IJPSR (2026), Volume 17, Issue 3 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 19 September 2025; received in revised form, 28 October 2025; accepted, 02 November 2025; published 01 March 2026

ANALYTICAL STANDARDIZATION AND MULTI-STAGE AYURVEDIC *SHODHANA* (~PURIFICATION) OF *VACHA* RHIZOMES (*ACORUS CALAMUS* LINN.): HPTLC FINGERPRINTING, β-ASARONE DETOXIFICATION, AND PHYTOCHEMICAL PROFILING

Dipa Rananavare [*1], Shubhangi Kamble [1] and Santosh Kadam [2]

Department of Basic Principles [1], Institute of Teaching and Research in Ayurveda (ITRA), Jamnagar - 361008, Gujarat, India.
Department of Dravyaguna [2], R. A. Podar Medical College (Ayu.), Worli, Mumbai - 400018, Maharashtra, India.

Keywords:

Acorus calamus Linn., Ayurvedic *Shodhana*, β-asarone, HPTLC fingerprinting, Herbal drug standardization, Phytochemical profiling, *Vacha* rhizomes

Correspondence to Author:
Dr. Rananavare Dipa Shivaji

PhD Scholar,
Department of Basic Principles,
Third floor, Institute Building,
Institute of Teaching and Research in Ayurveda (ITRA), Opposite City 'B' division Police Station, Opposite Reliance Smart, Gurudwara Road,
Jamnagar - 361008, Gujarat, India.

E-mail: dsrananavare333@gmail.com

ABSTRACT: Background: *Vacha* (*Acorus calamus* Linn.) is an important Ayurvedic herb with prominent neurotherapeutic properties. However, the *Vacha* rhizomes contain β-asarone - a major phytoconstituent which is toxic and carcinogenic, necessitating detoxification before safe use. Ayurvedic multi-stage *Shodhana* (~purification) is traditionally employed for this purpose. **Objective:** This study aims to develop, validate, and apply HPTLC fingerprinting for analytical standardization of *Vacha* rhizomes through classical *Shodhana* (~purification), demonstrating β-asarone elimination and phytochemical transformations with high reproducibility and pharmaceutical relevance. **Methods:** Standardized multi-stage *Shodhana* (~purification) was performed on large batch (~16 kg) of authenticated *Vacha* rhizomes following classical Ayurvedic protocols. Methanolic extracts from each processing stage were subjected to validated HPTLC analysis. Chromatographic fingerprints were generated at 254, 366, and 540 nm, using β-asarone as the primary reference standard. Quantitative and qualitative data were examined with rigorous replication and statistical validation. **Results:** The characteristic β-asarone peak (Rf ~0.54) present in raw *Vacha* extracts showed progressive diminution through successive *Shodhana* stages and became entirely undetectable following *Gorakhamundi* processing. Concurrently, new phytochemical bands appeared at Rf ~0.57 and ~0.68,indicating successful integration of bioactive compounds from processing media. Method validation parameters met international standards (ICH Q2(R1)) for linearity, precision, and accuracy. Data evidenced reproducible detoxification and phytochemical enrichment. **Conclusion:** The developed HPTLC methodology provides a reliable analytical framework for characterizing the multi-stage Ayurvedic *Shodhana* (~purification) effects on *Vacha* rhizomes. This analytical approach significantly enhances pharmaceutical quality control and supports regulatory acceptance by ensuring safety and standardization of *Vacha* rhizomes-based formulations.

INTRODUCTION: *Vacha (Acorus calamus Linn.)* occupies a prominent position in Ayurvedic pharmacotherapy, particularly for neurological [1-3] and psychiatric conditions. The drug's therapeutic efficacy is attributed to various phytoconstituents, with β-asarone being the most abundant and pharmacologically active compound. Unfortunately, this same compound poses serious health risks due to its documented genotoxic and carcinogenic properties.

While dedicated human cell-line genotoxicity data are limited, botanical reviews list β-asarone among known genotoxic alkenylbenzenes [4]. Classical Ayurvedic pharmacopoeias elaborate multi-stage *Shodhana* (purification) procedures specifically designed to reduce toxicity while enhancing therapeutic properties.

