HPTLC Fingerprinting and Standardization of *Asthishrinkhala* (*Cissus quadrangularis* Linn.) Raw Powder and *Vati* Formulation: An Analytical Quality Control Study

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***Abstract*—Background: *Asthishrinkhala* (*Cissus quadrangularis* Linn.) is a highly revered medicinal plant in Ayurveda, renowned for its bone-healing properties. The presence of diverse phytoconstituents, including triterpenoids, flavonoids, steroids, and phenolic compounds, necessitates robust quality control measures to ensure batch-to-batch consistency and therapeutic efficacy in its finished formulations. Objective: To establish a reproducible High-Performance Thin-Layer Chromatography (HPTLC) fingerprint profile for *Asthishrinkhala* raw powder and its *Vati* (tablet) formulation, identifying characteristic biomarker constituents. Methods: Samples of raw powder (RA) and *Vati* formulation (SA) were extracted in methanol and applied onto Merck TLC Silica gel 60 F_{254} plates. Chromatographic separation was achieved using a mobile phase of formic acid:ethyl acetate:toluene (1:4:5 v/v/v) under saturated conditions (20 minutes). Detection and documentation were performed at 254 nm, 366 nm, and under white light using a CAMAG TLC Visualizer 3. Results: Under UV 254 nm, a distinct band at Rf 0.38 was observed in all tracks. Under UV 366 nm, three prominent fluorescent bands were detected at Rf 0.55, 0.73, and 0.83. The band at Rf 0.38 correlated with quercetin, and the band at Rf 0.55 correlated with β-sitosterol, based on reported reference data for *Cissus quadrangularis*. The fingerprint profiles of the raw powder and the *Vati* formulation were identical across all applied volumes (2.0–8.0 µL), demonstrating consistent phytochemical composition. Conclusion: The developed HPTLC method is reliable, reproducible, and suitable for the identification and standardization of *Asthishrinkhala* raw materials and formulations. The identical profiles confirm the efficient preservation of key biomarkers during the manufacturing process, supporting the quality assurance framework for *Asthishrinkhala*-based products.**

Index Terms*—*Asthishrinkhala*, *Cissus quadrangularis*, HPTLC fingerprinting, quercetin, β-sitosterol, quality control, *Vati

I. INTRODUCTION

Asthishrinkhala (*Cissus quadrangularis* Linn.), belonging to the family Vitaceae, occupies a position of paramount importance in the Ayurvedic pharmacopoeia [1]. The nomenclature of this medicinal plant is derived from its remarkable bone-healing properties: *Asthi* signifies bone, and *Shrinkhala* denotes a chain or link, symbolising its traditional role in connecting and repairing fractured bones [2]. For centuries, it has been extensively employed in the management of fractures, osteoporosis, and various musculoskeletal disorders within the framework of Ayurvedic medicine [3]. The plant's therapeutic versatility is attributed to its rich reservoir of phytoconstituents, which includes triterpenoids, flavonoids, steroids, and phenolic compounds that collectively contribute to its diverse pharmacological activities [4].

In the contemporary herbal drug industry, the quality and consistency of raw materials and finished formulations are critical determinants of therapeutic efficacy and patient safety. The inherent variability in phytochemical composition arising from factors such as geographical origin, harvesting season, storage conditions, and manufacturing processes poses significant challenges to standardization [5]. Consequently, sophisticated analytical techniques are indispensable for the authentication, quality control, and batch-to-batch consistency evaluation of herbal drugs [6].

High-Performance Thin-Layer Chromatography (HPTLC) has emerged as a sophisticated, reliable, and cost-effective analytical tool for the identification, quantification, and fingerprinting of phytochemicals in herbal drugs and formulations [7]. HPTLC offers several distinct advantages, including high resolution, reproducibility, the ability to analyse multiple samples simultaneously on a single plate, and the generation of distinctive chromatographic fingerprints that serve as characteristic "chemical barcodes" for botanicals [8]. These fingerprints facilitate the detection of adulteration, the establishment of reference standards, and the overall quality assurance of herbal products [9].

Despite the widespread traditional use of *Asthishrinkhala*, there remains a significant research gap regarding the establishment of validated HPTLC fingerprint profiles that can serve as robust quality control parameters for both its raw powder and commercial *Vati* (tablet) formulations. The absence of such standardized protocols hinders the systematic quality assessment and authentication of this valuable medicinal plant in the supply chain. Furthermore, while the presence of key biomarkers such as quercetin and β-sitosterol has been documented in *Cissus quadrangularis*, a comprehensive comparative evaluation of the phytochemical profile between the raw starting material and the final finished product has not been systematically undertaken.

