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PHYTOCHEMICAL MARKER BASED EVALUATION OF RAJAPRAVARTINI VATI USING HPTLC

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Keywords:

Rajapravartini Vati, Marker compounds, HPTLC, Phytochemical evaluation, Quality, In-process changes

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ABSTRACT: Rajapravartini Vati is an Ayurvedic formulation prescribed for gynaecological and menstrual disorders such as amenorrhea and is comprised of raw materials, namely Shuddha Kasisa (purified green vitriol), Hing (purified *Ferula asafoetida*), Tankana (purified borax) and Kumari (*Aloe vera*) mixed in equal proportions. The present work focuses on HPTLC-based evaluation and phytochemical profiling of commercially available Rajapravartini Vati samples. The work proposes a phytobioactive compound detected in the commercial samples as a marker compound for evaluation of Rajapravartini Vati. It also establishes it as a compound different from conventional expected marker compounds such as aloin from *Aloe vera* and ferulic acid from *Ferula asafoetida*, which were not detected in the commercial samples but only in the in-house laboratory raw material mixture. Aloin and ferulic acid were subsequently quantified in the prepared sample at concentrations of 0.99 mg/g and 0.92 mg/g respectively. The findings of the study thus provide valuable insights into the phytochemical fingerprint of Rajapravartini Vati and will help propel future work on the marker compounds studied herein.

INTRODUCTION: In the last few years, an increasing shift towards the use of traditional medicinal systems is being observed. However, the lack of sufficient scientific validation with respect to the physicochemical and marker compound profile of such polyherbal formulations has deterred significant expansion with respect to their adoption. Thus, Ayurvedic remedies require scientific supplementation to ensure their authenticity, quality, safety and potency.

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Rajapravartini Vati is one such Ayurvedic formulation which has long been prescribed for gynaecological and menstrual disorders such as amenorrhea, categorised as Artavakshaya in Ayurvedic literature. In Sanskrit terminology, 'Rajas 'refers to the menstrual cycle while 'Pravartini' describes its stimulation [1, 2]. The principal raw materials and their associated therapeutic roles in Rajapravartini Vati are illustrated below in **Table 1** [3, 4].

While the individual components themselves do not have any documented standalone roles to play in treatment of menstrual disorders, synergistic harmony between their composite phyto-constituents is hypothesised to play a role in bringing about the therapeutic effects associated with Rajapravartini Vati.

This is attempted to be scientifically understood through quantification of specific phytobioactives to provide better insights about the formulation.

TABLE 1: RAW MATERIALS USED IN RAJAPRAVARTINI VATI AND THEIR ASSOCIATED ROLES IN THE FORMULATION

Raw Material	Role in the Formulation
Shuddha Kasisa (purified green vitriol)	Responsible for enhancing uterine blood circulation
Shuddha Hing (purified *Ferula asafoetida*)	Suppresses the release of progesterone
ShuddhaTankana (purified borax)	Responsible for removal of obstructions in uterine passageways
Kumari (*Aloe vera*)	Exhibits anti-prostaglandin and anti-spasmodic activity

This study thus comparatively evaluates commercially available formulations of Rajapravartini Vati by HPTLC fingerprinting and quantitation to provide valuable insights in to the phytobioactives in Rajapravartini Vati as well as the possible in-process changes in its phytochemical profiles. This is crucial due to it being a polyherbal formulation, where conventional marker compounds from the raw materials such as ferulic acid from *Ferula asafoetida* and aloin from *Aloe vera* are expected to be identified. Aloin is presently being explored for its anti-inflammatory, laxative, neuroprotective and anti-cancer properties [5, 6]. Ferulic acid is an established antioxidant compound with its pharmacological potential to serve as an anti-diabetic, anti-carcinogenic and radioprotectant properties under investigation [7]. These compounds by themselves may however not be reflective of the synergism between them in the final polyherbal formulation. Exploration for such marker compounds thus attempts to provide a comprehensive understanding of the potential of Rajapravartini Vati in therapeutics.

MATERIALS AND METHODS:

Sample Collection and Reagents: Five different commercially available brands of Rajapravartini Vati tablets were procured from local Ayurvedic stores in Mumbai and were labelled as RPV-1, RPV-2, RPV-3, RPV-4 and RPV-5 respectively.

Raw materials required for formulating the raw material mixture of Rajapravartini Vati such as *Ferula asafoetida* and *Aloe barbadensis* powder, and *Aloe vera* juice were procured from Ayurvedic stores. All chemicals and reagents which were used were of analytical grade. Standard aloin (Sigma Aldrich 1415-73-2), standard ferulic acid (Sigma Aldrich 537-98-4) and standard gallic acid monohydrate (Himedia 5995-86-8) were used for all analyses wherever applicable.