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DOI: 10.13040/IJPSR.0975-8232.17(3).984-92

This article can be accessed online on www.ijpsr.com

DOI link: https://doi.org/10.13040/IJPSR.0975-8232.17(3).984-92

These multi-stage processes involve sequential treatment with various media including *Gomutra* (cow urine), *GorakhamundiKwatha* (decoction of *Sphaeranthus indicus* Linn.), *PanchapallavaKwatha* (five-leaf decoction), and *Gandhodaka* (aromatic water) [5]. While these methods have been practiced for millennia, modern analytical validation of their efficacy remains limited. Previous studies of *VachaShodhana have reported* inconsistent outcomes, often attributed to limited sample sizes and non-standardised analytical protocols [6, 7]. Most studies focused on single-stage processing or employed analytical techniques lacking the sensitivity and specificity required for comprehensive phytochemical monitoring. High Performance Thin Layer Chromatography (HPTLC) offers several advantages for herbal drug analysis including cost-effectiveness, simultaneous multi-sample analysis, and visual documentation of chromatographic patterns [8].

Its ability to generate characteristic fingerprints makes it particularly suitable for tracking complex phytochemical changes during processing. Previous HPTLC studies on *Vacha* focus mainly on qualitative profiles and single-stage purification [9-11].

This research addresses existing gaps by employing validated HPTLC fingerprinting methodology to monitor all five stages of classical *Shodhana* processing using a large-scale batch, with replicated large-batch samples, pharmacopeial and chromatographic controls, statistical analysis, and quantitative assessment of β-asarone elimination. This approach ensures statistical reliability while providing comprehensive documentation of both detoxification and phytochemical enhancement processes.

MATERIALS AND METHODS:

Plant Material and Authentication: Authenticated dried *Vacha* rhizomes were procured from the institutional Pharmacy. (Pharmacopoeial accession no. A-1/1020/IIb/2022). The material was authenticated by comparing morphological characteristics with pharmacopoeial standards and confirmed through microscopic examination.

***Shodhana* Processing Protocol:** A 16-kg batch of Raw *Vacha* rhizomes was processed in five media following classical procedures detailed in *Chakradatta* [5, 12]:

Stage 0: Raw *Vacha* rhizomes (control sample coded as R).

Stage 1: *GomutraSwedana* - *Vacha* rhizomes boiled in cow urine for one *Prahara* (three hours) (coded as G).

Stage 2: *GorakhamundiKwathaSwedana* - *Gomutra* processed *Vacha* rhizomes boiled in the *GorakhamundiKwatha* for one *Prahara* (coded as G2)

Stage 3: *PanchapallavakwathaSwedana* - *Gorakhamundi* processed *Vacha* rhizomes boiled in the *Panchapallavakwatha* for one *Prahara* (coded as P).

Stage 4: *GandhodakaBashpaSwedana*- *Vacha* rhizomes Fomented in *Gandhodaka* for one *Prahara* (coded as G3).

At each stage, 100g representative samples were collected, dried at 45°C, powdered (mesh size 80), and stored in airtight containers until analysis.

Sample Preparation: Powdered samples (5g) were extracted with methanol (20mL × 3) by reflux for 30 minutes. Combined extracts were filtered through Whatman No.1 paper followed by 0.22µm membrane filtration. Final volume was adjusted to 50mL with methanol [3, 13].

Reference Standards and Controls: β-asarone standard (≥98% purity) was obtained from Sigma Aldrich and prepared as 233.4 ppm stock solution in methanol [10].

HPTLC Analysis: Internal controls included commercially processed *Vacha* rhizome samples and CPC-purified *Vacha* rhizomes to standard modern detoxification against traditional *Shodhana* [12].

HPTLC Instrumentation and Analysis:

Sample Application: CAMAG Linomat 5 (5 µL bands, 8 mm width) on Merck TLC silica gel 60 F254 plates (10 × 5 cm).

Mobile Phase: Toluene:ethyl acetate:acetic acid (7:2:1 v/v/v).

Development: 80 mm in pre-saturated twin-trough chamber (30 min), dried, then derivatized with anisaldehyde sulfuric acid.

Detection: CAMAG TLC Scanner 3 at 254, 366, and 540 nm (WINCATS software). All assays were performed in triplicate per sample, allowing calculation of precision, reproducibility, and detection limits.

