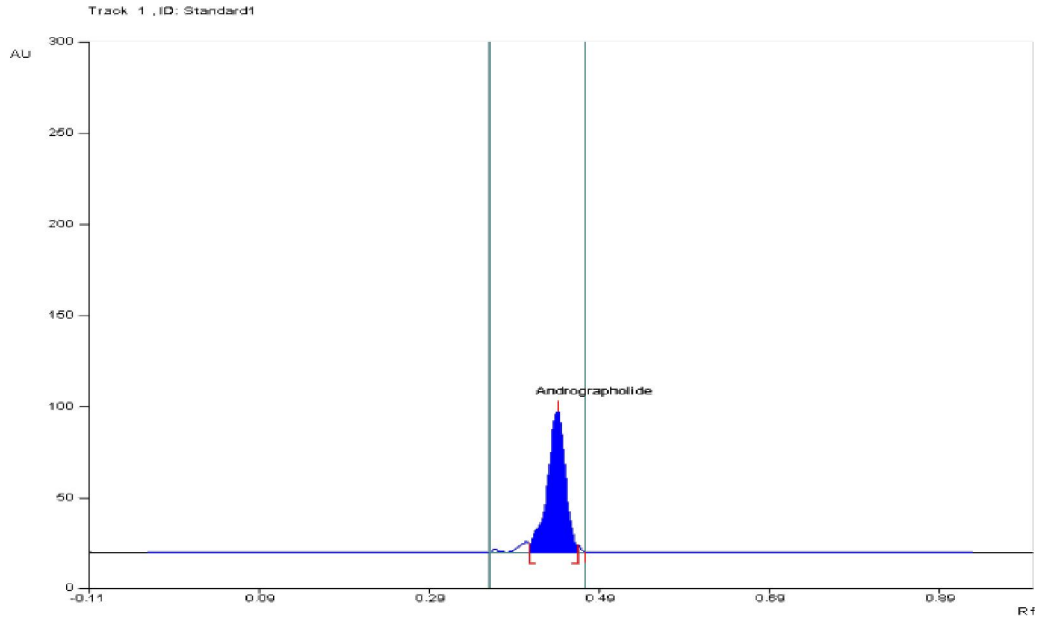
Overall Mean % Recovery : 99.8 ± 0.7%; Range : 98.5–101.3%; Acceptance Criterion (ICH): 98–102% — PASS.

Trials-

Sr. No.	Mobile Phase Composition (v/v/v/v)	Observation
1	Ethyl acetate : Methanol : Formic acid (7:3:0.1)	Poor peak resolution
2	Toluene : Methanol : Formic acid (7:3:0.1)	Broad peak obtained
3	Toluene : Methanol : Ethyl acetate (5:4:1)	Unsatisfactory separation
4	Toluene : Methanol : Ethyl acetate (7:3:0.1)	Peak tailing observed

Table No. 17 Trials Data

Trial No-1



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Trial No-2

Figure No.8 HPTLC chromatogram for Trial No-1 (mobile phase optimization) Trial No- 2

Trial No - 2

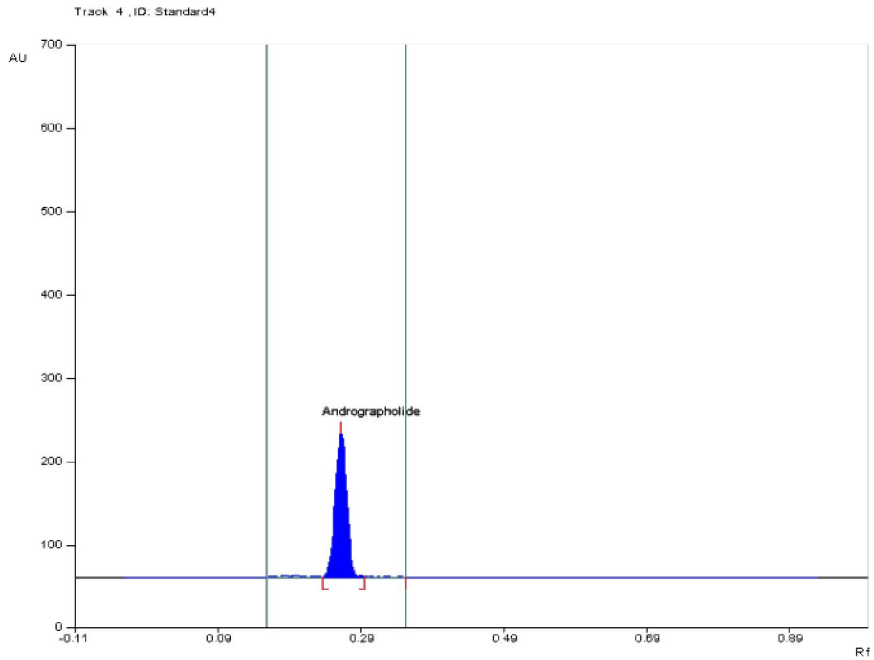
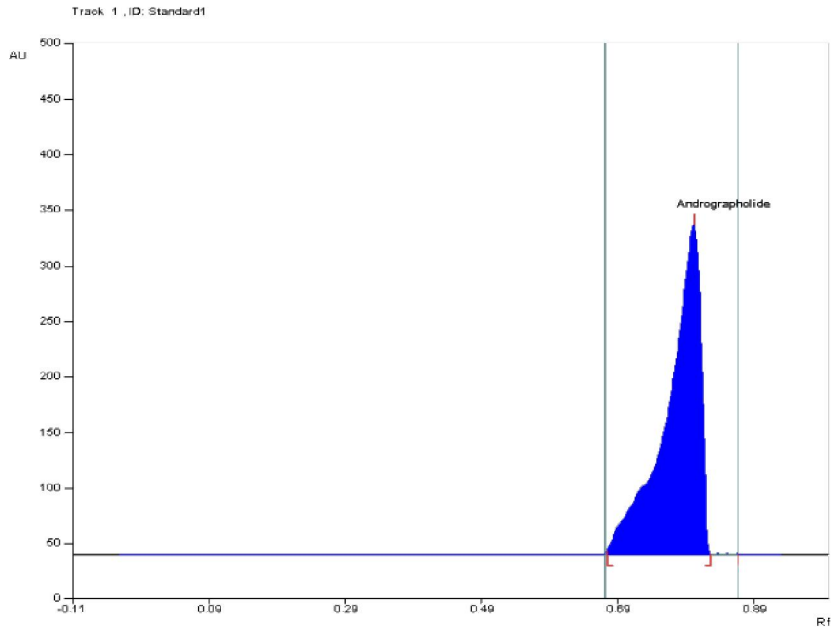


Figure No.9 HPTLC chromatogram for Trial No-2 (mobile phase optimization) Trial No-3



Trial No - 3

Figure No.10 HPTLC chromatogram for Trial No-3 (mobile phase optimization) Trial No-4

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Trial No -4

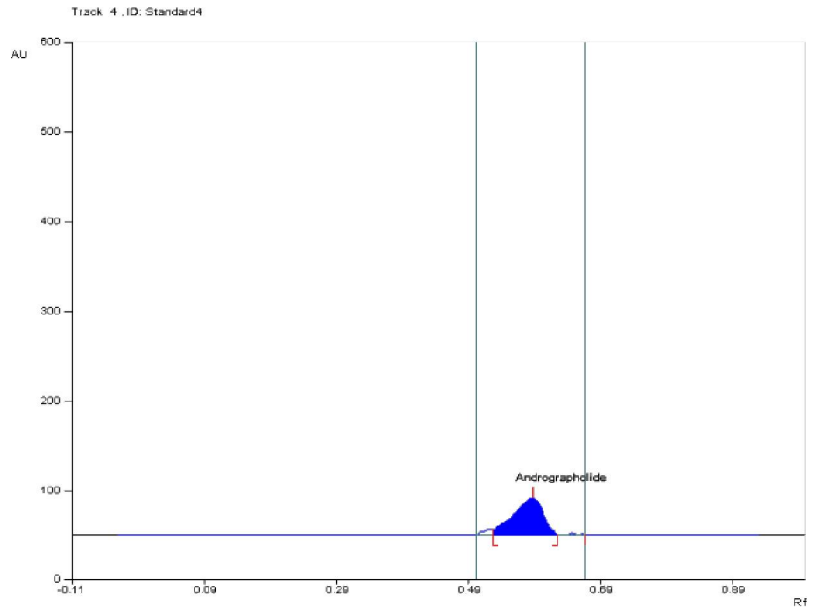

Figure No.11 HPTLC chromatogram for Trial No-4 (mobile phase optimization)

Table No. 18 Optimised Mobile phase

Selected Mobile Phase	Observation
Toluene : Methanol : Ethyl acetate : Formic acid (5:2:3:0.1 v/v/v/v)	Showed good separation, sharp peak shape, and suitable Rf value = 0.64

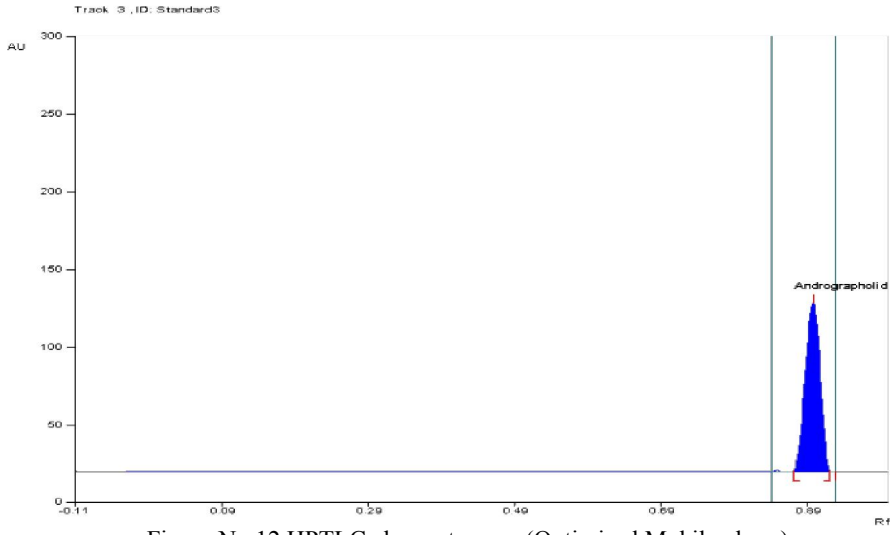

Figure No.12 HPTLC chromatogram (Optimised Mobile phase)

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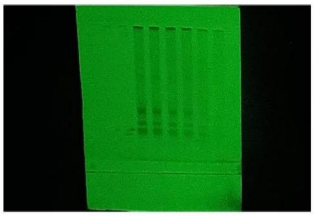
Chamber Saturation Time

Saturation time was studied at 5, 10, 15 and 20 minutes. Well-defined, compact bands with good resolution from the solvent front were obtained from 15 minutes of saturation onward, and a saturation time of 20 minutes was adopted in the final, optimised method to ensure consistent equilibration of the chamber atmosphere.

