

A Comparative High-Performance Thin-Layer Chromatographic Fingerprint Analysis of Two Classical Ayurvedic Kwaths: Unveiling the Phytochemical Basis of Therapeutic Action

Running Title: HPTLC Fingerprinting of Venugranthikultthadi and *HiberadiKwath*

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Abstract

Introduction: Ayurvedic polyherbal formulations, such as *Kwaths* (decoctions), represent complex mixtures whose therapeutic efficacy is attributed to a synergistic interplay of multiple phytoconstituents. Standardization of these formulations using modern analytical techniques is crucial for ensuring quality, consistency, and establishing a scientific basis for their traditional use. This study aims to develop and compare the high-performance thin-layer chromatography (HPTLC) fingerprint profiles of two classical Ayurvedic formulations: *VenugranthikultthadiKwath* and *HiberadiKwath*.

Methods: The two Kwath samples were prepared via standardized extraction using methanol. HPTLC was performed on pre-coated silica gel 60 F254 plates using a mobile phase of Toluene: Ethyl Acetate: Methanol: Formic Acid (3:2:1:0.5 v/v/v/v). The chromatograms were developed and visualized under UV light at 254 nm (short wave) and 366 nm (long wave) before and after derivatization. The retention factor (R_f) values of resolved bands were recorded and interpreted to identify probable classes of phytoconstituents based on their chromatographic behavior.

Results: The chromatographic conditions yielded well-resolved, reproducible bands. *VenugranthikultthadiKwath* (Track 4) revealed 7 bands at 254 nm and 3 bands at 366 nm. *HiberadiKwath* (Track 3) exhibited 7 bands at 254 nm and 6 bands at 366 nm. At 254 nm, both formulations shared bands with similar R_f values (e.g., ~0.90-0.91), indicating the presence of common non-polar terpenoids. At 366 nm, *HiberadiKwath* displayed a significantly richer fluorescence pattern. Probable phytoconstituent classes identified across both formulations included polar phenolics (gallic acid, R_f ~0.07-0.11), flavonoid glycosides (rutin, R_f ~0.33-0.44), triterpenes (β-sitosterol, R_f ~0.68), and non-polar terpenoids (R_f ~0.88-0.92).

Conclusion: The HPTLC fingerprint profiles provide a visual and analytical basis for distinguishing between the two formulations. *VenugranthikultthadiKwath*'s profile, dominated by UV-absorbing tannins and triterpenes, aligns with its traditional use in managing venous disorders (Granthi). *HiberadiKwath*'s diverse fluorescent profile suggests a higher abundance of flavonoids and coumarins, pointing towards its potential for broader anti-inflammatory and metabolic actions. This study successfully establishes distinct chemical fingerprints for these formulations, serving as a critical step in their pharmacognostic standardization and offering a foundation for correlating phytochemical composition with observed therapeutic activities.

Keywords: HPTLC, Ayurveda, *VenugranthikultthadiKwath*, *HiberadiKwath*, Phytochemical Fingerprinting, Standardization, Flavonoids, Triterpenoids.

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1. Introduction

Ayurveda, the ancient Indian system of medicine, relies heavily on polyherbal formulations for their holistic and synergistic therapeutic effects [1]. Among these, *Kwaths* or *Kashayas* (decoctions) are fundamental dosage forms, prepared by boiling

herbs in water to extract water-soluble active principles [2]. The therapeutic efficacy of these formulations is not attributed to a single compound but to the complex interplay of hundreds of phytoconstituents, including alkaloids, flavonoids, tannins, saponins, and terpenoids [3]. This complexity presents a significant challenge for quality control and standardization, which are

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essential for ensuring batch-to-batch consistency, reproducibility of therapeutic effects, and integration into modern healthcare systems [4].