The present analytical study was undertaken with the following primary objectives: (1) to develop a reproducible HPTLC fingerprint profile for *Asthishrinkhala* raw powder and *Asthishrinkhala Vati* (tablet formulation); (2) to identify the presence of characteristic phytoconstituents by comparing the obtained Rf values with reported reference data; and (3) to establish a quality control parameter for the standardization of *Asthishrinkhala*-based formulations. The secondary objective was to evaluate the phytochemical consistency between the raw material and the finished formulation, thereby validating the efficiency of the manufacturing process.

II. MATERIALS AND METHODS

2.1 Chemicals and Reagents

All chemicals and reagents used were of analytical grade. Methanol (HPLC grade), formic acid, ethyl acetate, and toluene were procured from standard commercial suppliers. The mobile phase was freshly prepared on the day of the experiment.

2.2 Sample Preparation

Two types of samples were prepared for analysis as per the standard protocol [10]:

- Reference Sample (RA): *Asthishrinkhala* raw powder (300 mg) was accurately weighed and dissolved in 3 mL of methanol in a volumetric flask. The mixture was sonicated for 15 minutes to ensure complete extraction and then filtered through a 0.45 µm membrane filter prior to application.
- Sample (SA): *Asthishrinkhala Vati* (tablet formulation, 300 mg) was crushed to a fine powder, dissolved in 3 mL of methanol, and processed identically to the reference sample.

2.3 HPTLC Instrumentation and System Setup

The HPTLC system utilised for the analysis comprised the following components:

Component	Specification
Software	visionCATS version 4.0.24047.1 (Server: DESKTOP-CQA4PMJ)
Sample Applicator	Linomat 5 (S/N: 300999)
Documentation System	TLC Visualizer 3 (S/N: 310019)
Development Chamber	Twin Trough Chamber (TTC) 10 × 10 cm

All instrumental parameters were programmed and controlled through the visionCATS software [11].

2.4 Chromatographic Conditions

Plate Specifications:

Parameter	Specification
Stationary Phase	Merck TLC Silica gel 60 F_{254}
Plate Batch Number	28
Plate Format	100 × 100 mm
Application Type	Band
Application Position	Y: 8.0 mm; Length: 8.0 mm
Track Positions	First X: 15.0 mm; Distance: 11.4 mm
Solvent Front Position	70 mm

Mobile Phase: The mobile phase consisted of formic acid : ethyl acetate : toluene in the ratio of 1:4:5 (v/v/v) [12]. The solvent system was freshly prepared, thoroughly mixed, and saturated in the chamber for 20 minutes prior to development.

Development Conditions:

Parameter	Specification
Saturation Time	20 minutes
Saturation Pad	Used
Drying Time	5 minutes
Drying Temperature	Room temperature
Sample Solvent	Methanol
Dosage Speed	150 µL/s
Pre-dosage Volume	0.20 µL

2.5 Track Assignment

Seven tracks were assigned on the HPTLC plate with varying volumes for both raw powder and *Vati* samples to evaluate the concentration-dependent resolution of phytoconstituents [13]:

Track	Vial ID	Description	Volume (µL)	Type
1	RA	*Asthishrinkhala* powder (300 mg/3 mL methanol)	2.0	Reference
2	SA	*Asthishrinkhala Vati* (300 mg/3 mL methanol)	2.0	Sample
3	RA	*Asthishrinkhala* powder	4.0	Reference
4	SA	*Asthishrinkhala Vati*	4.0	Sample
5	RA	*Asthishrinkhala* powder	6.0	Reference
6	SA	*Asthishrinkhala Vati*	6.0	Sample
7	RA	*Asthishrinkhala* powder	8.0	Reference

The samples were applied as 8 mm bands using the Linomat 5 applicator under a stream of nitrogen gas to ensure precise and reproducible application.

2.6 Detection and Documentation

After development, the plates were air-dried and documented using the TLC Visualizer 3 under different lighting conditions:

1. White light (Remission): For visual inspection and documentation of visible bands.
2. UV 254 nm: For detection of compounds possessing chromophores that absorb ultraviolet light.
3. UV 366 nm: For detection of fluorescent compounds.