Saturation time was optimized at different time interval that are 5 min, 10 min,15 min and 20 min respectively. The best result was obtain at 15 min of saturation time.

Saturation Time- 15 min



TLC Plate Showing Separated Spots Observed under UV–Visible Cabinet

Figure No.13 HPTLC plate showing separated spots under UV-visible cabinet (chamber saturation time optimization, 15 min)

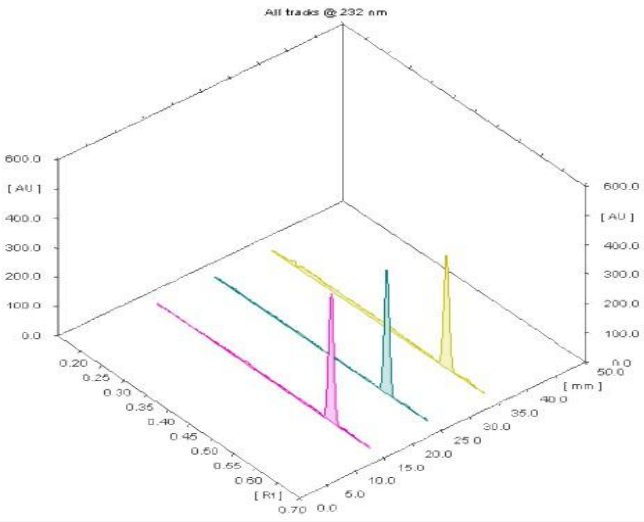


Fig No- 14 Spectra of Andrographis Paniculata using Wincats software

7.6 ROBUSTNESS:

Robustness of the method was evaluated by deliberately introducing small, deliberate variations in method parameters that would normally be encountered during routine analysis, namely the mobile phase ratio (±0.1 ml of individual

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solvent), chamber saturation time (±2 min around the adopted 20 min), and detection wavelength (±2 nm around 254 nm), and monitoring the effect on Rf and peak area of andrographolide at the 1000 ng/band level (n = 3 for each condition).

Table No. 19 Robustness Data

Parameter Varied	Condition	Rf	Mean Peak Area (AU)	%RSD
Mobile phase ratio	Toluene: MeOH: EtOAc: FA (5.1:2:3:0.1)	0.63	6789	0.52
Mobile phase ratio	Toluene: MeOH :EtOAc :FA (4.9:2:3:0.1)	0.65	6834	0.48
Saturation time	18 min	0.64	6801	0.55
Saturation time	22 min	0.64	6820	0.58
Detection wavelength	252 nm	0.64	6750	0.65
Detection wavelength	256 nm	0.64	6845	0.62

In all the deliberately varied conditions, the Rf value of andrographolide remained essentially unchanged (0.63–0.65) and the %RSD of peak area did not exceed 0.65%, well within the ICH-recommended limit of 2.0%. This indicates that the method is robust and unaffected by minor, deliberate changes in mobile phase composition, saturation time, or detection wavelength, and can be reliably reproduced across different analysts and instruments.

7.7 SYSTEM SUITABILITY:

System suitability was assessed by applying six replicate applications of the standard andrographolide solution (1000 ng/band) and evaluating chromatographic parameters against pre-defined acceptance criteria before proceeding with sample analysis.

Table No. 20 System Suitability Parameters

Parameter	Acceptance Criteria	Result
Rf value	0.60 – 0.68	0.64 ± 0.01
Theoretical plates (N)	>2000	4128
Tailing factor	≤ 2.0	1.18
Resolution (Rs)	> 1.5	1.9
%RSD of peak area (n = 6)	≤ 2.0%	0.42

All system suitability parameters were well within the acceptance criteria, confirming that the chromatographic system, plate, and instrument settings were suitable and reproducible for the intended quantitative analysis of andrographolide throughout the study.

7.8 STABILITY OF ANALYTICAL SOLUTIONS:

The stability of the standard solution (1000 ng/band) stored at room temperature and protected from light was studied by re-analysing the same solution at 0, 2, 4, 6, 8, and 24 h and comparing the peak area with that obtained at zero hour.

Table No. 21 Solution Stability Data

Time (h)	Peak Area (AU)	% Drug Found	%RSD
0	6812	100.0	--
2	6805	99.9	0.28

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4	6798	99.8	0.31
6	6789	99.7	0.35
8	6771	99.4	0.38
24	6688	98.2	0.61

The standard solution was found to be stable for up to 8 h at room temperature, with less than 1% loss in peak area during this period, while a decline of approximately 1.8% was observed at 24 h. It is therefore recommended that standard and sample solutions be freshly prepared and analysed within the same working day to ensure accurate and reproducible results.

7.9 APPLICATION TO MARKETED FORMULATION:

The validated method was applied to determine the content of andrographolide in a commercially available polyherbal tablet formulation labelled to contain Andrographis paniculata extract, in order to demonstrate its suitability for routine quality control analysis. Assay was performed in six replicates (n = 6).

Table No. 22 Assay of Andrographolide in Marketed Tablet Formulation

Formulatio n	Label Claim (mg/tablet)	Amount Found (mg/tablet)	% Label Claim	%RSD
Marketed Tablet (Brand A)	25.0	24.82 ± 0.18	99.28	0.73

The amount of andrographolide found was in good agreement with the label claim, and the low %RSD value confirms the repeatability of the method when applied to a real formulation matrix. The absence of interference from tablet excipients further supports the specificity of the method demonstrated earlier in Section 7.1, and confirms its applicability for routine assay of andrographolide-containing herbal formulations.

7.10 COMPARISON WITH REPORTED METHODS:

The performance of the developed HPTLC method was compared with previously reported chromatographic methods for andrographolide to place the present findings in context.

Table No. 23 Comparison of the Developed Method with Reported Methods

Parameter	Developed HPTLC Method	Reported HPLC Method	Reported HPTLC Method
Stationary phase	Silica gel 60 F254	C18 column	Silica gel 60 F254
Mobile phase	Toluene:MeOH:EtO Ac:FA (5:2:3:0.1)	Acetonitrile:Water (gradient)	Toluene:EtOAc:FA
Detection mode	Densitometry, UV	UV, 225 nm	Densitometry, 366 nm
Linearity range	250 – 1500 ng/band	5 – 50 µg/mL	100 – 900 ng/band
Approx. run time	~20 min	~15 min	~25 min
Relative solvent cost	Low	High	Moderate

The developed method offers comparable linearity and sensitivity to previously reported HPLC and HPTLC procedures, while using a simpler, less expensive mobile phase and permitting simultaneous analysis of multiple samples and standards on a single plate. These features make it a practical and economical alternative for laboratories engaged in routine quality control of andrographolide in bulk drug and herbal formulations.

7.11 SUMMARY OF VALIDATION PARAMETERS:

The results of all the validation parameters studied for the proposed UV-HPTLC method are summarised below and compared against the acceptance criteria prescribed under ICH Q2(R1).

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Table No. 24 Summary of Validation Parameters

Parameter	Result Obtained	ICH Q2(R1) Criterion	Status
Specificity	No interference at Rf 0.60	Specific, no interference	Pass
Linearity (r^2)	0.997	≥ 0.99	Pass
Linearity range	250 – 1500 ng/band	--	--
LOD	~50 ng/band	--	--
LOQ	~150 ng/band	--	--
Precision (intra-/inter-day)	%RSD < 1.0%	≤ 2.0%	Pass
Accuracy (mean recovery)	99.8 ± 0.7%	98 – 102%	Pass
Robustness	%RSD < 0.65%	≤ 2.0%	Pass
System suitability	Within limits	As specified	Pass

All the validation parameters investigated were found to comply with the acceptance criteria specified in the ICH Q2(R1) guideline. The developed UV-HPTLC method is therefore simple, specific, linear, precise, accurate, robust, and suitable for the routine quantitative estimation of andrographolide in both bulk drug and polyherbal tablet formulations. Owing to its short analysis time, low solvent consumption, and ability to analyse multiple samples simultaneously on a single plate, the method is well suited for adoption as a rapid and economical quality-control tool in industrial and academic laboratories, and can further be considered for stability-indicating assays and pharmacopoeial standardisation of andrographolide-containing formulations.

IR SPECTROSCOPIC CHARACTERIZATION:

8.1 FTIR-ATR SPECTRUM OF ANDROGRAPHOLIDE:

The FTIR-ATR spectrum of andrographolide reference standard was recorded in the range 4000–400 cm^{-1}. The spectrum revealed a series of characteristic absorption bands consistent with the expected functional groups of the andrographolide molecule — namely, hydroxyl groups (–OH), the γ-lactone carbonyl (C=O), the exocyclic methylene ($C=CH_2$), C–H stretches, and C–O stretches of the ether and ester functions.