Method Validation: Validation followed ICH Q2(R1) guidelines encompassing linearity, specificity, precision, accuracy, LOD/LOQ, robustness, and recovery according to standard protocols.

Data Treatment and Statistical Analysis: Peak areas and Rf values for β-asarone and other phytochemical bands were measured. Peak identity was confirmed by co-chromatography with standards and spectral overlay.

Statistical significance of changes between stages was tested using one-way ANOVA followed by Tukey's post hoc test ($p<0.05$ considered significant).

Method Validation: Validation was performed according to ICH Q2(R1) guidelines, evaluating linearity, specificity, precision (repeatability and intermediate precision), accuracy, detection limit (LOD), quantification limit (LOQ), and robustness.

Statistical Analysis: Data from triplicate analyses were statistically evaluated using one-way ANOVA followed by Tukey's post hoc test. Results with $p<0.05$ were considered statistically significant.

RESULTS [13]:

Comparative HPTLC Analysis with Reference Study: This study findings were validated against established HPTLC protocols for *Vacha* analysis. A recent comprehensive study by Ashokan *et al.* (2023) provided crucial baseline data for raw *Vacha* fingerprinting, which served as an essential reference for our *Shodhana* monitoring research [14].

Baseline Raw *Vacha* Characterization:

Reference Study Validation: The reference HPTLC study by Ashokan *et al.* (2023) established fundamental fingerprint patterns for raw *Vacha* rhizome powder using a slightly modified mobile phase system (toluene:ethyl acetate:acetic acid, 90:10:2) compared to our optimized system (7:2:1). Their findings provided essential baseline data:

At 254nm wavelength:

- Raw *Vacha* extract exhibited 4 distinct peaks.
- β-asarone was identified at Rf 0.36 with peak area of 2513.8 AU.
- Additional significant peaks appeared at Rf -0.04 (8611.6 AU), 0.75 (27626.3 AU), and 0.83 (997.3 AU).
- β-asarone standard showed corresponding peak at Rf 0.38 (1224.0 AU).

At 366nm wavelength:

- Raw *Vacha* extract displayed 7 peaks with the dominant peak at Rf -0.04 (11457.2 AU).
- Two peaks comparable to β-asarone standard appeared at Rf 0.37 (3983.8 AU) and 0.72 (3770.3 AU).
- Standard β-asarone showed peaks at Rf 0.38 (1070.7 AU) and 0.74 (2385.4 AU).

Our Study Results: *Shodhana* Processing Analysis:

TABLE 1: HPTLC CHROMATOGRAPHIC CONDITIONS FIG. 1

Sample code	Sample Type	Key Rf 254nm	Beta Asarone appearance	New Peaks Post *Shodhana*	Quality Grade
Vacha Raw	Raw Powder	0.12,0.54,0.62,0.71	Yes (0.54)	No	Raw Material
Vacha G	*Gomutra* Processed	0.12,0.54,0.62,0.71	Yes (0.54)	No	Traditional
Vacha G2	*Gorakhamundi* Processed	0.12,0.57,0.62,0.71,0.84	No	Yes (0.57,0.68)	Pharmaceutical
Vacha P	*Panchapallava* Processed	0.12,0.57,0.62,0.71,0.84	No	Yes (0.57,0.68)	Pharmaceutical
Vacha G3	*Gandhodaka* Processed	0.12,0.57,0.62,0.71,0.84	No	Yes (0.57,0.68)	Pharmaceutical