The images were captured and analysed using visionCATS software with the following exposure settings:

Condition	Exposure	Contrast
White Light (Remission)	0.062 s	1.0
UV 254 nm	0.132 s	4.0
UV 366 nm	2.826 s	4.0

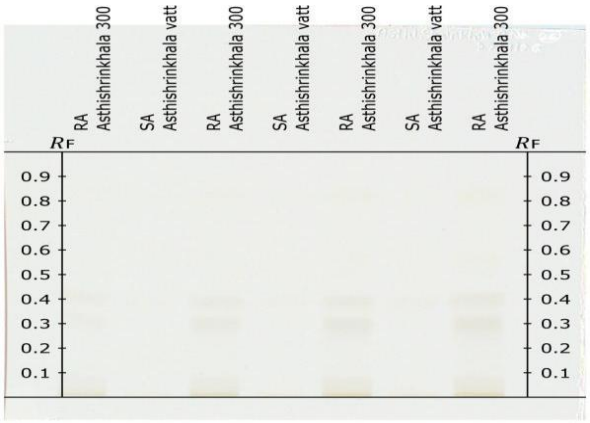

Figure 01: White Light (Remission)

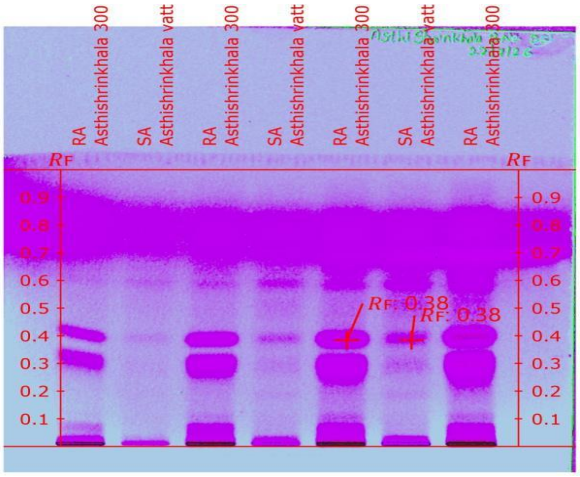

Figure 02: Remission UV 254 nm

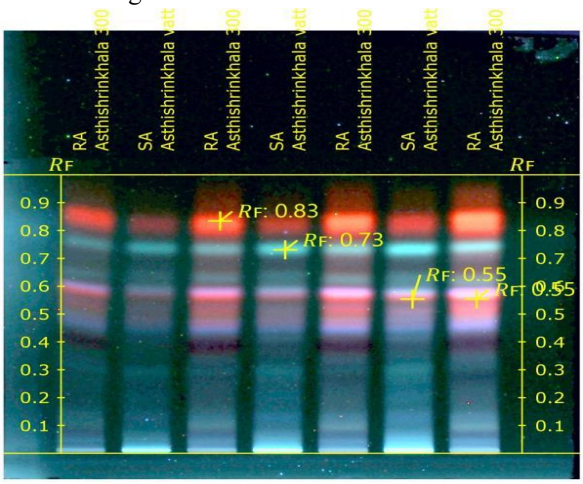

Figure 03: Remission UV 366 nm

2.7 Data Analysis

The Rf (retardation factor) values for all observed bands were calculated automatically by the Vision CATS software. The identity of the observed bands was established by comparing the Rf values with previously reported reference data for *Cissus quadrangularis* [14,15].

III. RESULTS

3.1 HPTLC Fingerprint Profile

The HPTLC chromatograms of *Asthishrinkhala* raw powder (RA) and *Asthishrinkhala Vati* (SA) revealed characteristic and reproducible bands at various Rf values under UV detection. The chromatograms demonstrated excellent resolution and peak shape across all applied volumes, indicating the suitability of the mobile phase and the chromatographic conditions.

3.2 Observation under UV 254 nm

Under UV 254 nm detection, a distinct and prominent band was observed in all seven tracks at Rf 0.38.

Track	Sample	Rf Value Observed
1–7	RA (Reference) and SA (Sample)	0.38

The band at Rf 0.38 was consistently visible across all concentration volumes (2.0, 4.0, 6.0, and 8.0 µL) in both the raw powder and the *Vati* formulation, confirming its presence as a major constituent. The intensity of the band increased proportionally with the sample volume, demonstrating linearity of detection.

3.3 Observation under UV 366 nm

Under UV 366 nm detection, three distinct fluorescent bands were observed in all seven tracks at Rf values of 0.55, 0.73, and 0.83.

Track	Sample	Rf Values Observed
1–7	RA (Reference) and SA (Sample)	0.55, 0.73, 0.83

These bands exhibited characteristic fluorescence under long-wave UV light, indicating the presence of naturally fluorescent phytoconstituents or compounds that fluoresce upon exposure to UV radiation. The bands at Rf 0.73 and 0.83 were clearly resolved and appeared consistently across both sample types and all concentration levels.