HPTLC Analysis: The detailed HPTLC method parameters are illustrated herein in **Table 2.**

TABLE 2: COMMON METHOD PARAMETERS FOR HPTLC ANALYSIS

Parameter	Description
Stationary Phase	Merck $60F_{254}$ Silica Gel TLC Plates
Development Chamber	CAMAG Twin-Trough Chamber
Chamber Saturation	20 minutes
Sample Applicator	CAMAG Linomat 5 Applicator
Syringe	CAMAG Linomat 695.0014, 100.0 µL
Rate of Sample Application	120 nL/sec
Development Distance	80.0 mm
Densitometric Scanning	CAMAG TLC Scanner 4 with winCATS Planar Chromatography Manager Software (Version 1.4.7)
Lamp and Wavelength	D2 and Hg for 254 nm and 366 nm respectively

Preliminary Screening: HPTLC screening was carried out for preliminary identification of marker compounds across the commercial Vati samples. Methanolic extracts of the samples (RPV-1 to RPV-5) at a concentration of 10000 ppm were subjected to HPTLC separation in presence of standard gallic acid, standard aloin and standard ferulic acid solutions, each prepared in methanol at a concentration of 1000 ppm. The mobile phase composition for separation of aloin was optimised after referring to multiple literature sources based on similar chromatographic separations of aloin and aloin-related compounds [8]. Two duplicate plates (20 cm × 10 cm) were run and derivatised with different reagents as described in **Table 3.**

TABLE 3: PRELIMINARY SCREENING OF COMMERCIAL RAJAPRAVARTINI VATI SAMPLES FOR MARKER COMPOUNDS

	Plate 1	Plate 2
Plate Size	20 cm × 10 cm	20 cm × 10 cm
Samples	Methanolic extracts of RPV-1, RPV-2, RPV-3, RPV-4 and RPV-5 (10000 ppm)	
Standards Used for Screening	Standard gallic acid (1000 ppm), standard aloin (1000 ppm) and standard ferulic acid (1000 ppm)	
Mobile Phase	Toluene : Ethyl Acetate : Formic Acid (2:7:1) (v/v)	Ethyl Acetate : Methanol : Water (10:1.45:1) (v/v)
Derivatising Agent	1% $FeCl_3$ reagent	10% methanolic H_3BO_3 (w/v), followed by heating at 100°C for 10 minutes [9]

Fingerprinting of Raw Materials and Finished Formulations: To gain preliminary insights into the differences between the phytochemical profiles of commercial samples and freshly formulated raw material mixtures, a raw material mixture sample of Rajapravartini Vati in the laboratory was prepared. Each of the four raw material powders – Shuddha Tankana (purified borax), Shuddha Kasisa (purified green vitriol or ferrous sulphate), Shuddha Hing (*Ferula asafoetida* powder) and Kumari (*Aloe barbadensis* powder) of Ayurvedic grade were thoroughly pulverised and ground in a mortar and pestle to obtain a homogenous mixture in equal proportions. This was labelled as RPV-L. Based on further literature review, 60% ethanol was chosen as the extracting solvent for subsequent analyses [10]. 60% ethanolic extracts of all commercial samples, the freshly formulated mixture and individual raw materials at 5% w/v concentration were prepared and subjected to HPTLC fingerprinting as illustrated in **Table 4.**

In addition, to gain further understanding of the impact of commercial manufacturing on phytochemical profiles, 60% ethanolic extracts (5% w/v) of commercial *Aloe* gel and *Aloe* juice were also subjected to HPTLC fingerprinting.

TABLE 4: FINGERPRINTING OF RAW MATERIALS AND FINISHED FORMULATIONS OF RAJAPRAVARTINI VATI

Plate Size	20 cm × 10 cm and 10 cm × 10 cm
Samples	60% ethanolic extracts (5% w/v concentration) of: **a.** Commercial samples: RPV-1, RPV-2, RPV-3, RPV-4 and RPV-5 **b.** Freshly formulated raw material mixture: RPV-L **c.** Raw materials: *Ferula asafoetida* powder, *Aloe barbadensis* powder **d.** Commercial *Aloe* gel **e.** Commercial *Aloe* juice
Standards	Standard aloin (1000 ppm) and standard ferulic acid (1000 ppm)
Mobile Phase	Ethyl acetate : Methanol : Water (10:1.45:1) (v/v)

Quantitation of Aloin and Ferulic Acid from Raw Material Mixture: Qualitative detection of aloin and ferulic acid markers in the freshly formulated raw material mixture was followed by quantitative estimation of aloin and ferulic acid in the mixture using calibration curve method by HPTLC, as the first step towards preliminary quality control. This is illustrated in **Table 5.**