Traditional methods of quality assessment, such as organoleptic evaluation, are subjective and insufficient for the rigorous standards of modern pharmacognosy. Therefore, sophisticated analytical techniques are required to create a "chemical fingerprint" that can holistically characterize these complex mixtures [5]. High-Performance Thin-Layer Chromatography (HPTLC) has emerged as a powerful and cost-effective tool for this purpose [6]. HPTLC offers several advantages over other chromatographic techniques, including the ability to run multiple samples simultaneously under identical conditions, high sensitivity, and the visual representation of the entire phytochemical profile, which allows for a direct comparison of samples [7, 8].

The present study focuses on two classical Ayurvedic formulations: *VenugranthikultthadiKwath* and *HiberadiKwath*. *VenugranthikultthadiKwath* is traditionally indicated in the management of *Granthi* (which can be correlated with conditions like venous thromboembolism, fibroids, or other mass-like growths) and is believed to possess properties that act on the venous channels (*Siraj Granthi*) [9]. Its pharmacodynamics are thought to be mediated by its *Tridosahara* (balancing all three doshas) and *Shothahara* (anti-inflammatory) actions. *HiberadiKwath*, on the other hand, is a formulation traditionally used for conditions like obesity, diabetes, and other metabolic disorders, attributed to its *Medohara* (lipid-lowering) and *Deepana-Pachana* (digestive-stimulating) properties [10]. While both are Kwaths, their indications suggest distinct phytochemical compositions and therapeutic targets.

A comparative phytochemical analysis of these two formulations has not been previously reported. Therefore, the primary objectives of this study were:

1. To develop and validate a robust HPTLC method for the comparative fingerprint analysis of *VenugranthikultthadiKwath* and *HiberadiKwath*.
2. To identify the major phytoconstituent classes present in each formulation based on their chromatographic behavior (R_f values) under UV light (254 nm and 366 nm).
3. To interpret the observed phytochemical profiles in the context of their respective traditional therapeutic uses.

This research aims to provide a scientific foundation for the standardization of these Ayurvedic formulations, linking their traditional knowledge with modern analytical chemistry.

2. Materials and Methods

2.1. Sample Collection and Preparation

The samples of *VenugranthikultthadiKwath* (Sample ID: 04) and *HiberadiKwath* (Sample ID: 03) were obtained from the Pharmacy of the Parul Institute of Ayurved, Vadodara, Gujarat, prepared according to classical Ayurvedic texts. For the HPTLC analysis, 5 grams of each Kwath sample were accurately weighed and extracted with 100 mL of methanol. The mixture was sonicated for 15 minutes and then filtered through Whatman filter paper No. 1. The resulting methanolic extracts were concentrated and used as the test solutions for chromatography.

2.2. Chromatographic Conditions

HPTLC was performed on pre-coated silica gel 60 F254 aluminum sheets (MERCK, Germany) of 20 x 10 cm dimension. The stationary phase was activated by heating the plates at 100°C for 30 minutes prior to sample application.

2.2.1. Sample Application

The test solutions (10.0 μ L each) were applied as 8 mm bands onto the HPTLC plates using a CAMAG Linomat 5 (S/N: 280008) semi-automatic applicator equipped with a 100 μ L Hamilton syringe. The application parameters were: band length 8 mm, distance from side edge 20 mm, and distance from bottom edge 15 mm. The track spacing was maintained at 21.4 mm.

2.2.2. Mobile Phase and Development

The plates were developed in a CAMAG TLC Twin Trough Chamber (20 x 10 cm) pre-saturated with the mobile phase for 30 minutes at 25°C $\pm$ 2°C. The mobile phase consisted of a mixture of Toluene: Ethyl Acetate: Methanol: Formic Acid in a ratio of 3:2:1:0.5 (v/v/v/v). The development distance was 80 mm from the point of application. After development, the plates were removed and dried in a current of air.

2.2.3. Detection and Visualization

The dried plates were visualized under UV light at 254 nm (short wave) and 366 nm (long wave) using a CAMAG UV cabinet. For further visualization, the plates were derivatized by dipping in a suitable reagent (e.g., anisaldehyde-sulfuric acid) using a CAMAG Dip Tank for approximately 1 minute. The plates were then heated on a TLC Plate Heater at 100 $\pm$ 5°C for 3 minutes to allow full color development. The chromatograms were documented using a CAMAG Reprostar 3 system.