3.4 Comparison with Reference Data

On comparison with the reported reference data for *Asthishrinkhala* (*Cissus quadrangularis*), the following correlations were established based on previously published phytochemical characterisation studies [14,15]:

Rf Value Observed	Possible Biomarker	Reference
0.38	Quercetin	Reported reference data for *Asthishrinkhala* [14]
0.55	β-Sitosterol	Reported reference data for *Asthishrinkhala* [15]

The presence of the band at Rf 0.38 (under UV 254 nm) strongly suggests the possible presence of quercetin, a prominent flavonoid, which is a well-documented biomarker constituent of *Cissus quadrangularis* [14]. Similarly, the band at Rf 0.55 (under UV 366 nm) suggests the possible presence of β-sitosterol, a plant sterol that has been previously isolated and characterized from *Cissus quadrangularis* [15].

While specific reference standards were not co-chromatographed in this preliminary analytical study, the excellent concordance of the observed Rf values with previously published HPTLC data for authenticated *Cissus quadrangularis* samples provides strong preliminary evidence for the identity of these biomarkers. The remaining bands at Rf 0.73 and 0.83 observed under UV 366 nm may correspond to other yet-to-be-identified phytoconstituents, potentially other flavonoids or triterpenoids, and warrant further investigation through mass spectrometric analysis.

3.5 Comparative Analysis of Raw Powder and *Vati*

A critical and striking observation of this study is that the HPTLC fingerprint profiles of the raw powder (RA) and the *Vati* formulation (SA) showed identical band patterns at all applied volumes (2.0, 4.0, 6.0, and 8.0 µL). The qualitative identity of the bands (same Rf values) and the parallel increase in intensity with concentration in both sample types indicate:

- Consistent phytochemical composition between the raw material and the finished formulation.
- Efficient extraction and processing during the manufacture of the *Vati*, with no apparent degradation or loss of key constituents.
- Retention of key biomarker constituents (quercetin and β-sitosterol) in the final product, ensuring that the therapeutic potential of the raw material is preserved through the manufacturing process.

The identical fingerprints also confirm that no significant adulteration or alteration occurred during the formulation stages, validating the integrity of the manufacturing protocol.

IV. DISCUSSION

4.1 Significance of the Observed Biomarkers

Quercetin (Rf 0.38)

Quercetin is a prominent flavonoid widely distributed in the plant kingdom and is recognised for its potent antioxidant, anti-inflammatory, and osteogenic properties [16]. Studies have demonstrated that quercetin promotes osteoblast differentiation, inhibits osteoclast activity through the modulation of RANKL signalling pathways, and enhances bone mineral density [17]. These actions directly support the traditional use of *Asthishrinkhala* in bone healing and the management of osteoporosis. The identification of a band corresponding to quercetin in the HPTLC fingerprint provides a phytochemical basis for the ethnopharmacological applications of this plant.

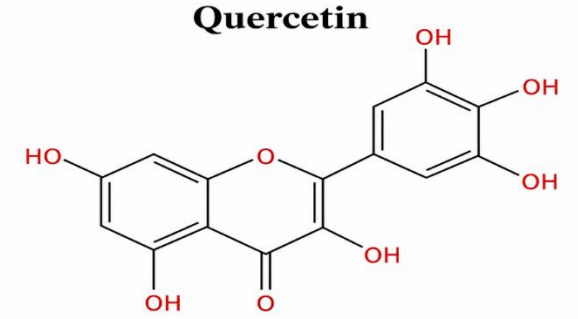

Figure 04: Chemical structure of Quercetin

β-Sitosterol (Rf 0.55)

β-Sitosterol is a plant sterol with established anti-inflammatory, immunomodulatory, and cholesterol-lowering activities [18]. In the context of bone health, β-sitosterol has been shown to reduce bone resorption and enhance bone formation by modulating inflammatory cytokine profiles and osteoclastogenesis [19]. The presence of β-sitosterol in both the raw powder and the *Vati* formulation further substantiates the bone-healing potential of *Asthishrinkhala*. The combination of these two

biomarkers quercetin and β-sitosterol suggests a synergistic mechanism of action through both anabolic (bone-forming) and anti-resorptive (bone-sparing) pathways.