TABLE 5: QUANTITATION OF ALOIN AND FERULIC ACID FROM THE FRESHLY FORMULATED RAW MATERIAL MIXTURE OF RAJAPRAVARTINI VATI

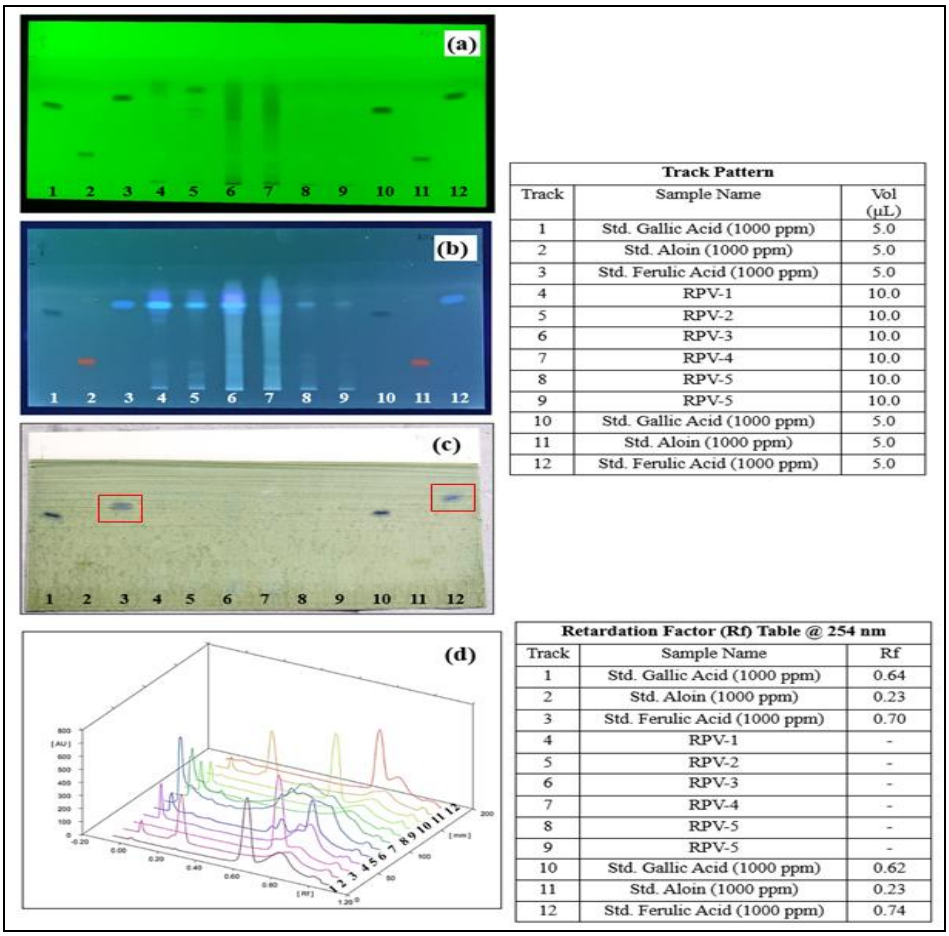
Plate Size	20 cm × 10 cm
Samples	60% ethanolic extracts (5% w/v concentration) of the freshly formulated raw material mixture (RPV-L)
Standards	Standard aloin (1000 ppm) and standard ferulic acid (1000 ppm)
Mobile Phase	Ethyl acetate : Methanol : Water (10:1.45:1) (v/v)

RESULTS AND DISCUSSION: Preliminary screening of the commercially available samples of Rajapravartini Vati for presence of prospective marker compounds did not show presence of ferulic acid or aloin in any of the methanolic extracts of the samples, when compared with the separation profiles of standard ferulic acid and standard aloin and confirmed with derivatisation techniques, as is illustrated in **Fig. 1** and **2.**

The chosen mobile phase composition for aloin separation was subsequently determined and optimised to be suitable for separation of ferulic acid, allowing for simultaneous analysis of both aloin and ferulic acid in subsequent analyses. The peak shapes for the two standard components for the optimised mobile phase are illustrated in **Fig. 3.** These findings are illustrative of the fact that phytobioactive profiles of the commercially available formulations are likely to be influenced by synergistic in-process changes which might result in non-detection of expected marker compounds characteristic of their raw materials.

To establish whether marker compounds such as aloin and ferulic acid are detectable in raw material mixtures of Rajapravartini Vati which have not been subjected to commercial manufacturing, a fingerprinting analysis was carried out with 60% ethanolic extracts of the commercial samples.

In addition, a freshly prepared raw material mixture of Rajapravartini Vati using Ayurvedic grade raw materials was also subjected to extraction with 60% ethanol and used for fingerprinting, along with individual raw materials, commercial *Aloe* gel and *Aloe* juice samples. When subjected to chromatographic separation with the previously optimised mobile phase and standards, aloin was not detected in any of the commercial samples, including *Aloe* gel and *Aloe* juice, further highlighting the possibility of in-process phytochemical profile changes during commercial manufacturing. Aloin was however detected in *Aloe barbadensis* powder as well as the freshly formulated raw material mixture of Rajapravartini Vati. Ferulic acid was similarly detected in *Ferula asafoetida* as well as the freshly formulated mixture. This is illustrated through the images shown in **Fig. 4** and **5.**