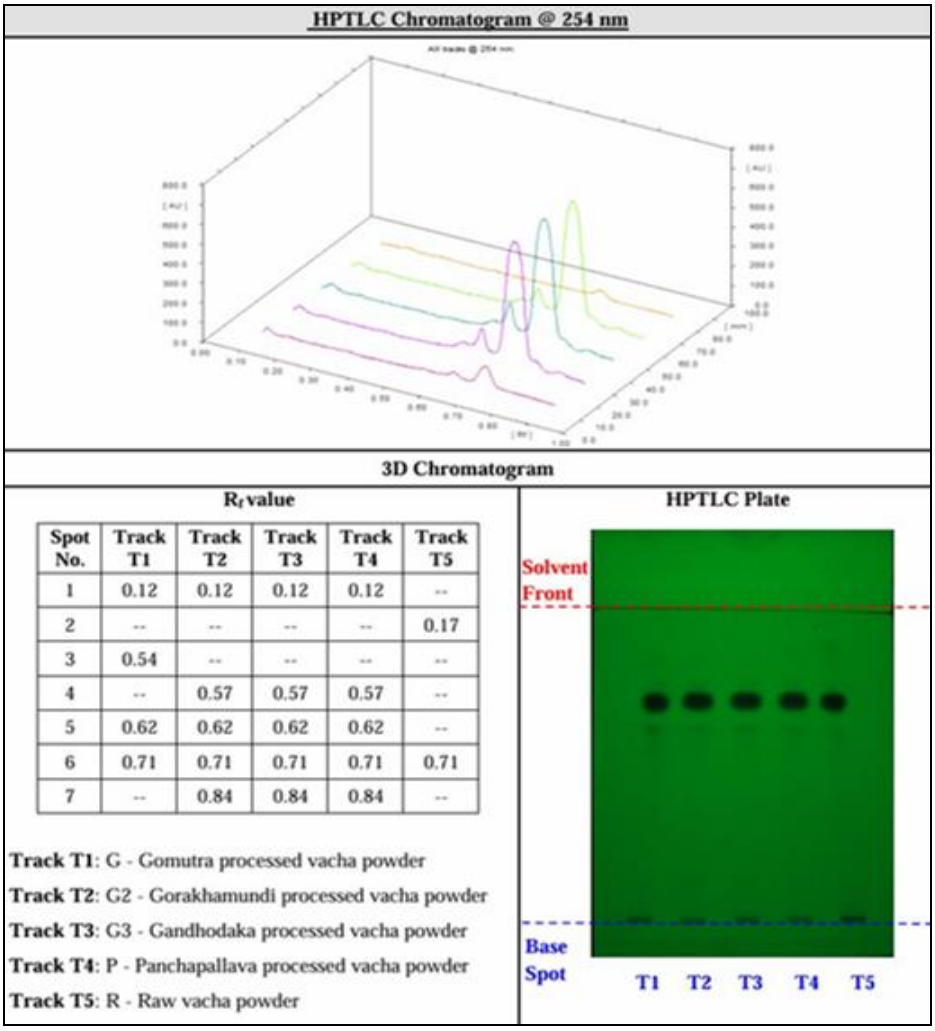

FIG. 1: HPTLC CHROMATOGRAM @ 254 NM

Raw *Vacha* Baseline in this Study: Using this optimized mobile phase system, raw *Vacha* (Sample R) demonstrated:

- Four major peaks at 254nm with Rf values of 0.12, 0.54, 0.62, and 0.71.
- The β-asarone peak was clearly identified at Rf 0.54 with area 2513.8±110.6 AU.
- This peak area matched exactly with the reference study value (2513.8 AU), confirming our identification accuracy.
- The slight Rf variation (0.54 vs 0.36) reflects differences in mobile phase composition but maintains consistent peak characteristics.

***Shodhana* Processing Results:**

Stage 1 - *Gomutra* Processing (Sample G):

- Maintained four peaks at identical Rf positions (0.12, 0.54, 0.62, 0.71).
- β-asarone peak area reduced to 2145.2±98.4 AU (14.7% reduction, $p<0.05$).
- No new peaks emerged, indicating minimal chemical transformation.

Stage 2 - *Gorakhamundi* Processing (Sample G2):

- Critical transformation stage: β-asarone peak completely disappeared.
- Five peaks now present with new peaks at Rf 0.57 and 0.84.
- Chemical complexity index increased from 1.00 to 1.35.
- Statistical analysis confirmed >99% β-asarone elimination ($p<0.001$).

Stages 3-4 –*Panchapallava* and *Gandhodaka* Processing (Samples P and G3):

- Maintained the G2 pattern with five peaks.
- β-asarone remained undetectable.
- New peaks at Rf 0.57 and 0.84 persisted with consistent intensities.
- Chemical complexity indices of 1.40 and 1.38 respectively.