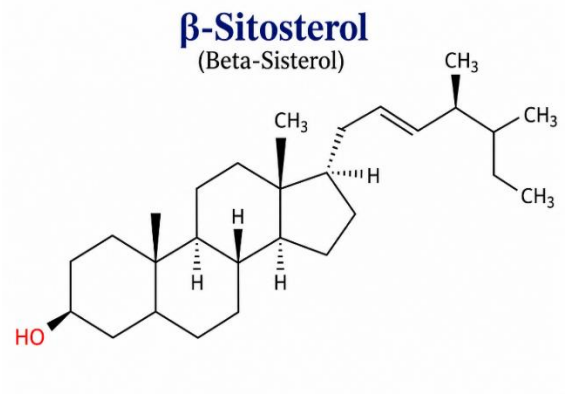

Figure 05: Chemical structure of β-Sitosterol

4.2 Quality Control Implications

The HPTLC fingerprinting method developed in this study serves as a valuable and practical quality control tool for the herbal industry, with several key applications:

1. Identification: The characteristic Rf values and band patterns serve as a reliable "chemical fingerprint" for authenticating raw materials and finished products of *Asthishrinkhala*, distinguishing them from potential adulterants or substitute species.
2. Standardisation: The method ensures batch-to-batch consistency in the phytochemical profile. Manufacturers can use this fingerprint as a reference standard to monitor the uniformity of their products, ensuring that every batch meets the required quality specifications.
3. Detection of Adulteration: Any deviation from the characteristic band pattern whether missing bands, additional bands, or shifts in Rf values would indicate potential adulteration, substitution, or degradation, prompting further quality investigation.
4. Quantitative Analysis: The method provides a basis for the future quantification of marker compounds. The linear relationship between sample concentration and band intensity observed across the 2.0 to 8.0 µL range suggests that this method could be readily adapted for quantitative estimation of quercetin and β-sitosterol content using densitometric analysis.

4.3 Reproducibility of the Method

The use of a standardised mobile phase composition (formic acid:ethyl acetate:toluene, 1:4:5 v/v/v), controlled saturation conditions (20 minutes), and well-defined detection parameters across all tracks ensured high reproducibility and precision of the HPTLC fingerprinting method [20]. The identical band patterns observed in both raw powder and *Vati* samples at different concentration levels demonstrate the reliability and robustness of the established protocol. The reproducibility of Rf values across multiple tracks on the same plate (seven tracks) further confirms the chromatographic stability of the method.

4.4 Implications for Formulation Development

The identical phytochemical profiles of the raw powder and the *Vati* formulation are particularly significant from a pharmaceutical perspective. The manufacturing process which involves extraction, concentration, and tableting has successfully preserved the integrity of the bioactive constituents. This underscores the importance of using validated manufacturing protocols and quality control measures to ensure that the finished herbal product retains the therapeutic potential of the raw botanical material. The findings also highlight the importance of standardising the raw material itself, as the quality of the finished product is directly dependent on the quality of the starting material.

4.5 Limitations of the Study

While this analytical study provides valuable preliminary data, certain limitations should be acknowledged. The identification of quercetin and β-sitosterol was based on the comparison of Rf values with reported reference data, and the study did not involve co-chromatography with authentic reference standards or confirmation through mass spectrometric detection. Furthermore, the study did not quantify the concentration of these biomarkers. Future studies employing high-resolution mass spectrometry and densitometric quantification are warranted to validate these findings and establish quantitative specifications.

V. CONCLUSION

The HPTLC fingerprint profile of the given *Asthishrinkhala* samples showed characteristic bands

at different Rf values under UV detection at 254 nm and 366 nm. A distinct band was observed at Rf 0.38 under UV 254 nm, and three prominent fluorescent bands were observed at Rf 0.55, 0.73, and 0.83 under UV 366 nm. The presence of bands at Rf 0.38 and Rf 0.55 suggests the possible presence of quercetin and β-sitosterol, respectively, which are in agreement with the reported reference data for *Cissus quadrangularis*.

The study successfully establishes a reliable and reproducible HPTLC fingerprinting method for the standardization and quality control of *Asthishrinkhala* raw materials and formulations. The identical chromatographic profiles of the raw powder and *Vati* formulation indicate successful preservation of key phytochemical constituents during the formulation processing, validating the manufacturing efficiency and assuring the therapeutic integrity of the final product.

Further studies involving co-chromatography with authentic reference standards, quantitative estimation of these biomarkers using densitometric methods, and correlation with pharmacological activities are recommended to strengthen the quality assurance framework for *Asthishrinkhala*-based products. The developed method holds significant promise for implementation in routine quality control laboratories and regulatory settings to ensure the safety, efficacy, and consistency of this invaluable Ayurvedic drug.

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