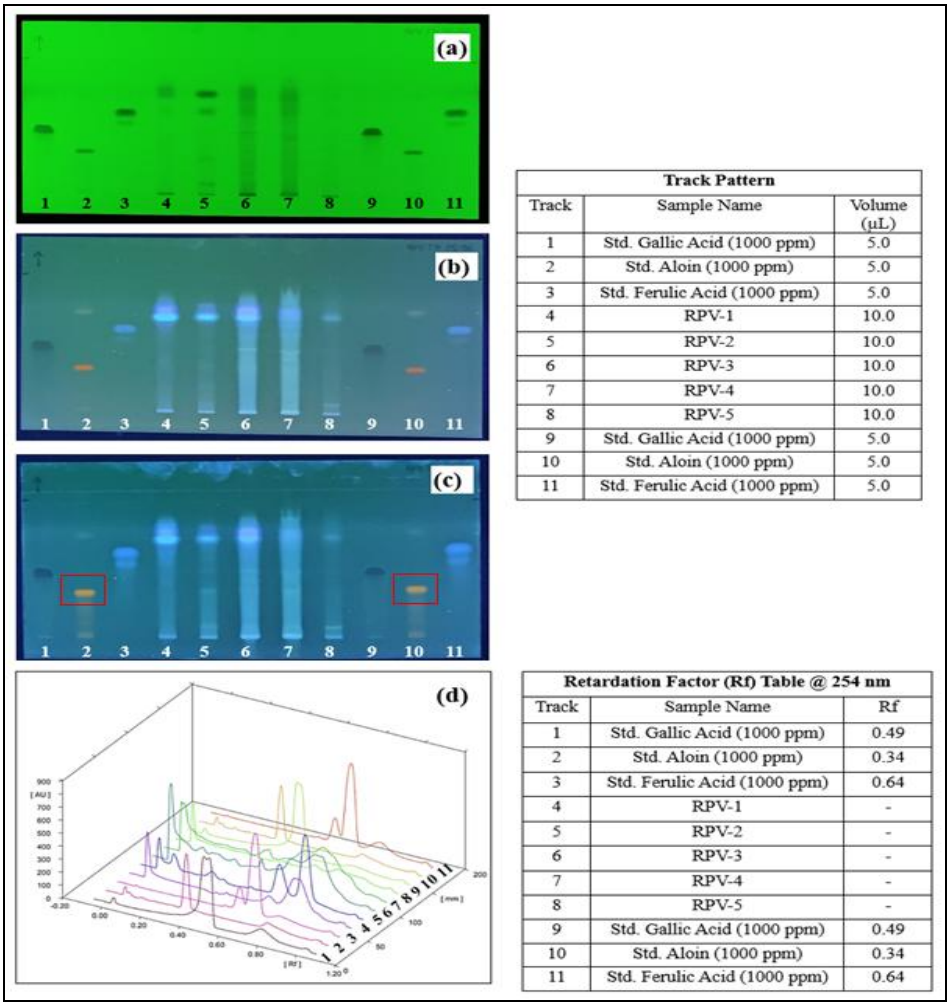
Track Pattern

Track	Sample Name	Vol (µL)
1	Std. Gallic Acid (1000 ppm)	5.0
2	Std. Aloin (1000 ppm)	5.0
3	Std. Ferulic Acid (1000 ppm)	5.0
4	RPV-1	10.0
5	RPV-2	10.0
6	RPV-3	10.0
7	RPV-4	10.0
8	RPV-5	10.0
9	RPV-5	10.0
10	Std. Gallic Acid (1000 ppm)	5.0
11	Std. Aloin (1000 ppm)	5.0
12	Std. Ferulic Acid (1000 ppm)	5.0

Retardation Factor (Rf) Table @ 254 nm

Track	Sample Name	Rf
1	Std. Gallic Acid (1000 ppm)	0.64
2	Std. Aloin (1000 ppm)	0.23
3	Std. Ferulic Acid (1000 ppm)	0.70
4	RPV-1	-
5	RPV-2	-
6	RPV-3	-
7	RPV-4	-
8	RPV-5	-
9	RPV-5	-
10	Std. Gallic Acid (1000 ppm)	0.62
11	Std. Aloin (1000 ppm)	0.23
12	Std. Ferulic Acid (1000 ppm)	0.74

FIG. 1: HPTLC PLATES AND TRACK PATTERN VISUALISED FOR PRELIMINARY SCREENING OF FERULIC ACID VISUALISED AT (A) 254 NM, (B) 366 NM AND (C) VISIBLE LIGHT POST-DERIVATISATION WITH 1% $FECL_3$ REAGENT. THE MOBILE PHASE COMPOSITION USED WAS TOLUENE : ETHYL ACETATE : FORMIC ACID (2:7:1) (V/V). PHENOLIC COMPONENTS SUCH AS FERULIC ACID FORM A DEEP BLUE COMPLEX WITH THE DERIVATISATION REAGENT (HIGHLIGHTED IN RED BOXES). (D) COMPARATIVE CHROMATOGRAM VIEW AND RF TABLE OF THE TRACKS SCREENED AT 254 NM.



Track Pattern

Track	Sample Name	Volume (µL)
1	Std. Gallic Acid (1000 ppm)	5.0
2	Std. Aloin (1000 ppm)	5.0
3	Std. Ferulic Acid (1000 ppm)	5.0
4	RPV-1	10.0
5	RPV-2	10.0
6	RPV-3	10.0
7	RPV-4	10.0
8	RPV-5	10.0
9	Std. Gallic Acid (1000 ppm)	5.0
10	Std. Aloin (1000 ppm)	5.0
11	Std. Ferulic Acid (1000 ppm)	5.0

Retardation Factor (Rf) Table @ 254 nm

Track	Sample Name	Rf
1	Std. Gallic Acid (1000 ppm)	0.49
2	Std. Aloin (1000 ppm)	0.34
3	Std. Ferulic Acid (1000 ppm)	0.64
4	RPV-1	-
5	RPV-2	-
6	RPV-3	-
7	RPV-4	-
8	RPV-5	-
9	Std. Gallic Acid (1000 ppm)	0.49
10	Std. Aloin (1000 ppm)	0.34
11	Std. Ferulic Acid (1000 ppm)	0.64

FIG. 2: HPTLC PLATES AND TRACK PATTERN VISUALISED FOR PRELIMINARY SCREENING OF ALOIN VISUALISED AT (A) 254 NM, (B) 366 NM AND (C) 366 NM POST-DERIVATISATION WITH 10% METHANOLIC BORIC ACID. THE MOBILE PHASE COMPOSITION USED WAS ETHYL ACETATE : METHANOL : WATER (10:1.45:1) (V/V). ALOIN POSSESSES A CHARACTERISTIC RED COLOURED FLUORESCENCE WHICH TURNS ORANGE AFTER DERIVATISATION (HIGHLIGHTED IN RED BOXES). (D) COMPARATIVE CHROMATOGRAM VIEW AND RF TABLE OF THE TRACKS SCREENED AT 254 NM