2.2.4. R_f Value Calculation

The retention factor (R_f) values for each resolved

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band were calculated based on the distance traveled by the solute divided by the distance traveled by the solvent front.

3. Results

The developed HPTLC method provided well-resolved, sharp, and reproducible bands for both formulations, enabling a comprehensive fingerprint analysis.

3.1. Chromatographic Profile at 254 nm (UV Short Wave)

At 254 nm, which primarily detects compounds with conjugated double bonds (e.g., unsaturated terpenoids, aromatic compounds), both formulations displayed seven distinct bands. The R_f values for these bands are presented in Table 1.

Table 1: R_f Values of Bands Observed at 254 nm

Spot No.	Track 04: <i>VenugranthikultthadiKwath</i>	Track 03: <i>HiberadiKwath</i>
1	0.07	0.11
2	0.33	0.19
3	0.40	0.26
4	0.52	0.34
5	0.62	0.44
6	0.68	0.64
7	0.90	0.91

A notable observation at this wavelength is the presence of a common high-R_f band in both formulations (0.90 and 0.91, respectively), indicating the likely presence of a shared class of non-polar terpenoids.

3.2. Chromatographic Profile at 366 nm (UV Long Wave)

At 366 nm, which detects compounds with inherent fluorescence or those that become fluorescent after derivatization (e.g., coumarins, certain flavonoids), a distinct difference was observed. *VenugranthikultthadiKwath* showed only 3 fluorescent bands, whereas *HiberadiKwath* showed 6. The R_f values are detailed in Table 2.

Table 2: R_f Values of Bands Observed at 366 nm

Spot No.	Track 04: <i>VenugranthikultthadiKwath</i>	Track 03: <i>HiberadiKwath</i>
1	0.02	0.03
2	0.61	0.15

3	0.92	0.28
4	---	0.63
5	---	0.76
6	---	0.88

3.3. Interpretation of Probable Phytoconstituents

The interpretation is based on the R_f values, their position on the plate, and the behavior with the specific mobile phase (moderately polar with an acidic modifier, selective for flavonoids, terpenes, and phenolic acids). The probable classes and likely compounds are summarized in Table 3.

Table 3: Interpretation of Phytoconstituents Based on R_f Values

Track	Wavelength	Rf Value	Probable Phytoconstituent Class	Likely Compound(s)
Track 4	254 nm	0.07	Polar Phenolics / Saponins	Gallic acid, saponin glycosides
		0.33	Phenolic / Flavonoid Glycosides	Rutin, chlorogenic acid
		0.40	Flavonoids / Tannins	Quercetin glycoside, ellagic acid
		0.52	Flavonoids / Coumarins	Kaempferol, scopoletin
		0.62	Terpenoids / Flavonoid Aglycones	Luteolin, ursolic acid
		0.68	Triterpenes / Sterols	Beta-sitosterol, betulinic acid
		0.90	Non-Polar Terpenoids / Fatty Acids	Diterpenoids, long-chain fatty acids
	366 nm	0.02	Polar Fluorescent Compound	Coumarin glucoside or phenolic acid

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		0.61	Flavonoid / Coumarin	Quercetin , umbelliferone
		0.92	Non-Polar Terpenoid / Sterol	Triterpenoid ester
Track 3	254 nm	0.11	Polar Phenolics / Amino Acids	Gallic acid, protocatechuic acid
		0.19	Organic Acids / Glycosides	Caffeic acid, phenolic glycosides
		0.26	Tannins / Polar Flavonoids	Catechin, ellagic acid derivatives
		0.34	Flavonoid Glycosides	Rutin, vitexin
		0.44	Phenolic Acids / Flavonoids	Ferulic acid, apigenin glycoside
		0.64	Flavonoid Aglycones / Coumarins	Quercetin , scopoletin
		0.91	Non-Polar Terpenoids	Diterpenoids, waxes
	366 nm	0.03	Polar Coumarin / Phenolic Glycoside	Aesculetin, chlorogenic acid
		0.15	Flavonoid Glycoside	Rutin, hyperoside
		0.28	Tannin / Phenolic	Catechin, epicatechin
		0.63	Flavonoid Aglycone	Quercetin , luteolin
		0.76	Coumarin / Terpenoid	Coumarin derivative ,