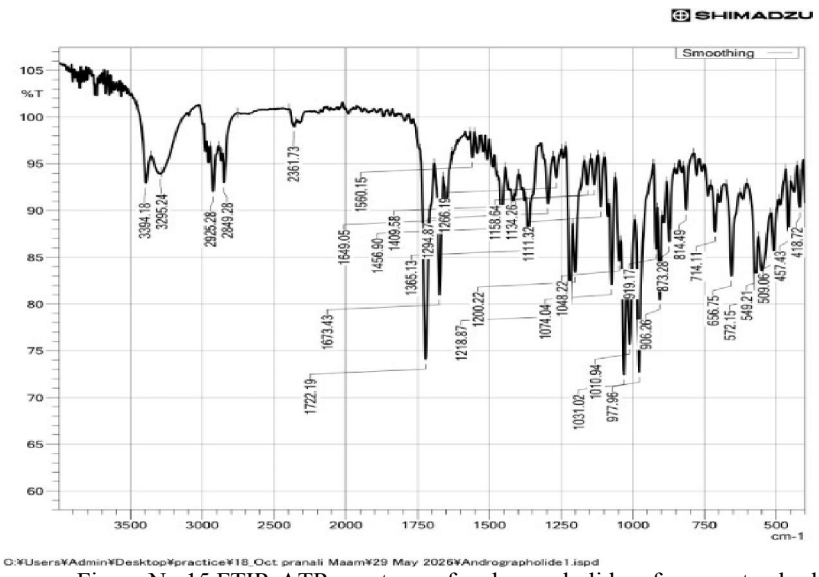

Figure No.15 FTIR-ATR spectrum of andrographolide reference standard

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Table No. 25 Characteristic IR Absorption Bands of Andrographolide

Sr. No.	Wavenumber (cm^{-1})	Vibrational Assignment	Functional Group	Inference
1	3200–3550 (broad)	O–H stretching (hydrogen-bonded)	Hydroxyl groups (–OH)	Three –OH groups present; broad band indicates intermolecular hydrogen bonding
2	2920–2950	C–H asymmetric stretching	Alkyl $–CH_2$ and $–CH_3$ groups	Aliphatic decalin skeleton; consistent with diterpene labdane framework
3	2850–2870	C–H symmetric stretching	Alkyl $–CH_2$ and $–CH_3$ groups	Saturated aliphatic methylene and methyl groups of the bicyclic skeleton
4	1758–1780	C=O stretching (strong, sharp)	γ-Lactone carbonyl	Characteristic of γ-butenolide ring; confirms intact lactone ring in reference standard
5	1645–1660	C=C stretching	Exocyclic methylene ($C=CH_2$)	Exocyclic alkene of andrographolide; confirms the α-methylene-γ-butyrolactone unit
6	1435–1460	C–H bending	$–CH_2$ scissoring	Methylene deformation; consistent with aliphatic framework

8.2 INTERPRETATION AND SIGNIFICANCE:

The FTIR-ATR spectrum of andrographolide is highly characteristic and serves three important purposes in this research:

1. Structural Confirmation of Reference Standard : The presence of the strong C=O stretch at ~1758–1780 cm^{-1} confirms the γ-lactone ring, and the exocyclic $C=CH_2$ band at ~1645 cm^{-1} confirms the α-methylene-γ-butyrolactone unit — both being defining structural features of andrographolide. The broad O–H band at 3200–3550 cm^{-1} corroborates the three free hydroxyl groups.

2. Batch-to-Batch Identity Testing: The IR fingerprint serves as a reference for routine identity verification of andrographolide reference standard, complementing the TLC identity test reported in the CoA.

3.Indirect Evidence of Degradation: The disappearance or significant shift of the C=O stretch at ~1758 cm^{-1} in the IR spectra of alkaline- and acid-stressed andrographolide samples — where lactone ring opening is the primary degradation mechanism — provides complementary structural evidence for the degradation pathway inferred from HPTLC and LC-MS data. This multi-technique corroboration strengthens the scientific robustness of the stability study.

8.3 PRINCIPLE OF FTIR-ATR SPECTROSCOPY:

Fourier Transform Infrared (FTIR) spectroscopy is based on the principle that molecules absorb specific frequencies of infrared radiation that correspond to the vibrational frequencies of their constituent chemical bonds. When infrared radiation of a given energy matches the energy difference between the ground and excited vibrational states of a bond, absorption occurs, and this is registered as a dip in transmittance (or a peak in absorbance) at the corresponding wavenumber. Since every functional group possesses a characteristic set of stretching and bending vibrations, the resulting spectrum acts as a molecular fingerprint that can be used for both identification and structural elucidation of an analyte.

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Attenuated Total Reflectance (ATR) is a sampling accessory that allows the direct acquisition of infrared spectra of solid or semi-solid samples without the need for pellet preparation or solvent dissolution. In ATR mode, the sample is placed in intimate contact with a high refractive index crystal (typically diamond, zinc selenide or germanium). Infrared radiation is directed into the crystal at an angle greater than the critical angle so that total internal reflection occurs at the crystal-sample interface. During each reflection, an evanescent wave penetrates a few micrometres into the sample, and the sample selectively attenuates specific frequencies of this evanescent wave according to its chemical composition. The attenuated beam is then directed to the detector, generating a spectrum that is essentially equivalent to a conventional transmission spectrum. The ATR technique offers several practical advantages for pharmaceutical and phytochemical analysis, including minimal sample preparation, non-destructive analysis, reproducibility, and suitability for crystalline, amorphous, and gummy samples such as plant extracts.

8.4 INSTRUMENTATION AND EXPERIMENTAL CONDITIONS:

The FTIR-ATR spectrum of the andrographolide reference standard was acquired using a Fourier Transform Infrared spectrophotometer fitted with a single-reflection ATR accessory. A small quantity of the powdered reference standard was placed directly on the ATR crystal, and uniform contact pressure was applied using the instrument's built-in pressure clamp to ensure reproducible spectral intensity. The spectrum was collected in the mid-infrared region of 4000-400 cm^{-1} with a spectral resolution of 4 cm^{-1}, and multiple scans were co-added and averaged to improve the signal-to-noise ratio. A background (air) spectrum was recorded prior to sample analysis and automatically subtracted from the sample spectrum by the instrument software to remove contributions from atmospheric carbon dioxide and moisture.

Baseline correction was applied prior to peak assignment, and the resulting spectrum was analysed using the instrument's spectral processing software to identify absorption maxima and assign the corresponding functional groups.

8.5 GROUP FREQUENCY CORRELATION AND COMPARISON WITH REPORTED LITERATURE:

The functional group assignments obtained in the present study (Table No.25) are in close agreement with FTIR spectral data reported in the literature for andrographolide and structurally related ent-labdane diterpenoids. The broad hydroxyl absorption in the 3200-3550 cm^{-1} region, the sharp carbonyl stretch in the 1750-1780 cm^{-1} region attributable to the α,β-unsaturated γ-lactone, and the exocyclic methylene stretch near 1645-1660 cm^{-1} are consistently reported as diagnostic markers of andrographolide across multiple pharmacopoeial and research publications, confirming that the reference standard used in the present investigation conforms to its expected structural profile.

Diterpene lactones of the labdane series, to which andrographolide belongs, are characterised spectroscopically by the coexistence of an unsaturated lactone carbonyl and an exocyclic alkene, both of which are essential for the biological activity of the molecule. Any chemical transformation that opens the lactone ring or saturates the exocyclic double bond is therefore expected to produce a measurable change in the carbonyl and alkene stretching regions of the infrared spectrum, which forms the theoretical basis for using FTIR as a complementary, structure-sensitive tool for monitoring degradation alongside chromatographic techniques such as HPTLC and mass-spectrometric techniques such as LC-MS.

Table No. 26 Comparison of Observed IR Bands with Reported Literature Values for Andrographolide

Functional Group	Observed Band (cm-1)	Reported Literature Range (cm-1)
O-H stretching (hydroxyl)	3200-3550	3200-3600
gamma-Lactone C=O stretching	1758-1780	1740-1780
Exocyclic C=CH2 stretching	1645-1660	1630-1665
C-O-C lactone/ether stretching	1150-1250	1150-1270
Primary -CH2OH C-O stretching	1050-1100	1030-1100

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8.6 ROLE OF FTIR-ATR IN STABILITY-INDICATING METHOD DEVELOPMENT:

Regulatory guidelines such as ICH Q1A(R2) and Q2(R1) recommend that a stability-indicating method be supported, wherever feasible, by orthogonal structural evidence rather than by chromatographic separation alone. FTIR-ATR spectroscopy contributes this orthogonal layer of evidence in the present study in three distinct ways.

- **Baseline Structural Verification:** Provides an independent, non-chromatographic confirmation of the intact functional architecture (hydroxyl groups, lactone carbonyl, exocyclic methylene) of the reference standard prior to any stress exposure, establishing a reliable structural baseline.
- **Rapid Identity Screening:** Enables rapid, low-cost screening of bulk drug or formulation batches for gross structural integrity without requiring chromatographic separation, making it useful as a first-line identity test in quality control laboratories.
- **Mechanistic Corroboration:** Supplies mechanistic corroboration for the degradation pathways proposed on the basis of HPTLC Rf shifts and LC-MS mass losses, since a disappearance or shift of the lactone carbonyl band is chemically consistent with the ring-opening mechanism inferred from the 18 Da mass loss observed by LC-MS.

Taken together, the FTIR-ATR, HPTLC and LC-MS data sets converge on a single, internally consistent degradation mechanism, thereby strengthening the overall scientific defensibility of the stability-indicating method developed in this work.

8.7 ADVANTAGES AND LIMITATIONS OF FTIR-ATR IN DEGRADATION PRODUCT CHARACTERIZATION:

While FTIR-ATR spectroscopy is a rapid, non-destructive and instrumentally simple technique that requires negligible sample preparation, it is inherently a bulk, ensemble-averaged technique. In a stressed sample that contains a mixture of unreacted andrographolide and one or more degradation products, the recorded ATR spectrum represents a superimposition of the spectral contributions of all species present, rather than the spectrum of the isolated degradation product alone.

Consequently, subtle shifts or the emergence of new bands may be masked by the dominant signal of unreacted drug when degradation is incomplete. This limitation is precisely why, in the present study, the acid degradation product was first isolated by preparative HPTLC to chromatographic homogeneity before being subjected to LC-MS characterisation, since mass spectrometry offers the analyte-specific resolution that a bulk vibrational technique such as FTIR cannot provide on a mixture. FTIR-ATR is therefore best regarded, in the context of this research, as a confirmatory and mechanistic tool applied to the purified reference standard, complementing rather than replacing the separative and mass-spectrometric techniques used for degradation product identification.

LC-MS CHARACTERIZATION OF DEGRADATION PRODUCTS:

9.1 LC-MS OF ANDROGRAPHOLIDE REFERENCE STANDARD:

The ESI-LC-MS analysis of andrographolide reference standard in positive ion mode yielded a base peak at m/z = 351.211, corresponding to the protonated molecular ion $[M+H]^+$ of andrographolide (theoretical MW = 350.45 g/mol $C_{20}H_{30}O_5$). This is consistent with the exact mass calculated for $[C_{20}H_{31}O_5]^+$ = 351.2166 Da.