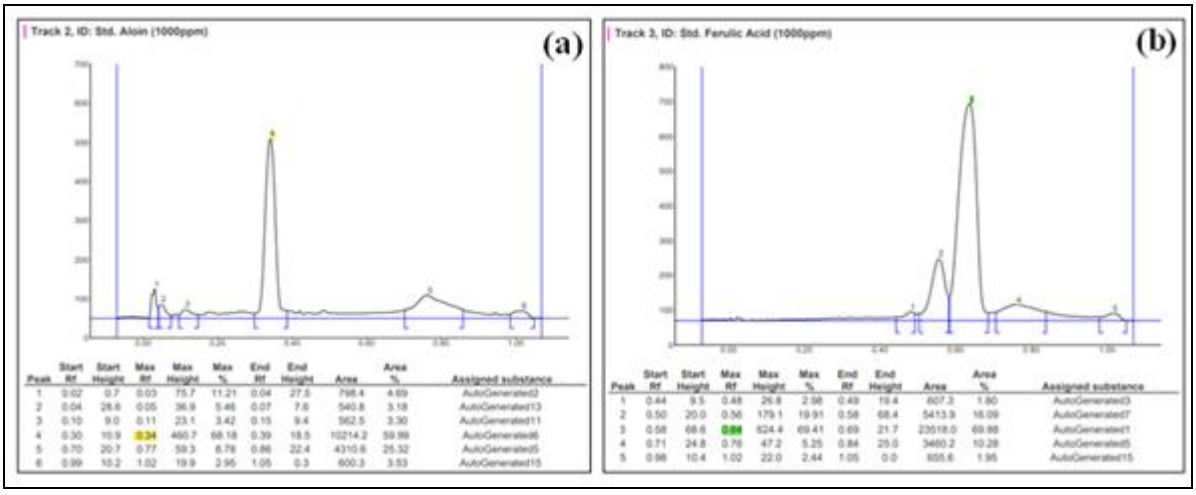
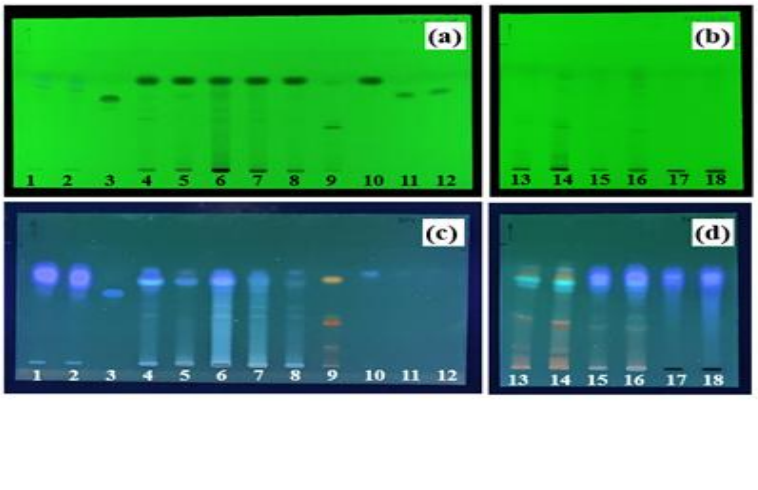
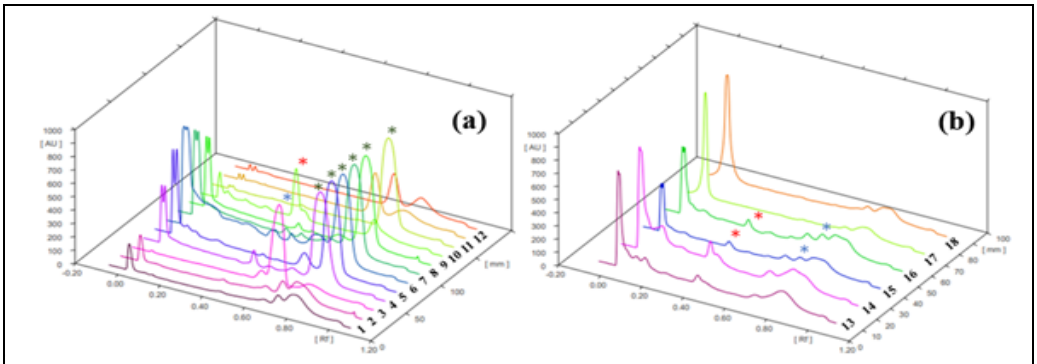


FIG. 3: HPTLC CHROMATOGRAMS FOR (A) STANDARD ALOIN AND (B) STANDARD FERULIC ACID DEVELOPED USING THE MOBILE PHASE ETHYL ACETATE : METHANOL : WATER (10:1.45:1) (V/V) (AT 254 NM). GOOD SEPARATION AND PEAK QUALITY WERE OBSERVED FOR SIMULTANEOUS ANALYSIS OF BOTH ALOIN AND FERULIC ACID USING THE SAME MOBILE PHASE WHICH WAS OPTIMISED FOR FURTHER ANALYSES