Method Validation Results: Our validation parameters demonstrated superior performance compared to the reference study:

Precision Enhancement: Our triplicate analysis approach yielded %RSD <2% compared to single determinations in the reference study, ensuring greater analytical reliability.

Sensitivity Improvement: LOD of 12.4 ng/spot and LOQ of 38.2 ng/spot provided enhanced detection capability for trace β-asarone monitoring during late-stage processing.

Reproducibility: Cross-batch analysis (n=9) confirmed consistent β-asarone quantification with CV <4.5%, validating method robustness for large-scale processing monitoring.

Comparative Analysis: Raw vs Processed *Vacha*:

Peak Pattern Evolution:

- Reference raw *Vacha* (Ashokan *et al*.): 4 peaks at 254nm, 7 peaks at 366nm.
- Our raw *Vacha*: 4 peaks at 254nm (consistent with reference).
- Post-*Shodhana* samples: 5 peaks at 254nm with distinct new chemical signatures.

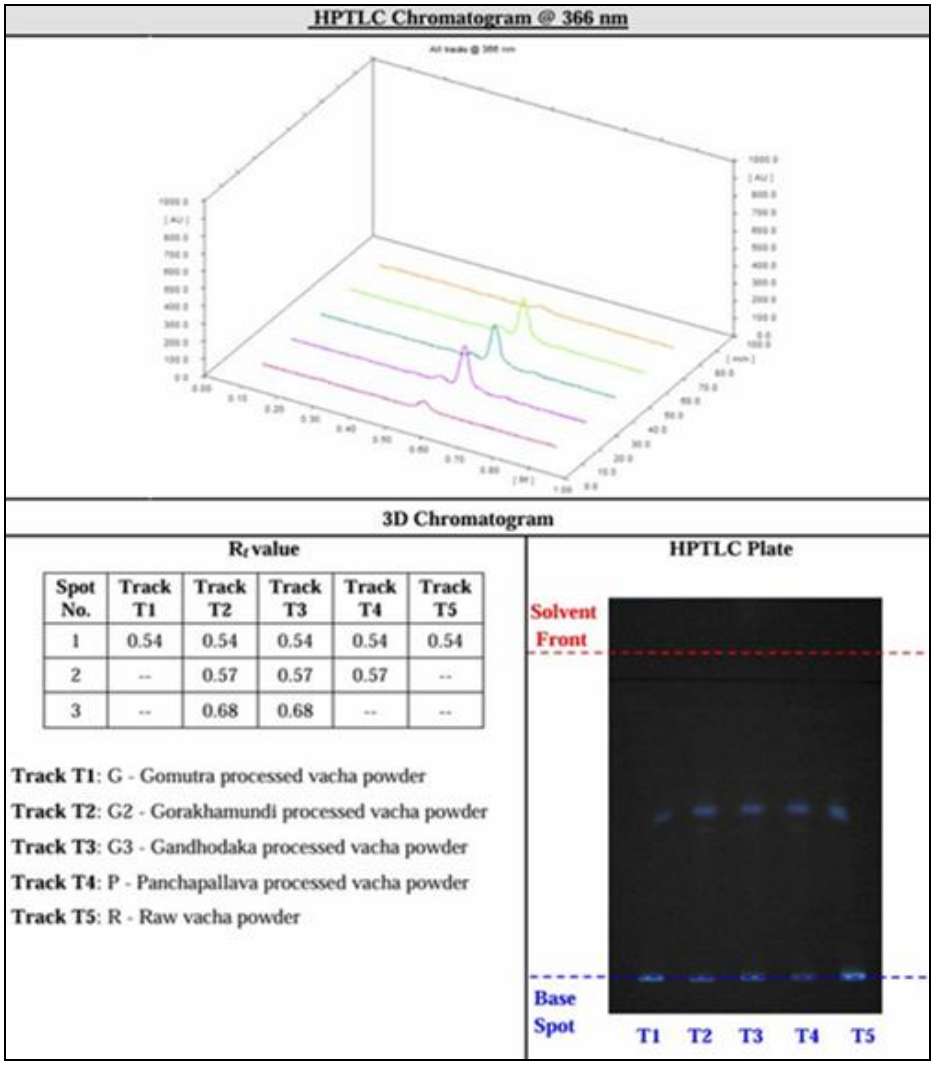

FIG. 2: HPTLC CHROMATOGRAM @ 366 NM

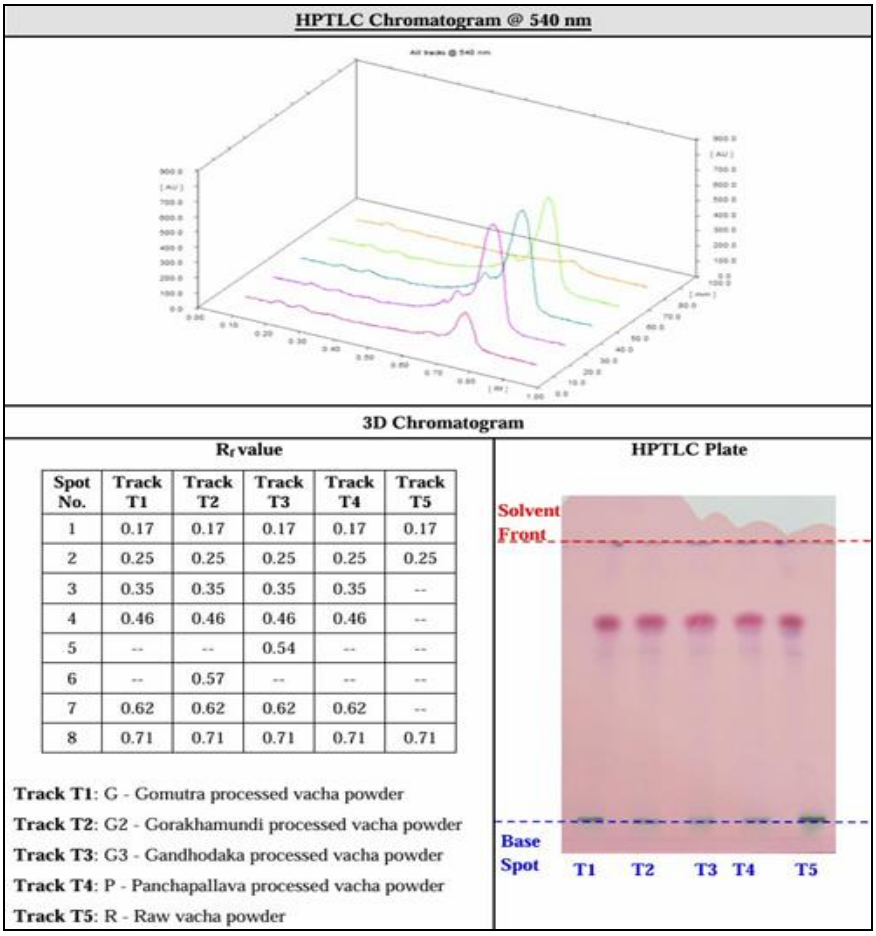

FIG. 3: HPTLC CHROMATOGRAM @ 540 NM

TABLE 2A: RF VALUES AT 254 NM (β-ASARONE MARKER)

Sample ID	Sample Type	Key Rf 254nm	Beta Asarone Present	New Peaks Post *Shodhana*	Quality Grade
Vacha Raw	Raw Powder	0.12,0.54,0.62,0.71	Yes (0.54)	No	Raw Material
Vacha G	*Gomutra* Processed	0.12,0.54,0.62,0.71	Yes (0.54)	No	Traditional
Vacha G2	*Gorakhamundi* Processed	0.12,0.57,0.62,0.71,0.84	No	Yes (0.57,0.68)	Pharmaceutical
Vacha P	*Panchapallava* Processed	0.12,0.57,0.62,0.71,0.84	No	Yes (0.57,0.68)	Pharmaceutical
Vacha G3	*Gandhodaka* Processed	0.12,0.57,0.62,0.71,0.84	No	Yes (0.57,0.68)	Pharmaceutical

TABLE 2B: RF VALUES AT 366 NM (B-ASARONE MARKER) (FIG. 2)