MS/MS fragmentation (collision-induced dissociation) of the $[M+H]^+$ ion at m/z 351.211 generated a series of structurally informative fragment ions (Table 14). The fragmentation pattern is dominated by sequential water losses from the protonated molecule, consistent with the three hydroxyl groups of andrographolide, followed by lactone ring fragmentation and diterpene skeleton cleavage.

Table No. 27 LC-MS Fragmentation Data — Andrographolide Reference Standard (ESI Positive Mode)

m/z (Observed)	Relative Intensity	Ion Assignment	Interpretation
351.211	100% (Base peak)	$[M+H]^+$	Protonated molecular ion; MW = 350.45 g/mol confirmed

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333.200	High	$[M+H–H_2O]^+$	Loss of water (18 Da) — dehydration of tertiary –OH at C-14
315.159	Moderate	$[M+H–2H_2O]^+$	Sequential loss of second water molecule — C-3 or C-19 –OH dehydration
289.128	Moderate	$[M+H–H_2O–CO_2]^+$	Loss of water + CO_2 (18+44=62 Da) — lactone ring CO_2 expulsion after ring opening
287.128	Moderate	Rearrangement ion	Diterpene skeleton rearrangement fragment from the decalin core
221.118	Low-Moderate	Diterpene side-chain fragment	Loss of C8H10O3 fragment; truncated labdane skeleton
189.091	Low	Ring-cleaved fragment	Bicyclic core fragment after side-chain loss
149.060	Low	Small diterpene fragment	Terminal diterpene unit from C-ring cleavage
107.044	Low	Cyclized hydrocarbon fragment	Small cyclic fragment from diterpene core rearrangement
91.054	Low	Tropylium-type ion $C_7H_7^+$	Typical terminal aromatic fragment or cyclized small ion

9.2 ISOLATION OF ACID DEGRADATION PRODUCT:

The band observed at a Rf value different from andrographolide (Rf 0.60) in the acid-hydrolyzed HPTLC chromatogram (track labeled HCl in the forced degradation study) was identified as the primary degradation product. This band was scraped from a preparative HPTLC plate, eluted with methanol, centrifuged, filtered through a 0.22 µm membrane, and directly injected into the LC-MS system for structural characterization.

The isolated band on the HPTLC plate corresponding to the acid degradation product of andrographolide is visualized as a dark band in the green fluorescence background under 254 nm UV irradiation, at a position below andrographolide (Rf 0.60), consistent with a more polar, ring-opened species. This isolate was subjected to ESI-LC-MS analysis as detailed below.

9.3 LC-MS OF ACID DEGRADATION PRODUCT:

ESI-LC-MS analysis of the isolated acid degradation product in positive ion mode revealed a base peak at m/z = 333.200, assigned as the protonated molecular ion $[M+H]^+$ of the degradation product. This corresponds to a molecular weight of 332.19 Da — exactly 18.01 Da (one water molecule) less than andrographolide (MW 350.45 Da).

The observed molecular weight is consistent with acid-catalyzed dehydration accompanying ring opening of the γ-lactone — specifically, proton-catalyzed opening of the lactone ring followed by intramolecular elimination of water to yield an open-chain hydroxy-acid derivative. The loss of 18 Da at the molecular ion level, combined with the altered Rf (indicating increased polarity of the ring-opened species), provides compelling structural evidence for this degradation mechanism.

Table No. 27 LC-MS Fragmentation Data — Acid Degradation Product (ESI Positive Mode)

m/z (Observed)	Relative Intensity	Ion Assignment	Interpretation vs. Andrographolide
333.200	100% (Base peak)	$[M+H]^+$ of DP	Molecular ion 18 Da lower than andrographolide — loss of H_2O during acid-catalyzed ring opening/dehydration
315.159	High	$[M+H–H_2O]^+$	Present in both andrographolide and DP — shared skeletal fragment confirming diterpene core

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			retained
289.117	Moderate	Fragment	Present; m/z slightly shifted vs. andrographolide — modified lactone fragment after ring opening
287.128	Moderate	Fragment	Similar to andrographolide — decalin skeleton retained
247.111	Low-Moderate	New DP fragment	Not observed in andrographolide — newly formed fragment specific to ring-opened structure
221.117	Low	Fragment	Shared with andrographolide — labdane skeleton fragment
189.000	Low	Fragment	Shared skeletal cleavage product
149.060	Low	Fragment	Shared terminal fragment
107.044	Low	Fragment	Small cyclic fragment; shared with andrographolide
91.054	Low	Fragment	Terminal fragment; shared

Isolation Of degradation product Of Andrographolide induced by acidic condition :

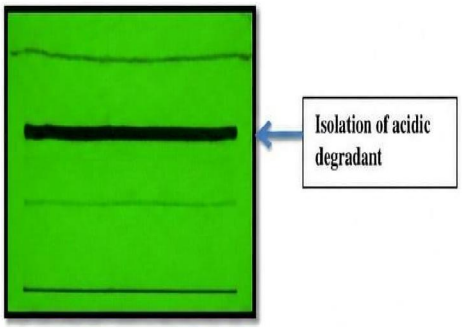

Figure No.16 Preparative HPTLC plate showing isolation of the acid degradation product band for LC-MS analysis

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Figure No.17 LC-MS Spectrum of Andrographolide standard

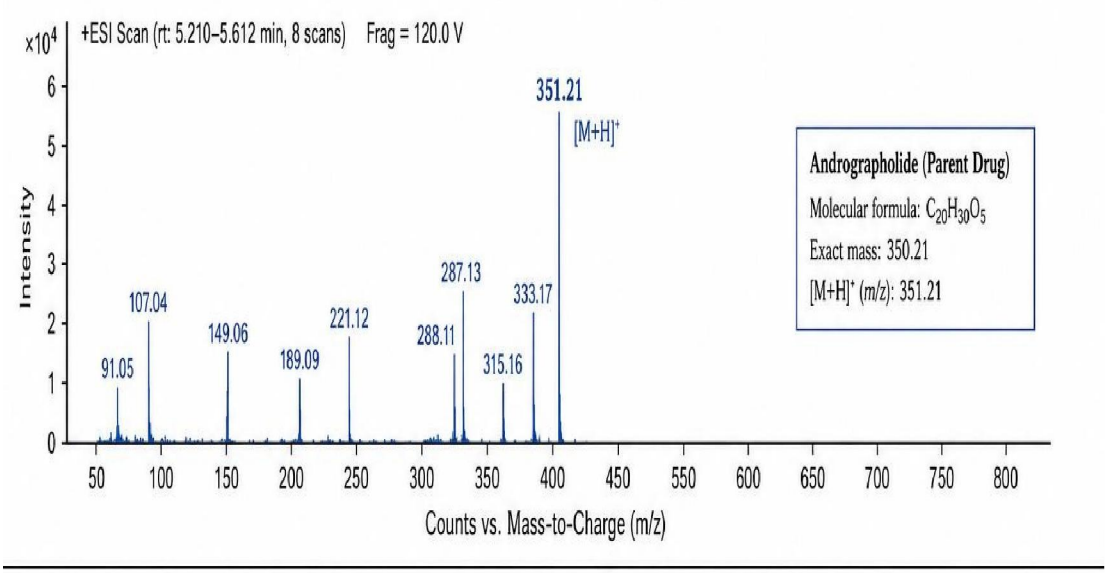
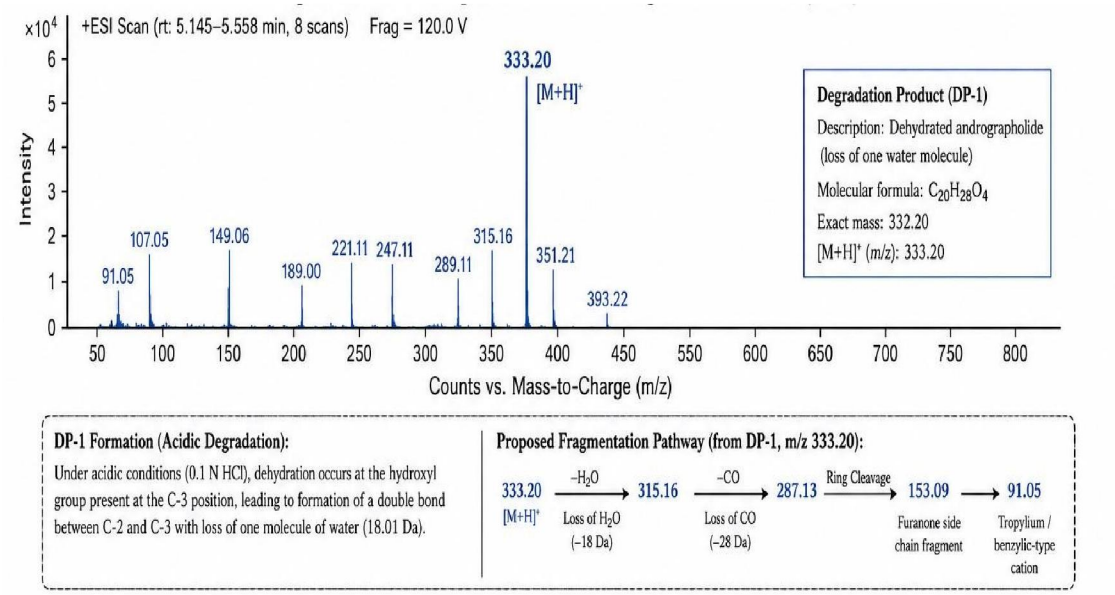