Track Pattern		
Track	Sample Name	Vol (µL)
1	*Ferula asafoetida* Extract	10.0
2		10.0
3	Std. Ferulic Acid (1000 ppm)	5.0
4	RPV-1	10.0
5	RPV-2	10.0
6	RPV-3	10.0
7	RPV-4	10.0
8	RPV-5	10.0
9	Std. Aloin (1000 ppm)	5.0
10	Commercial *Aloe* Gel Sample	10.0
11	Commercial *Aloe* Juice Sample	10.0
12		10.0
13	*Aloe barbadensis* Powder Extract	5.0
14		10.0
15	RPV-L (60% EtOH)	10.0
16		20.0
17	RPV-L (MeOH)	10.0
18		20.0

FIG. 4: HPTLC FINGERPRINTS OF RAW MATERIALS AND FINISHED FORMULATIONS VISUALISED AT (A) AND (B) 254 NM, AND (C) AND (D) 366 NM. 60% ETHANOLIC EXTRACTS OF ALL SAMPLES WERE USED



Component	Aloin		Ferulic Acid		Aloe Constituent	
	Standard	Lab Formulation (RPV-L)	Standard	Lab Formulation (RPV-L)	Commercial *Aloe* Gel	Marketed Formulations (RPV-1 to RPV-5)
Track Number(s)	9	15, 16	3	15, 16	10	4 – 8
Retardation Factor (Rf)	0.38	0.33	0.62	0.69	0.76	0.76 - 0.77

FIG. 5: (A) AND (B) COMPARATIVE CHROMATOGRAM VIEW OF RAW MATERIALS AND FINISHED FORMULATIONS VISUALISED AT 254 NM (PLATE IMAGES ILLUSTRATED IN FIGURE 4). THE PRESENCE OF ALOIN (HIGHLIGHTED BY RED ASTERISK) AND FERULIC ACID (HIGHLIGHTED BY BLUE ASTERISK) IS NOTED IN THE FORMULATED VATI RAW MATERIAL MIXTURE. ADDITIONALLY, A COMMON *ALOE* MARKER COMPOUND (HIGHLIGHTED BY GREEN ASTERISK) IS NOTED IN THE COMMERCIAL FORMULATIONS AND *ALOE* GEL

A common unidentified *Aloe* marker compound was observed to be present in all of the commercial Vati samples as well as *Aloe* gel, confirmed through spectral scan overlay as shown in **Fig. 6.** This component requires characterisation through further sophisticated techniques of analysis such as mass spectrometry. It is likely that this compound is reflective of the in-process changes or effect of certain other supplementary manufacturing procedures in these samples. In addition, effect of certain Ayurvedic procedures such as Bhavana and Shodhana, which are likely to be carried out as part of commercial production of Rajapravartini Vati, where certain other plant extracts are employed for enriching the raw material powders through sudation or trituration, may also play a role in impacting their phytochemical marker profiles [11].