				sesquiterpene
		0.88	Non-Polar Terpenoid	Triterpenoid, sterol

3.4. Overall Phytochemical Profile Summary

- **Venugranthikultthadi Kwath (Track 4):** This formulation's profile (7 bands at 254 nm vs. 3 at 366 nm) indicates a dominance of UV-absorbing compounds over strongly fluorescent ones. Its composition is rich in tannins and phenolic acids (low-Rf), flavonoid glycosides (mid-Rf), and triterpenes/sterols (high-Rf).
- **Hiberadi Kwath (Track 3):** This formulation shows a broader diversity with 7 bands at 254 nm and 6 at 366 nm. The richer fluorescence pattern indicates a higher content of naturally fluorescent compounds such as coumarins and specific flavonoids. Its profile features low-Rf water-soluble glycosides, mid-Rf flavonoid aglycones, and high-Rf non-polar terpenoids.

4. Discussion

The primary aim of this study was to develop a robust HPTLC fingerprinting method to compare the phytochemical compositions of two important Ayurvedic Kwaths and to interpret the findings in the context of their traditional therapeutic applications. The choice of the mobile phase—Toluene: Ethyl Acetate: Methanol: Formic Acid (3:2:1:0.5 v/v/v/v)—was strategic. The moderate polarity with an acidic modifier (formic acid) is classically employed for the optimal separation of flavonoids, phenolic acids, and triterpenoids, which are the key bioactive groups expected in such polyherbal decoctions [11, 12]. The results successfully yielded distinct, reproducible fingerprints for both formulations, highlighting both their shared characteristics and unique phytochemical signatures.

4.1. Shared Phytochemical Features and Their Significance

A striking similarity is the presence of high-Rf bands (Rf 0.90-0.92) in both formulations under both wavelengths. This strongly suggests the presence of common non-polar terpenoids, possibly triterpenoids or sterols [13]. Such compounds, like β -sitosterol, are ubiquitous in many medicinal plants and are known for their anti-inflammatory, anti-hyperlipidemic, and membrane-stabilizing properties [14]. Their presence in both Kwaths suggests a foundational anti-inflammatory action, albeit likely expressed differently due to the other co-existing compounds. Additionally, low-Rf spots

(0.02-0.11) across both tracks indicate a commonality in highly polar phenolic compounds, such as gallic acid, which are potent antioxidants [15]. This shared antioxidant base could contribute to the overall *Rasayana* (rejuvenating) potential often attributed to Ayurvedic formulations.

4.2. Distinct Phytochemical Signature of *VenugranthikultthadiKwath*

The fingerprint of *VenugranthikultthadiKwath* is characterized by a strong UV absorption profile at 254 nm (7 spots) but a relatively simple fluorescence profile at 366 nm (3 spots). This pattern is indicative of a formulation rich in tannins, condensed phenolic compounds, and triterpenes [16].

The presence of significant bands corresponding to tannins and triterpenoids (Rf 0.07, 0.40, 0.68) is particularly noteworthy. Tannins are well-documented for their astringent and venotonic properties [17]. They strengthen vascular walls, reduce capillary permeability, and exhibit anti-inflammatory activity by modulating the arachidonic acid pathway [18]. This directly aligns with the classical use of *VenugranthikultthadiKwath* for *Granthis* (venous swellings/thrombotic conditions). Triterpenes like betulinic and ursolic acid, suggested by the band at Rf 0.68, possess potent anti-inflammatory, anti-edematous, and wound-healing properties, further supporting its role in managing inflammatory swellings [19]. The presence of flavonoid glycosides (Rf 0.33, 0.52) like rutin, known for its anti-thrombotic and vascular-protective effects, synergistically reinforces the formulation's venotropic action [20].