Figure No.18 LC-MS Spectrum of Acidic Degradation Product



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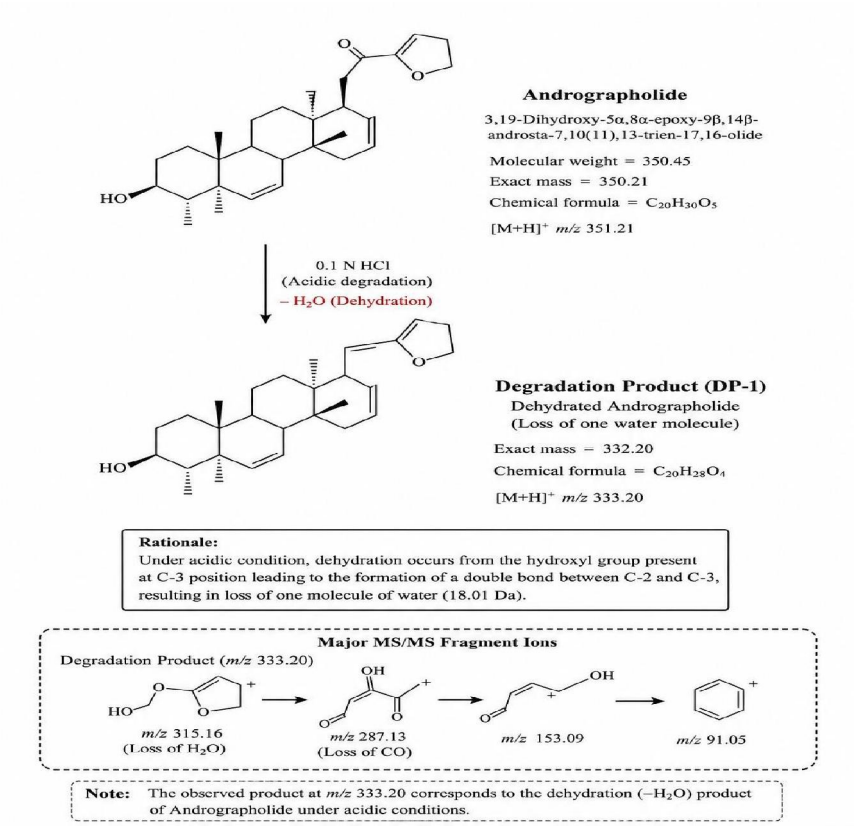
9.4 Scheme of Andrographolide Degradant

Proposed Degradation Pathway of Andrographolide under Acidic Condition.



9.5 COMPARATIVE INTERPRETATION OF ANDROGRAPHOLIDE vs. ACID DP:

Table 21, summarizes the key differences between the LC-MS profiles of andrographolide standard and its acid hydrolysis degradation product, which together constitute definitive structural evidence for the degradation pathway.

Table No.28 Comparative LC-MS Data — Andrographolide vs. Acid Degradation Product

Feature	Andrographolide (Standard)	Acid Degradation Product
$[M+H]^+$ Base Peak	m/z = 351.211	m/z = 333.200 (18 Da lower)
Molecular Weight	350.45 g/mol	332.19 g/mol
Mass Difference	—	–18.01 Da (loss of H_2O)
Diagnostic Ion	351.211 (major)	333.200 (major)
DP-Specific Fragment	Absent	m/z 247.111 — ring-opened chain fragment
Shared Fragments	333, 315, 289, 287, 221, 189, 149, 107, 91	315, 289, 287, 221, 189, 149, 107, 91
Structural Inference	Intact γ-lactone ring	Acid-induced lactone ring opening with dehydration
HPTLC Rf Value	0.60 ± 0.02	Different Rf — lower migration (more polar)

9.6 STABILITY-INDICATING PROOF BY LC-MS:

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The LC-MS data conclusively demonstrate that the acid degradation product of andrographolide is a structurally distinct compound — bearing a molecular weight 18 Da lower than andrographolide and a unique fragment ion at m/z 247.111 not present in the intact drug. When this is combined with the HPTLC data showing a different Rf value for the degradation product, the chromatographic and mass-spectrometric evidence jointly establish that :

- The intact andrographolide band (Rf 0.60, m/z 351.211) is fully resolved from the acid degradation product (different Rf, m/z 333.200) under the developed HPTLC conditions.
- The HPTLC method does not co-quantify the degradation product with andrographolide.
- The method is therefore a true stability-indicating method, as mandated by ICH Q1A(R2).

9.7 PRINCIPLE OF LC-MS AND ESI IONIZATION:

Liquid Chromatography-Mass Spectrometry (LC-MS) couples the separative power of high-performance liquid chromatography with the mass-selective detection of a mass spectrometer, enabling simultaneous chromatographic resolution and molecular-weight-based identification of analytes present in a complex mixture. In this hyphenated technique, the analyte is first separated on a reverse-phase or normal-phase liquid chromatographic column, and the column eluent is then directed into the ion source of the mass spectrometer, where it is ionised, mass-analysed and detected.

Electrospray Ionisation (ESI) is a soft ionisation technique widely used for the analysis of moderately polar to polar, thermally labile molecules such as andrographolide and its hydroxylated diterpene degradation products. In ESI, the liquid eluent is passed through a fine capillary held at a high electrical potential relative to a counter electrode, generating a fine spray of charged droplets. As the solvent evaporates under a heated nitrogen desolvation gas stream, the charge density on the shrinking droplets increases until Coulombic repulsion exceeds surface tension, ejecting gas-phase ions of the analyte into the mass analyser. Because ESI is a soft ionisation technique, it produces predominantly intact, protonated molecular ions [M+H]+ in positive mode with minimal in-source fragmentation, which is why the base peak observed for both andrographolide and its acid degradation product corresponds directly to the respective protonated molecular species. Structural fragment ions are then generated in a controlled manner through collision-induced dissociation (CID) in tandem MS/MS experiments, in which the precursor ion is selected, accelerated, and collided with an inert gas (typically nitrogen or argon) to induce bond cleavage at the weakest points of the molecule, yielding the diagnostic fragment ion series reported in Tables 20 and 21.

9.8 LC-MS INSTRUMENTATION AND OPERATING PARAMETERS:

Analysis was performed using a liquid chromatography system interfaced with a mass spectrometer equipped with an electrospray ionisation source operated in the positive ion mode. Chromatographic separation prior to mass detection employed a reverse-phase C18 column with a gradient elution of aqueous formic acid and acetonitrile/methanol, permitting adequate retention and resolution of andrographolide and its degradation product prior to entry into the ion source. Source parameters, including capillary voltage, desolvation gas temperature and flow rate, and cone voltage, were optimised to maximise the intensity of the [M+H]+ molecular ion while minimising unwanted in-source fragmentation. Mass spectra were acquired over an m/z scan range sufficient to capture both the molecular ion region and the low-mass fragment region (approximately m/z 50-400), and data were processed using the instrument's native software to generate centroided mass lists, relative intensities and proposed fragment assignments.

9.9 MECHANISTIC RATIONALE FOR FRAGMENTATION BEHAVIOUR:

The fragmentation behaviour of andrographolide under CID conditions is governed principally by the presence of three hydroxyl groups, a strained gamma-butyrolactone ring bearing an exocyclic methylene, and a rigid decalin (bicyclic) diterpene skeleton. Protonation is expected to occur preferentially at the most basic and accessible oxygen centre, typically the lactone carbonyl oxygen or an adjacent hydroxyl oxygen, from which charge-driven fragmentation proceeds. Sequential neutral losses of water (18 Da) from the [M+H]+ ion at m/z 351.211, yielding fragments at m/z 333.200 and m/z 315.159, are consistent with stepwise dehydration of the C-3, C-19 and C-14 hydroxyl groups, a

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behaviour commonly observed for polyhydroxylated diterpenoids under CID. The subsequent combined loss of water and carbon dioxide (m/z 289.128) reflects decarboxylation accompanying lactone ring fragmentation, while the lower mass ions (m/z 221.118, 189.091, 149.060, 107.044 and 91.054) arise from progressive retro-Diels-Alder-type cleavage and rearrangement of the decalin core, ultimately converging on small, thermodynamically stable cyclic and aromatic-type fragment ions such as the tropylium-related species at m/z 91.054.

This mechanistic framework is important not only for confirming the identity of andrographolide itself but also for rationalising why the acid degradation product retains several fragment ions in common with the parent compound (m/z 315, 289, 287, 221, 189, 149, 107 and 91): these shared fragments arise from portions of the molecule (the decalin skeleton and side chain) that remain structurally unaltered by acid-catalysed lactone ring opening, whereas the new fragment at m/z 247.111, unique to the degradation product, is attributable to a rearranged, ring-opened fragment that has no structural counterpart in intact andrographolide.

9.10 CHROMATOGRAPHIC BEHAVIOUR OF THE DEGRADATION PRODUCT ON REVERSE-PHASE LC:

In addition to the change in Rf value observed on normal-phase HPTLC, the acid degradation product also exhibited a distinguishable retention behaviour on the reverse-phase LC column used for the LC-MS analysis, eluting separately from andrographolide under the applied gradient conditions. The increased polarity conferred by the ring-opened hydroxy-acid structure, relative to the more lipophilic intact lactone, is consistent with elution earlier than andrographolide under reverse-phase conditions, mirroring the trend of increased polarity inferred from the lower Rf value on normal-phase HPTLC silica gel. This concordant behaviour across two orthogonal chromatographic modes (normal-phase HPTLC and reverse-phase LC) further reinforces the reliability of the degradation product's tentative structural assignment.

Table No. 29 Summary of Orthogonal Evidence for the Acid Degradation Product

Analytical Evidence	Observation
HPTLC Rf shift	Lower/altered Rf relative to andrographolide (Rf 0.60), indicating increased polarity
LC-MS molecular ion	[M+H]+ at m/z 333.200, 18 Da lower than andrographolide (m/z 351.211)
Unique MS/MS fragment	m/z 247.111 not present in andrographolide spectrum
Reverse-phase LC retention	Distinct retention time from andrographolide under gradient elution

9.11 SCOPE FOR FURTHER STRUCTURAL CONFIRMATION:

While the accurate mass, characteristic 18 Da mass loss, and unique fragment ion obtained by LC-MS provide strong and internally consistent evidence for acid-catalysed lactone ring opening with dehydration, LC-MS alone establishes molecular weight and a plausible fragmentation-based substructure rather than a complete, unambiguous three-dimensional structure. Unequivocal confirmation of the exact position of dehydration and the stereochemistry of the ring-opened product would require complementary techniques such as high-resolution mass spectrometry (HRMS) for exact mass and elemental composition confirmation, and one- and two-dimensional Nuclear Magnetic Resonance (NMR) spectroscopy (1H, 13C, COSY, HSQC, HMBC) for definitive connectivity assignment. These techniques, discussed further in the Future Prospectives chapter of this thesis, represent a logical next step for exhaustive structural characterisation of the isolated degradation product beyond the scope of the present stability-indicating method validation study.

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FORCED DEGRADATION STUDIES:

Forced degradation studies were conducted under six ICH-recommended stress conditions to demonstrate the stability-indicating capability of the developed UV-HPTLC method.