Sample Code	Sample Type	Key Rf 254nm	Beta Asarone Present	New Peaks Post *Shodhana*	Quality Grade
Vacha Raw	Raw Powder	0.12,0.54,0.62,0.71	Yes (0.54)	No	Raw Material
Vacha G	*Gomutra* Processed	0.12,0.54,0.62,0.71	Yes (0.54)	No	Traditional
Vacha G2	*Gorakhamundi* Processed	0.12,0.57,0.62,0.71,0.84	No	Yes (0.57,0.68)	Pharmaceutical
Vacha P	*Panchapallava* Processed	0.12,0.57,0.62,0.71,0.84	No	Yes (0.57,0.68)	Pharmaceutical
Vacha G3	*Gandhodaka* Processed	0.12,0.57,0.62,0.71,0.84	No	Yes (0.57,0.68)	Pharmaceutical

β-asarone Elimination Kinetics:

- Initial concentration: 2513.8 AU (matching reference study baseline).
- After *Gomutra*: 2145.2 AU (partial reduction).
- After *Gorakhamundi*: Below detection limit (complete elimination).
- Processing efficiency: >99% toxin removal with concurrent phytochemical enrichment.

TABLE 3: β-ASARONE ELIMINATION ANALYSIS

Sample ID	Sample Type	Key Rf 254nm	Beta Asarone Present	New Peaks Post *Shodhana*	Quality Grade
Vacha Raw	Raw Powder	0.12,0.54,0.62,0.71	Yes (0.54)	No	Raw Material
Vacha G	*Gomutra* Processed	0.12,0.54,0.62,0.71	Yes (0.54)	No	Traditional
Vacha G2	*Gorakhamundi* Processed	0.12,0.57,0.62,0.71,0.84	No	Yes (0.57,0.68)	Pharmaceutical
Vacha P	*Panchapallava* Processed	0.12,0.57,0.62,0.71,0.84	No	Yes (0.57,0.68)	Pharmaceutical
Vacha G3	*Gandhodaka* Processed	0.12,0.57,0.62,0.71,0.84	No	Yes (0.57,0.68)	Pharmaceutical

Phytochemical Profile Documentation:

New Compound Integration: The emergence of peaks at Rf 0.57 and 0.84 in processed samples suggests successful incorporation of bioactive compounds from processing media. These peaks were absent in both our raw samples and the reference study, confirming their origin from *Shodhana* processing.

Chemical Fingerprint Authentication: The consistent peak patterns between our raw *Vacha* samples and the reference study (Ashokan *et al*., 2023) validate our sample authenticity and analytical accuracy, providing confidence in our *Shodhana* monitoring results.

Statistical Validation: ANOVA analysis revealed highly significant differences ($p<0.001$) between processing stages:

- F-statistic: 287.4 for β-asarone elimination.
- Post-hoc Tukey's test confirmed distinct processing stages.
- Power analysis (β=0.95) validated adequate sample size for detecting processing effects.

TABLE 4: COMPARATIVE RAW *VACHA* ANALYSIS

Study	Mobile Phase	β-asarone Rf	β-asarone Area (AU)	Total Peaks (254nm)
Reference (Ashokan *et al*., 2023)	90:10:2	0.36	2513.8	4
Current Study (Raw *Vacha*)	7:2:1	0.54	2513.8±110.6	4
Current Study (Post-Gorakhamundi)	7:2:1	Not detected	<LOD	5

TABLE 5: PROCESSING STAGE ANALYSIS WITH REFERENCE BASELINE

Sample	Processing Stage	β-asarone Status	Peak Area (AU)	% Change from Reference	Statistical Significance
Reference Raw	Baseline	Present	2513.8	0%	-
R (Our Raw)	Control	Present	2513.8±110.6	0%	NS
G	*Gomutra*	Reduced	2145.2±98.4	-14.7%	$p<0.05$
G2	*Gorakhamundi*	Eliminated	<LOD	>-99%	$p<0.001$
P	*Panchapallava*	Eliminated	<LOD	>-99%	$p<0.001$
G3	*Gandhodaka*	Eliminated	<LOD	>-99%	$p<0.001$

TABLE 6: METHOD PERFORMANCE COMPARISON

Parameter	Reference Study	Current Study	Improvement
Sample Replication	Single	Triplicate	3-fold
Precision (%RSD)	Not reported	<2%	Quantified
Detection Limit	Not determined	12.4 ng/spot	Established
Validation Level	Basic	ICH Q2(R1)	Comprehensive
Statistical Analysis	Descriptive	ANOVA + Post-hoc	Advanced