4.3. Distinct Phytochemical Signature of *HiberadiKwath*

HiberadiKwath exhibits a markedly different profile, particularly its rich fluorescence pattern at 366 nm, which reveals six distinct bands compared to only three in the other formulation. This points towards a higher diversity and abundance of naturally fluorescent compounds, namely coumarins and specific flavonoid aglycones [21, 22].

The presence of coumarin derivatives (Rf 0.03, 0.76) and flavonoid aglycones like quercetin and luteolin (Rf 0.63) is of great significance. Coumarins are known to possess a wide range of pharmacological activities, including anti-inflammatory, anti-edematous, and hepatoprotective effects [23]. Flavonoid aglycones are generally more lipophilic than their glycosides, allowing for better absorption and exhibiting potent metabolic-regulating activities. Quercetin, for instance, is a well-known inhibitor of alpha-glucosidase and has anti-adipogenic effects, which are highly relevant to conditions like diabetes and obesity [24, 25]. The presence of catechin (Rf 0.28) further adds to its metabolic and antioxidant profile. This

phytochemical composition—rich in coumarins and flavonoid aglycones—provides a strong scientific rationale for the traditional use of *HiberadiKwath* in metabolic disorders, where anti-diabetic, lipid-lowering, and anti-inflammatory actions are paramount.

4.4. Methodological Implications for Quality Control

The successful resolution of distinct bands for both formulations demonstrates that HPTLC is an invaluable tool for the quality control of Ayurvedic Kwaths [26]. The established fingerprint can serve as a "chemical passport" for these formulations. Any batch that deviates from this standard fingerprint, in terms of the number of bands, their Rf values, or their relative intensity, can be considered non-compliant. This is particularly important given the variability that can arise from differences in raw material sourcing, manufacturing processes, and storage conditions [27]. Furthermore, the distinct profiles allow for the unequivocal differentiation between the two formulations, preventing potential misidentification.

4.5. Limitations and Future Directions

While this study provides a comprehensive phytochemical interpretation based on Rf values and chromatographic behavior, it is important to acknowledge its limitations. The identification of compounds is "probable" and based on correlating Rf values with known literature for the solvent system used [28]. Definitive confirmation of individual compounds requires co-chromatography with authentic reference standards (e.g., for gallic acid, rutin, quercetin) or the use of hyphenated techniques like HPTLC-MS/MS, which couples the separation power of TLC with the identification power of mass spectrometry [29].

Future studies should focus on:

1. **Bio-autography:** To directly correlate specific bands with their bioactivity, such as antioxidant or alpha-amylase inhibitory activity.
2. **HPTLC-MS/MS:** To unambiguously identify the key compounds corresponding to the major bands.
3. **Quantitative HPTLC:** To estimate the amount of key marker compounds (e.g., gallic acid, quercetin) in both formulations.
4. **In-vivo Studies:** To validate the correlation between the phytochemical profile and the claimed therapeutic actions in suitable animal models.

5. Conclusion

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This study successfully developed and validated a simple, precise, and reproducible HPTLC method for the comparative fingerprint analysis of *VenugranthikultthadiKwath* and *HiberadiKwath*. The results reveal that while both formulations share some common non-polar and polar phenolic constituents, they possess distinct phytochemical signatures. *VenugranthikultthadiKwath*'s profile, dominated by UV-absorbing tannins and triterpenes, strongly corroborates its traditional use in managing inflammatory conditions related to venous channels. Conversely, the rich fluorescent profile of *HiberadiKwath*, indicating a higher abundance of coumarins and flavonoid aglycones, provides a scientific rationale for its application in metabolic disorders. This work lays a crucial foundation for the pharmacognostic standardization of these formulations, bridging the gap between traditional Ayurvedic knowledge and modern analytical science. The established HPTLC fingerprints can be utilized as a robust quality control tool, ensuring the consistency, efficacy, and safety of these valuable classical remedies.

6. References

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