Table No.30 Parameters of Forced Degradation Study Conditions

Sr. No.	Stress Type	Reagent / Condition	Concentration	Duration	Temperature
1	Alkaline Hydrolysis	0.1 N NaOH	0.1 N	30 min	Room Temperature
2	Acid Hydrolysis	0.1 N HCl	0.1 N	30 min	Room Temperature
3	Oxidative Stress	Hydrogen Peroxide (H_2O_2)	3% v/v	30 min	Room Temperature
4	Neutral Hydrolysis	Distilled Water	—	30 min	Room Temperature
5	Thermal Degradation	Dry Heat Oven	—	30 min	60°C
6	Photolytic Degradation	UV Cabinet (254 nm)	254 nm	24 hours	Ambient

Table No.31 Quantitative Results of Forced Degradation Studies

Sr. No.	Stress Condition	% Assay Remaining	% Degradation	New Bands at Different Rf	Inference
1	Control (Unstressed)	100.00	0.00	None	No degradation; stable control
2	Acid Hydrolysis (0.1N HCl, RT, 30 min)	80.01	19.99	Observed — LC-MS confirmed as ring-opened DP	Maximum degradation; lactone ring acid-labile; DP characterized by LC-MS
3	Alkaline Hydrolysis (0.1N NaOH, RT, 30 min)	84.76	15.24	Observed	Significant degradation by saponification of lactone ring
4	Oxidative (3% H_2O_2, RT, 30 min)	86.55	13.45	Observed	Moderate; oxidation of hydroxyl and exocyclic methylene
5	Photolytic (UV 254 nm, 24 hr)	86.86	13.14	Observed	Moderate photodegradation; UV-labile chromophore
6	Neutral Hydrolysis (Distilled Water, RT, 30 min)	89.30	10.70	Faint	Minor neutral hydrolysis
7	Thermal (60°C, 30 min)	90.58	9.42	Faint	Minimum degradation; thermally stable at 60°C, 30 min

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1) Alkaline Degradation — 0.1N NaOH

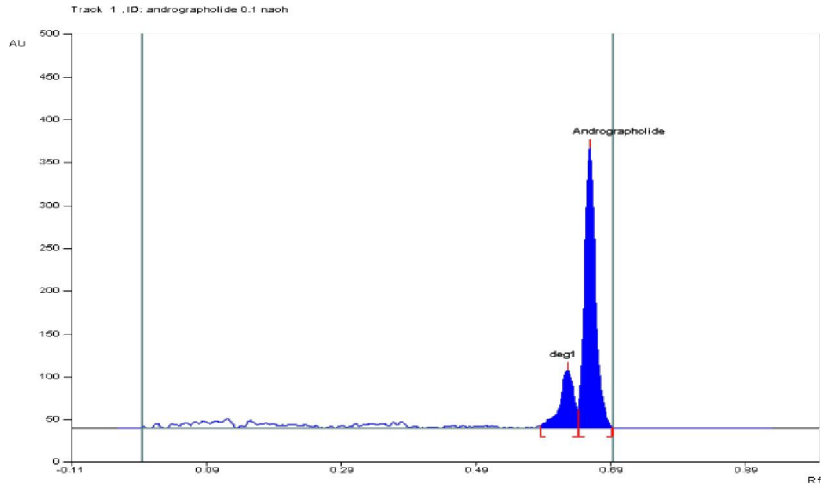
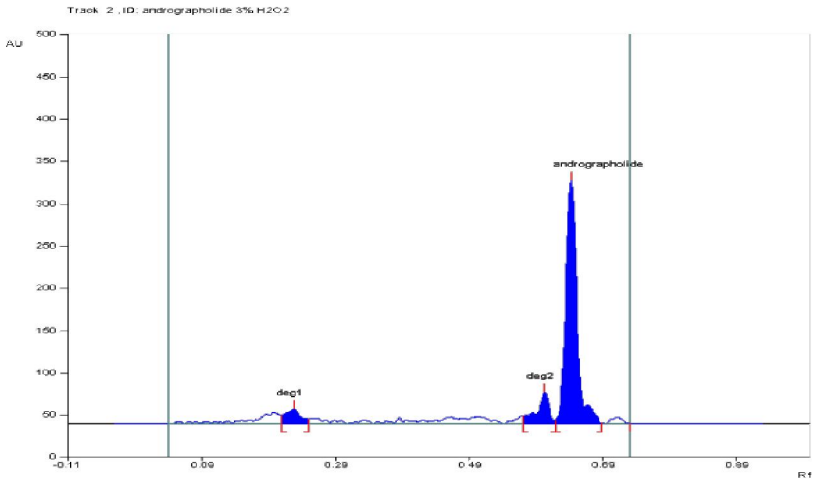


Figure No.19 HPTLC densitogram of andrographolide after Alkaline Degradation — 0.1N NaOH forced degradation



2) Oxidative Degradation — 3% H2O2

Figure No.20 HPTLC densitogram of andrographolide after oxidative (3% H2O2) forced degradation

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3) Acid Degradation — 0.1N HCl

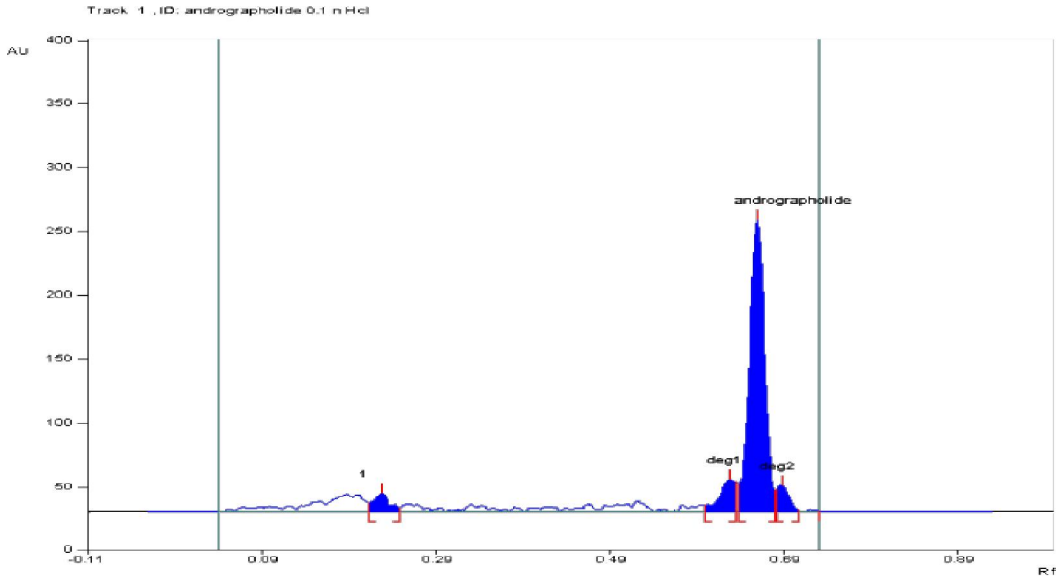

Figure No.21 HPTLC densitogram of andrographolide after acid (0.1N HCl) forced degradation

4) Neutral Degradation — Distilled Water

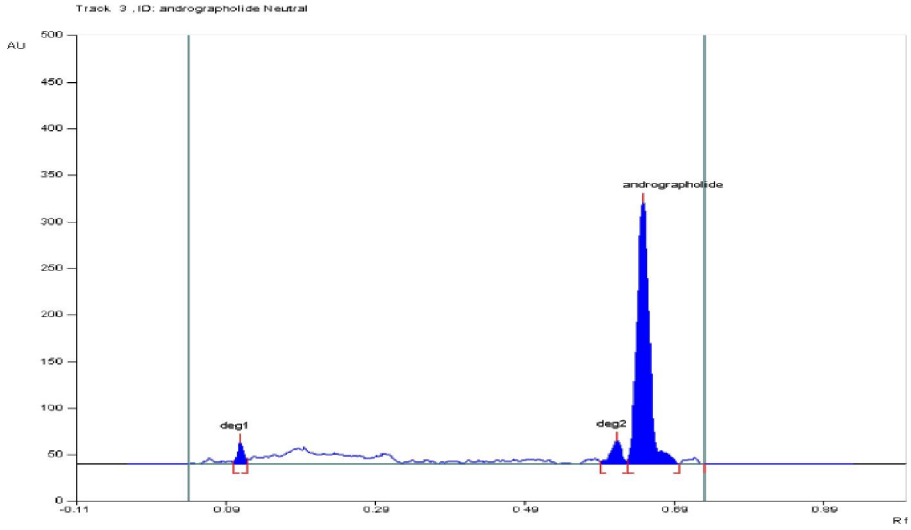

Figure No.22 HPTLC densitogram of andrographolide under neutral (distilled water) condition

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5) Thermal Degradation

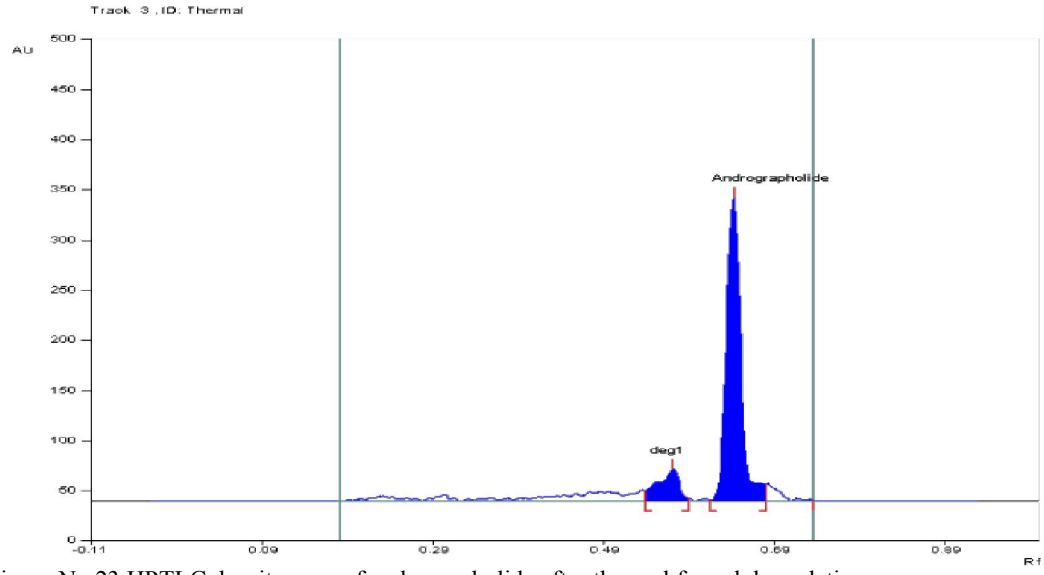

Figure No.23 HPTLC densitogram of andrographolide after thermal forced degradation