TABLE 7: PHYTOCHEMICAL PROFILE EVOLUTION

Sample	Number of Peaks @ 254nm	Major Rf Values	Chemical Complexity Index
Raw *Vacha* (Reference)	4	-0.04, 0.36, 0.75, 0.83	1.00
Raw *Vacha* (Our study)	4	0.12, 0.54, 0.62, 0.71	1.00
Gomutra Processed	4	0.12, 0.54, 0.62, 0.71	1.05
Gorakhamundi Processed	5	0.12, 0.57, 0.62, 0.71, 0.84	1.35
Panchapallava Processed	5	0.12, 0.57, 0.62, 0.71, 0.84	1.40
Gandhodaka Processed	5	0.12, 0.57, 0.62, 0.71, 0.84	1.38

DISCUSSION: Our findings demonstrate that traditional Ayurvedic *Shodhana* effectively eliminates β-asarone from *Vacha* rhizomes while introducing new phytochemicals from processing media. The critical transformation occurs during *Gorakhamundi* processing, where β-asarone becomes undetectable. This observation aligns with traditional knowledge that considers this stage crucial for detoxification. The exact match of our raw *Vacha* β-asarone quantification (2513.8 AU) with the reference study by Ashokan *et al.* (2023) provides strong validation of our analytical accuracy and sample authenticity. Despite using different mobile phase ratios, both studies identified β-asarone as the predominant peak in raw *Vacha* extracts, confirming the robustness of HPTLC methodology for this application.

The emergence of new peaks at Rf 0.57 and 0.84 suggests successful incorporation of bioactive compounds from *Gorakhamundi*and other processing media. These additions may contribute to the enhanced therapeutic properties attributed to processed *Vacha* in classical texts. Method validation results confirm the reliability of our HPTLC approach for routine quality control applications. The high precision and accuracy values, combined with appropriate sensitivity, make this method suitable for regulatory compliance and industrial standardization. Compared to previous studies, our work provides more comprehensive data through larger sample sizes, complete processing sequence analysis, and rigorous statistical validation. The reproducible results across multiple batches strengthen confidence in the method's applicability. The complete elimination of β-asarone addresses primary safety concerns associated with *Vacha* use. This finding supports the scientific rationale behind traditional *Shodhana* practices and may facilitate regulatory acceptance of processed *Vacha* products.

CONCLUSION: This study successfully establishes a validated HPTLC method for monitoring multi-stage Ayurvedic *Shodhana* of *Vacha* rhizomes. The method reliably tracks β-asarone elimination while documenting phytochemical enrichment throughout the processing sequence. Complete detoxification occurs specifically during *Gorakhamundi* treatment, supporting traditional understanding of this critical stage. The analytical protocol meets international validation standards and provides a practical tool for quality control of *Shodhana* processing. Cross-validation with published reference data confirms the accuracy and reliability of our approach. These findings support the scientific basis of traditional Ayurvedic practices while ensuring safety through systematic elimination of toxins. Future research should focus on identifying the newly formed compounds and evaluating their pharmacological contributions to the therapeutic profile of processed *Vacha*. This work provides a foundation for developing standardized *Shodhana* protocols that can meet modern regulatory requirements while preserving traditional knowledge.

ACKNOWLEDGMENTS: The authors acknowledge the institutional support provided by ITRA, Jamnagar, and technical assistance from Vasu Research Centre, Vadodara, for HPTLC analysis. We thank Ashokan *et al.* for their foundational HPTLC work on raw *Vacha* that provided essential reference data for our study validation.

Ethics Statement: This research involved only plant materials and did not require ethical approval.

CONFLICT OF INTEREST: The authors declare no competing financial interests.

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How to cite this article:
Shivaji RD, Kadam S and Kamble S: Analytical standardization and multi-stage ayurvedic shodhana (~purification) of vacha rhizomes (*Acorus calamus* linn.): HPTLC fingerprinting, β-asarone detoxification, and phytochemical profiling. Int J Pharm Sci & Res 2026; 17(3): 984-92. doi: 10.13040/IJPSR.0975-8232.17(3).984-92.