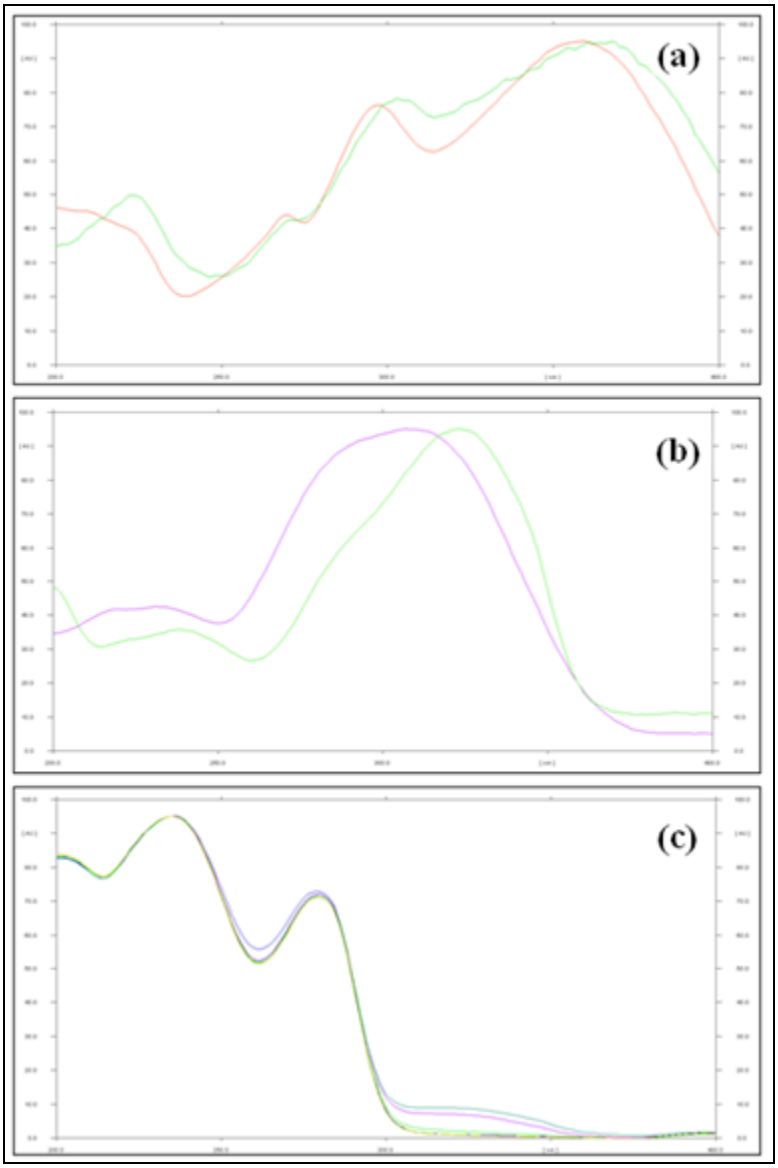
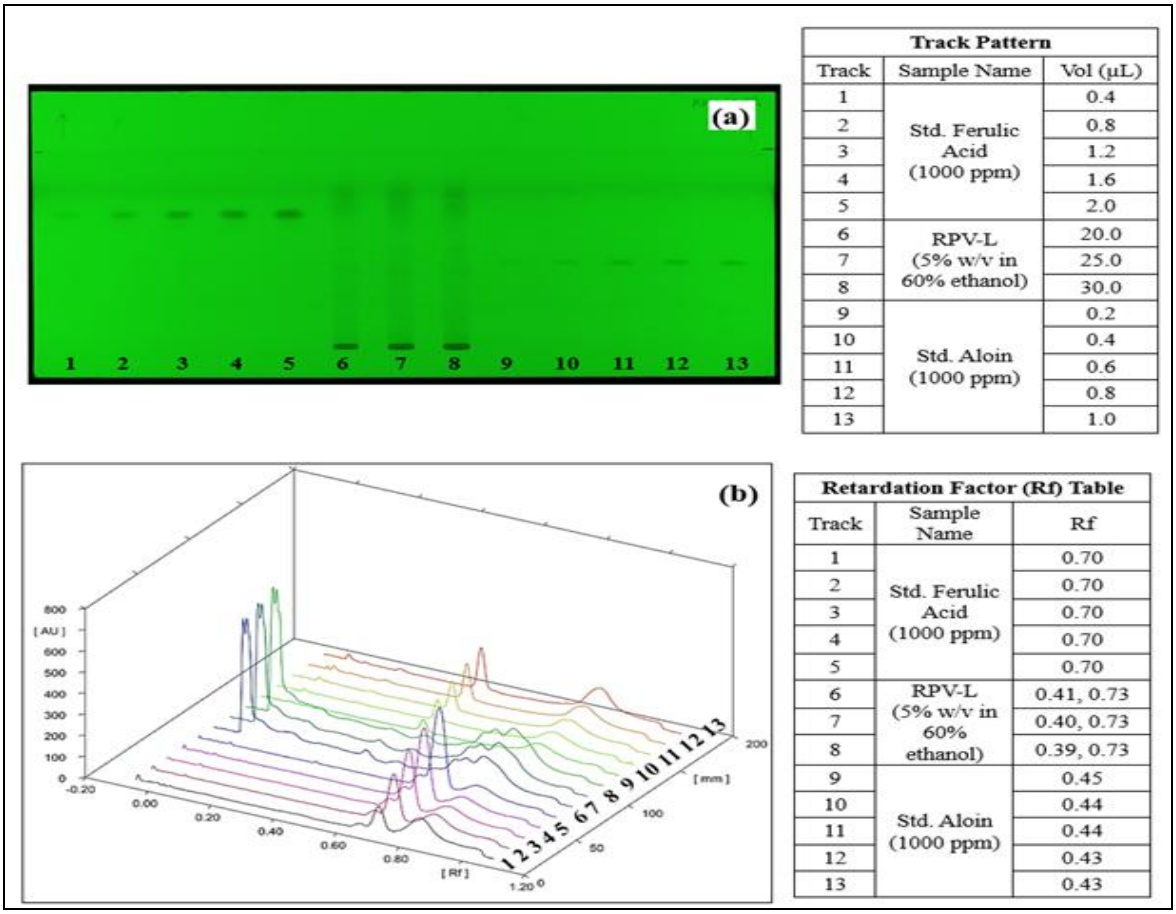


FIG. 6: UV SPECTRAL OVERLAY OF (A) STANDARD ALOIN (ORANGE) WITH ALOIN DETECTED IN THE FORMULATED VATI MIXTURE, (B) STANDARD FERULIC ACID (PURPLE) WITH FERULIC ACID DETECTED IN THE FORMULATED VATI MIXTURE AND (C) *ALOE* MARKER COMPOUND WHICH IS COMMON TO ALL MARKETED FORMULATIONS AND COMMERCIAL *ALOE* GEL

Quantitative analysis of aloin and ferulic acid was subsequently carried out from the freshly formulated raw material mixture as a preliminary quality control measure as well as to validate the developed method for simultaneous analysis of aloin and ferulic acid from such samples. Calibration curve method was utilised for quantitative analysis. A five-point volume series of standard aloin (1000 ppm) (0.2, 0.4, 0.6, 0.8, 1.0 µL) and standard ferulic acid (1000 ppm) (0.4, 0.8, 1.2, 1.6, 2.0 µL) (1000 ppm) were respectively used to quantitate the two markers from the freshly formulated Vati extract by HPTLC. The plate images are shown in **Fig. 7**. Appreciable linearity was observed for the two calibration curves with R^2 = 0.9895 (aloin) and R^2 = 0.9867 (ferulic acid) respectively. Using the σ/S approach, the LLOD and LLOQ of aloin using the developed method were established at 0.12 µg and 0.37 µg respectively. Similarly, the LLOD and LLOQ of ferulic acid were established at 0.28 µg and 0.85µg respectively (where µg represents the amount of analyte deposited on the plate). The sample extract was applied in triplicates in increasing volumes and the final concentration of the two components was computed as the average from the three sample tracks. These are illustrated in **Fig. 8**. Aloin and ferulic acid were subsequently quantitated at 0.99 mg/g and 0.92 mg/g in the raw material mixture respectively.