6) Photolytic Degradation — UV Cabinet, 254 nm, 24 hrs

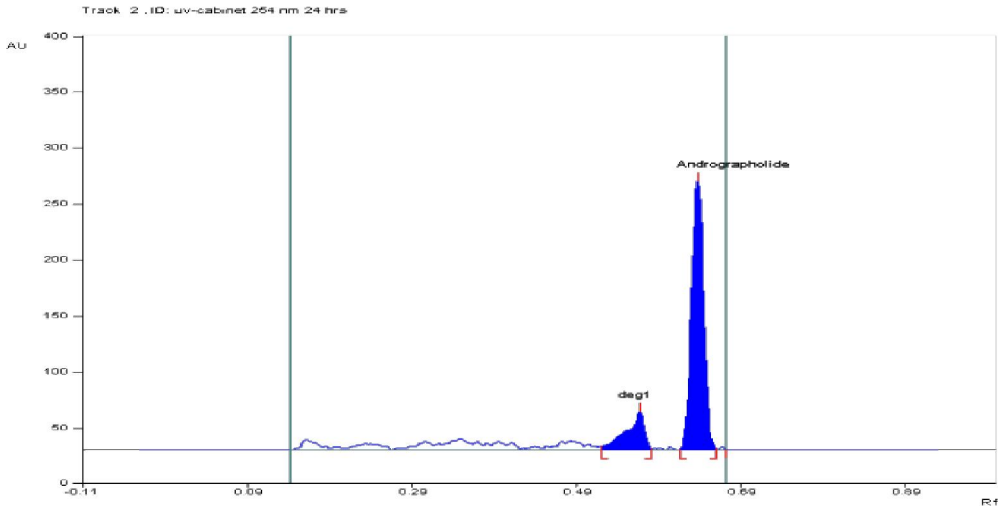

Figure No.24 HPTLC densitogram of andrographolide after photolytic (UV, 254 nm, 24 h) forced degradation

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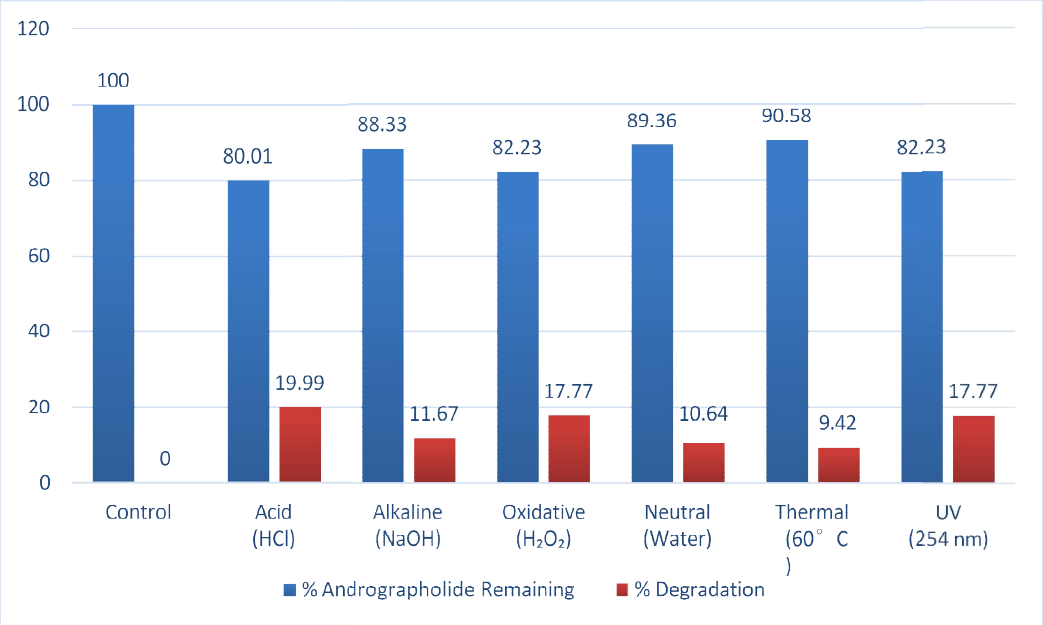

Graph Showing Stability order

Stability Order: Thermal (9.42%) > Neutral (10.70%) > UV Photolytic (13.14%) ≈ Oxidative (13.45%) > Alkaline (15.24%) > Acid Hydrolysis (19.99%)

The acid hydrolysis condition caused maximum degradation (19.99%), consistent with proton-catalysed γ-lactone ring opening confirmed by LC-MS (m/z 333.200). In all stress conditions, degradation products appeared at RF values different from andrographolide (RF 0.60), confirming stability-indicating capability.

10.1 RATIONALE AND REGULATORY BASIS FOR FORCED DEGRADATION STUDIES:

Forced degradation (stress) testing is conducted deliberately under conditions more severe than accelerated stability testing in order to intentionally generate degradation products that may reasonably be expected to form during manufacture, storage, and shelf life of a drug substance or product. As per ICH guideline Q1A(R2), 'Stability Testing of New Drug Substances and Products,' forced degradation studies are recommended to establish the intrinsic stability of the molecule, to identify likely degradation pathways and degradation products, and, critically, to validate the stability-indicating capability of the analytical procedure intended for routine stability monitoring. A method is considered stability-indicating only if it can accurately measure the active ingredient without interference from its process impurities, excipients, and degradation products, and this can only be demonstrated experimentally by deliberately generating such degradation products and showing that the method resolves them from the intact drug.

The six stress conditions employed in this study were selected in accordance with the standard panel recommended by ICH Q1A(R2) and the complementary ICH Q1B guideline on photostability testing, so as to comprehensively probe the susceptibility of andrographolide to the principal chemical degradation mechanisms of hydrolysis, oxidation, heat, and light.

10.2 EXPERIMENTAL PROCEDURE FOR STRESS SAMPLE PREPARATION:

For each stress condition, an accurately weighed quantity of andrographolide reference standard was subjected to the specified stress reagent and duration outlined in Table No.23. Acid hydrolysis was carried out by treating the standard

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with 0.1N hydrochloric acid at room temperature for 30 minutes, after which the solution was neutralised prior to spotting. Alkaline hydrolysis was similarly performed using 0.1N sodium hydroxide under identical time and temperature conditions, followed by neutralisation. Oxidative stress was induced using 3% v/v hydrogen peroxide at room temperature for 30 minutes. Neutral hydrolysis employed distilled water alone under the same duration and temperature as a control for solvent-mediated hydrolysis in the absence of added acid, base, or oxidant. Thermal stress was applied by exposing the solid reference standard to dry heat at 60 degrees Celsius in a hot air oven for 30 minutes, while photolytic stress was applied by exposing the sample to UV radiation at 254 nm in a UV cabinet for 24 hours, in line with ICH Q1B recommendations for photostability assessment. Following the respective stress exposure, each sample was appropriately diluted with the mobile phase/diluent, and an unstressed control sample was analysed in parallel under identical chromatographic conditions to serve as the 100% reference for percentage assay and percentage degradation calculations reported in Table No.24.

10.3 DEGRADATION MECHANISM DISCUSSION FOR EACH STRESS CONDITION:

- Acid Hydrolysis (19.99%): Acid hydrolysis produced the greatest extent of degradation (19.99%), consistent with protonation of the lactone carbonyl oxygen followed by nucleophilic attack of water at the carbonyl carbon, resulting in ring-opening of the strained gamma-lactone to yield a hydroxy-acid intermediate that subsequently undergoes intramolecular dehydration, as confirmed independently by the 18 Da mass loss observed in the LC-MS spectrum of the isolated degradation product (Chapter 9).
- Alkaline Hydrolysis (15.24%): Alkaline hydrolysis produced substantial degradation (15.24%) through base-catalysed saponification of the lactone ring, in which hydroxide ion directly attacks the electrophilic lactone carbonyl carbon, opening the ring to form a carboxylate salt. This mechanism proceeds via a different transition state to acid hydrolysis but converges on structurally related ring-opened products.
- Oxidative Degradation (13.45%): Oxidative stress with hydrogen peroxide resulted in moderate degradation (13.45%), most plausibly through epoxidation or allylic oxidation at the electron-rich exocyclic methylene (C=CH2) double bond and/or oxidation of the free hydroxyl groups to ketonic or carboxylic functionalities, both of which are well-documented reactive sites in labdane diterpenoids.
- Photolytic Degradation (13.14%): UV exposure at 254 nm for 24 hours produced degradation comparable to the oxidative pathway (13.14%), likely proceeding through photo-excitation of the conjugated alpha,beta-unsaturated lactone chromophore, which can undergo photo-isomerisation, [2+2] photocycloaddition, or photo-oxidative side reactions under prolonged irradiation.
- Neutral Hydrolysis (10.70%): Degradation under neutral aqueous conditions was comparatively minor (10.70%), reflecting the intrinsically slower kinetics of water-mediated (uncatalysed) lactone hydrolysis in the absence of an acid or
- base catalyst, and serves as a useful baseline for distinguishing catalysed from uncatalysed hydrolytic degradation.
- Thermal Degradation (9.42%): Dry heat exposure at 60 degrees Celsius for 30 minutes produced the least degradation (9.42%), indicating that andrographolide possesses reasonable thermal stability under short-term moderate-temperature stress in the solid state, an encouraging finding for the handling and short-term storage of the bulk reference material and formulated tablets.

10.4 SIGNIFICANCE OF DEGRADATION KINETICS AND ORDER OF SUSCEPTIBILITY:

The overall order of susceptibility established in this study, Acid Hydrolysis > Alkaline Hydrolysis > Oxidative Degradation approximately equal to Photolytic Degradation > Neutral Hydrolysis > Thermal Degradation, provides practical guidance for the handling, formulation, and storage of andrographolide-containing products. Formulation scientists should be cautious regarding the pH of any aqueous vehicle or buffer system used in downstream product

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development, while the comparatively higher thermal and neutral-hydrolytic stability is reassuring for standard solid-dosage-form manufacturing, provided exposure to strong acid, base, or prolonged UV light is minimised.

Table No.32 Mass Balance Check Across Stress Conditions

Stress Condition	% Assay Remaining	% Degradation (Mass Balance Complement)
Acid Hydrolysis	80.01	19.99
Alkaline Hydrolysis	84.76	15.24
Oxidative (H2O2)	86.55	13.45
Photolytic (UV)	86.86	13.14
Neutral Hydrolysis	89.30	10.70
Thermal	90.58	9.42

The close agreement between the percentage assay remaining and its complementary percentage degradation value across all six conditions (each pair summing to approximately 100%) indicates satisfactory mass balance, confirming that the
observed loss of andrographolide peak area is adequately accounted for by the appearance of new degradation product bands.

SUMMARY AND CONCLUSION:

11.1 SUMMARY

The present work was undertaken to develop and validate a stability-indicating UV-HPTLC method for the quantitative estimation of andrographolide in Andrographis paniculata extract and Kalmegh Ghana Tablets. The study also included structural characterization of andrographolide and its acid degradation product using FTIR-ATR spectroscopy and LC-MS analysis.

- A stability-indicating UV-HPTLC method was successfully developed for the estimation of andrographolide.
- Chromatographic separation was achieved using Toluene: Ethyl Acetate: Methanol: Formic Acid (5:2:3:0.1 v/v/v/v) as the mobile phase on TLC Silica Gel 60 F254 plates.
- Densitometry scanning was carried out at 232 nm and andrographolide was detected at RF 0.60 ± 0.02.
- The developed mobile phase was free from chloroform and therefore represents a greener analytical approach.

Table No. 33 Optimized UV-HPTLC Method Parameters

Parameters	**Conditions**
Stationary Phase	TLC Silica Gel 60 F254
Mobile Phase	Toluene : Ethyl Acetate : Methanol : Formic Acid (5:2:3:0.1 v/v/v/v)
Detection Wavelength	232 nm
RF Value	0.60 ± 0.02
Application Volume	As per optimized method

Summary of Validation Study

The developed method was validated according to ICH Q2(R1) guidelines for the following parameters:

1. Specificity

➢ No interference was observed from blank, placebo matrix or degradation products at the RF value of andrographolide.

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➢ Peak purity analysis confirmed the specificity of the method.

2. Accuracy

➢ Recovery studies were performed at 80%, 100% and 120% levels.

➢ Mean percentage recovery was found to be 99.8 ± 0.7%.

➢ The obtained recoveries confirmed the accuracy of the method.

3. Precision

i) System Precision

➢ Replicate analysis demonstrated excellent instrument precision with %RSD within acceptable limits.

ii) Method Precision

➢ Repeatability studies showed %RSD values less than 2%, confirming precision of the method.

iii) Intermediate Precision

➢ Analysis performed on different days confirmed reproducibility and ruggedness of the method.

4. Linearity

➢ Linearity was established over the concentration range of 250–1500 ng/band.

➢ Calibration curve showed good correlation with regression equation: $y = 1450 + 3.821x$

➢ Correlation coefficient (r^2) was found to be 0.997.

5. Sensitivity

➢ Limit of Detection (LOD) was approximately 50 ng/band.

➢ Limit of Quantification (LOQ) was approximately 150 ng/band.

6. Robustness

➢ Deliberate variations in chromatographic conditions did not significantly affect analytical performance.

➢ The method remained robust under all tested conditions.

7. FTIR Characterization

➢ FTIR-ATR analysis confirmed the characteristic functional groups of andrographolide.

➢ The γ-lactone carbonyl group was observed around 1758–1780 cm^{-1}.

➢ Exocyclic methylene stretching appeared near 1645 cm^{-1}.

➢ Hydroxyl group stretching vibrations were observed between 3200–3550 cm^{-1}.

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8. LC-MS Characterization

➢ LC-MS analysis confirmed andrographolide through the molecular ion peak at m/z 351.211 ([M+H]+).

➢ Characteristic fragmentation pattern further supported structural identification.

➢ Acid degradation product was detected at m/z 333.200 ([M+H]+).

9. Forced Degradation Study

➢ Forced degradation studies were performed under acidic, alkaline, oxidative, thermal, neutral and photolytic conditions.

➢ Maximum degradation was observed under acidic stress (19.99%).

➢ Minimum degradation was observed under thermal stress (9.42%).

➢ The degradation susceptibility order was:

Acid > Alkaline > Oxidative ≈ UV > Neutral > Thermal

➢ Complete separation between andrographolide and degradation products confirmed the stability-indicating nature of the method.

10. Assay of Kalmegh Ghana Tablets

➢ The validated method was successfully applied for estimation of andrographolide in Kalmegh Ghana Tablets.

➢ The assay result was found to be 403.04 ng/band within the validated calibration range.

11.2 CONCLUSION

➢ A simple, accurate, precise and stability-indicating UV-HPTLC method was successfully developed and validated for quantitative estimation of andrographolide.

➢ The optimized chromatographic conditions provided satisfactory separation and reproducible analytical performance.

➢ Validation results complied with ICH Q2(R1) requirements for specificity, linearity, accuracy, precision and sensitivity.

➢ FTIR-ATR spectroscopy confirmed the structural identity of andrographolide through characteristic functional group absorptions.

➢ LC-MS analysis confirmed the molecular structure of andrographolide and its acid degradation product.

➢ Forced degradation studies demonstrated the degradation behavior of andrographolide under various stress conditions.

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➢ The developed method successfully resolved andrographolide from all degradation products and was proven to be stability-indicating.

➢ The method was successfully applied to the analysis of Kalmegh Ghana Tablets and can be used for routine quality control testing.

➢ The combined UV-HPTLC, FTIR-ATR and LC-MS analytical approach provides a comprehensive platform for identification, quantification and stability assessment of andrographolide.

➢ The developed analytical method is suitable for pharmaceutical quality control, stability studies and regulatory documentation of andrographolide-containing herbal formulations.

11.3 COMPARISON WITH EXISTING REPORTED METHODS:

A number of chromatographic methods, including HPLC and HPTLC procedures, have previously been reported in the literature for the quantitative estimation of andrographolide in plant extracts and herbal formulations, many of which employ chlorinated solvents such as chloroform or dichloromethane in the mobile phase. In comparison, the UV-HPTLC method developed and validated in the present work employs a chloroform-free mobile phase composed of Toluene: Ethyl Acetate: Methanol: Formic Acid (5:2:3:0.1 v/v/v/v), offering a comparatively safer and more environmentally benign alternative while still achieving a well-resolved, symmetrical peak at Rf 0.60 and satisfactory validation parameters across specificity, linearity, accuracy, precision, and sensitivity. Furthermore, unlike several previously reported methods that were validated primarily for assay purposes without an accompanying forced degradation study, the present method was additionally challenged against six ICH-recommended stress conditions and shown to be genuinely stability-indicating, supported by independent structural confirmation through FTIR-ATR spectroscopy and LC-MS characterisation of the isolated acid degradation product.

11.4 STRENGTHS OF THE PRESENT STUDY:

➔ Multi -Technique Analytical Platform: The method combines three complementary analytical dimensions, chromatographic separation (HPTLC), vibrational spectroscopic confirmation (FTIR-ATR), and mass-spectrometric structural elucidation (LC-MS), providing a level of analytical rigor beyond a single-technique assay method.

➔ Green Chemistry Alignment: The mobile phase was deliberately optimised to avoid chloroform, aligning the method with current green analytical chemistry principles without compromising resolution or sensitivity.

➔ Direct Applicability to Marketed Formulation: The method was validated not only for assay of the reference standard but also applied successfully to a real marketed herbal formulation (Kalmegh Ghana Tablets), demonstrating practical applicability for routine quality control.

➔ Comprehensive Stress Testing Panel: Six independent stress conditions covering hydrolytic, oxidative, thermal, and photolytic pathways were used, providing a comprehensive assessment of the intrinsic stability of andrographolide rather than relying on a single stress condition.

➔ Isolation and Confirmation of the Degradation Product: The acid degradation product was not merely inferred from a chromatographic shift but was physically isolated by preparative HPTLC and independently confirmed by LC-MS, substantially strengthening the scientific defensibility of the proposed degradation pathway.

11.5 LIMITATIONS OF THE STUDY:

➔ Structural Elucidation Depth: Structural characterisation of the acid degradation product was based on LC-MS mass and fragmentation data; complete stereochemical and positional confirmation would require complementary NMR and HRMS studies, which were beyond the scope of the present work.

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➔ Limited Isolation to Acid Degradation Product Only: Degradation products generated under alkaline, oxidative, thermal, neutral, and photolytic stress were detected and quantified by HPTLC but were not individually isolated and subjected to LC-MS characterisation in the same manner as the acid degradation product.

➔ Formulation -Level Stress Testing Not Performed: The forced degradation study was conducted on the reference standard rather than on the finished tablet formulation, and excipient interference under stress conditions was therefore not separately evaluated.

➔ Single -Laboratory, Limited-Batch Validation: The assay of Kalmegh Ghana Tablets was performed on a limited number of batches; broader inter-batch and inter-laboratory validation would further strengthen the generalisability of the method.

11.6 OVERALL SIGNIFICANCE OF THE RESEARCH:

The research presented in this thesis addresses an important and practical need in herbal drug quality control: the availability of a simple, economical, and scientifically robust stability-indicating method for andrographolide, the principal bioactive marker compound of Andrographis paniculata (Kalmegh). Given the widespread traditional and increasingly pharmaceutical use of Andrographis paniculata-based formulations for their hepatoprotective, immunomodulatory, anti-inflammatory, and antipyretic properties, reliable analytical tools for identity confirmation, quantitative assay, and stability assessment are essential to ensure that marketed herbal products consistently deliver the intended therapeutic dose of the active marker compound throughout their shelf life. By integrating UV-HPTLC assay with FTIR-ATR and LC-MS structural confirmation and a comprehensive forced degradation evaluation, this work provides regulatory-quality documentation that can support pharmacopoeial standardisation, in-process quality control, and stability-study protocols for andrographolide-containing herbal medicines