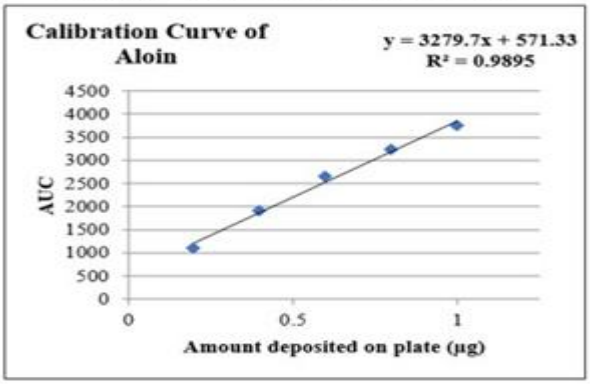
Track Pattern		
Track	Sample Name	Vol (µL)
1	Std. Ferulic Acid (1000 ppm)	0.4
2		0.8
3		1.2
4		1.6
5		2.0
6	RPV-L (5% w/v in 60% ethanol)	20.0
7		25.0
8		30.0
9	Std. Aloin (1000 ppm)	0.2
10		0.4
11		0.6
12		0.8
13		1.0

Retardation Factor (Rf) Table		
Track	Sample Name	Rf
1	Std. Ferulic Acid (1000 ppm)	0.70
2		0.70
3		0.70
4		0.70
5		0.70
6	RPV-L (5% w/v in 60% ethanol)	0.41, 0.73
7		0.40, 0.73
8		0.39, 0.73
9	Std. Aloin (1000 ppm)	0.45
10		0.44
11		0.44
12		0.43
13		0.43

FIG. 7: (A) HPTLC PLATE VISUALISED AT 254 NM AND TRACK PATTERN FOR QUANTITATION OF ALOIN ANDFERULIC ACID FROM THE FORMULATED VATI RAW MATERIAL MIXTURE AND (B) COMPARATIVE CHROMATOGRAM VIEW AND RF TABLE OF THE TRACKS SCREENED AT 254 NM

(a) Quantitation of Aloin				
Sample Name	Sample Concentration	Volume (µL)	Amount deposited on plate (µg)	AUC
Std. Aloin	1000 ppm	0.2	0.2	1131.4
		0.4	0.4	1898.6
		0.6	0.6	2681.1
		0.8	0.8	3248.3
		1.0	1.0	3736.2
RPV-L (5% w/v)		20		1631.4
		25		1934.6
		30		2720.6
Based on the calibration curve, aloin was quantitated at **0.99 mg/g** of the formulated raw material mixture.				

(b) Quantitation of Ferulic Acid				
Sample Name	Sample Concentration	Volume (µL)	Amount deposited on plate (µg)	AUC
Std. Ferulic Acid	1000 ppm	0.4	0.4	3848.1
		0.8	0.8	7139.9
		1.2	1.2	9460
		1.6	1.6	11414
		2.0	2.0	13394.1
RPV-L (5% w/v)		20		8023.1
		25		8919.5
		30		10841.2
Based on the calibration curve, ferulic acid was quantitated at **0.92 mg/g** of the formulated raw material mixture.				



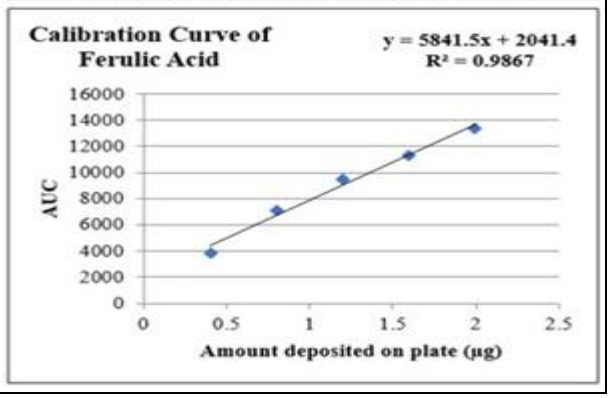


FIG. 8: CALIBRATION CURVES OF (A) ALOIN AND (B) FERULIC ACID FOR QUANTITATION OF THE TWO COMPONENTS FROM THE LABORATORY FORMULATED VATI RAW MATERIAL MIXTURE

CONCLUSION: HPTLC fingerprinting of the raw materials and formulations of Rajapravartini Vati provides a convenient and precise means for establishing their phytochemical marker profiles. In addition, it is also beneficial for gaining valuable preliminary insights into how in-process changes during commercial manufacturing and Ayurvedic enrichments such as Bhavana and Shodhana samskaras may serve as key factors which impact their phytochemical profiles. This is evident through the differences in the phytochemical profiles between the freshly formulated raw material mixture and the commercial formulations. Quantitative analysis of marker compounds such as aloin and ferulic acid from the raw material mixture serves as the first step towards quality control and raw material authentication in the production of Rajapravartini Vati. The findings provide a significant boost to knowledge insights on traditional Indian systems of medicine, besides opening up future avenues for exploration of these phytochemical profiles through more sensitive techniques of analysis, as well as for providing evidences of the mechanisms of action of phytobioactives in the Rajapravartini Vati.

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CONFLICTS OF INTEREST: The authors declare that there are no conflicts of interest.

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