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Standardization of Kankayan Vati: An Ayurvedic Polyherbal Formulation

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Abstract

Ayurvedic formulations have reached extensive acceptability as therapeutic agents for several diseases. In order to have a good coordination between the quality of raw materials, in process materials and final products, it has become essential to develop reliable, specific and sensitive quality control methods using combination of classical and modern instrumental methods of analysis. Standardization of herbal formulation is essential in order to assess quality, purity, safety and efficacy of drugs. Kankayan Vati is an Ayurvedic formulation containing seventeen herbs and is official in Ayurvedic Formulary of India. It is used in the treatment of piles, bloating, intestinal worms etc. and also used to treat abdominal lumps, haemorrhoids and heart diseases. In the present work, an attempt has been made to develop method for standardization of Kankayan Vati. Ferulic acid in *Ferula asafoetida* and plumbagin in *Plumbago zeylanica* are active constituents in this formulation and can be considered as marker components. A new, simple, rapid and precise High Performance Thin Layer Chromatography (HPTLC) method was developed for standardization of Kankayan Vati by simultaneous quantification of marker constituents. The separation was carried out on TLC aluminium plate precoated with silica gel 60 $F_{254,}$ using Toluene: Ethyl acetate: Formic acid (6.5:3.5:0.1 v/v/v) as mobile phase. The densitometric analysis of ferulic acid (0.32±0.03) and plumbagin (0.73±0.03) was carried at 281 nm. The proposed method has been validated as per ICH guidelines.

Key-Words: Ayurvedic formulations, Kankayan Vati, Ferulic acid, Plumbagin, HPTLC

Introduction

India has a rich heritage of traditional medicines constituting with its different components like Ayurveda, Siddha and Unani[1]. Standardization of drug means confirmation of its identity and determination of its quality and purity. The quality assessment of herbal formulations is of paramount importance in order to justify their acceptability in modern system of medicines. Also one of the major problem faced by herbal drug industry is unavailability of rigid quality control profile for herbal materials and their formulations[2]. Standardization of herbal formulation comprises of preliminary phytochemical screening, conducting various physicochemical studies on the plants used in it and quantification of the marker constituents by various modern analytical techniques. Over the few years chromatographic techniques such as High Performance Thin Layer Chromatography (HPTLC), High Performance Liquid Chromatography (HPLC), etc. have emerged as valuable tools for the qualitative and quantitative analysis of herbal drugs and formulations.

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Ayurvedic formulations are mainly available in the form of solid dosage form (Vati, Gutika, Churna, Bhasma), Liquid dosage form (Asava, Arishta, Arka, Taila) and Semisolid dosage form (Ghrita, Avleha, Lepa)[3,4]. Vati and Gutika are medicines prepared in the form of tablets or pills which are made of one or more drugs of plant, animal or mineral origin. Kankayan Vati is an Ayurvedic formulation which is official in Ayurvedic Formulary of India[5] and contains seventeen ingredients of plant origin hingu shuddha (*Ferula asafoetida*), amlavetsa (*Garcinia pedunculata*), yavkhar (*Hordeum vulgare*), chitrak (*Plumbago zeylanica*), pushkarmool (*Inula racemosa*), trivirit (*Operculina turpethum*), vacha (*Acorus calamus*), ardraka (*Zingiber officinale*), arhar (*Cajanus cajan*), kachur (*Curcum zedoria*), dantimool (*Baliospermum montanum*), ajmoda (*Apium graveolens*), Krishna jeeraka (*Carum carvi*), dhanyaka (*Coriandrum sativum*), maricha (*Piper nigrum*), jeera (*Cuminum cyminum*) and yavani (*Trichyspermum ammi*)[6]. It is used in the treatment of piles, bloating, intestinal worms etc. and also used to treat abdominal lumps, haemorrhoids and heart diseases. Modern analytical methods are not yet reported for standardization of Kankayan Vati. As it is difficult to estimate each and

every ingredient for its chemical constituents, two of the main ingredients of Kankayan Vati have been identified and standardized. *Ferula asafoetida* and *Plumbago zeylanica* are the major constituents of this formulation. Ferulic acid (Figure 1) from *Ferula asafoetida* and plumbagin (Figure 2) from *Plumbago zeylanica* are selected as marker constituents for standardization of the formulation. Literature survey reveals that few HPTLC, RP-HPLC and UV methods are reported for estimation of ferulic acid[7-11] and plumbagin[12-16] individually as well as in combination with other constituents. However no HPTLC method has been reported for simultaneous quantification of ferulic acid and plumbagin, which can be further applied for standardization of Kankayan Vati. The present work deals with the development and validation of HPTLC method for standardization of Kankayan Vati by simultaneous quantification of marker constituents ferulic acid and plumbagin from three marketed formulations (M1, M2, M3) and in-house formulation (D).

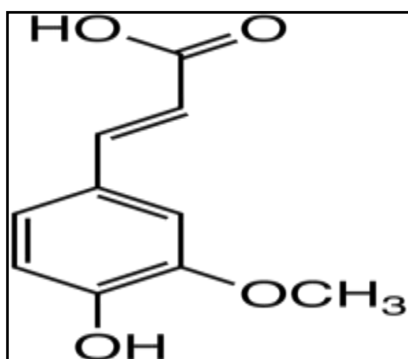

Fig. 1: Chemical structure of ferulic acid

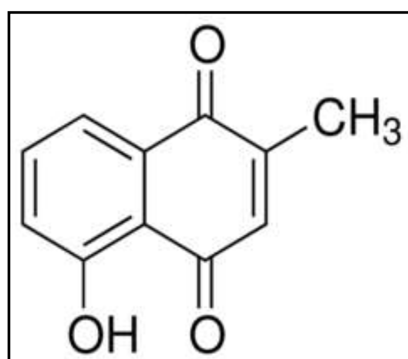

Fig. 2: Chemical structure of plumbagin

Material and Methods

Raw materials and marketed formulations

The raw materials (powders) used in the preparation of Kankayan Vati were procured from the local market of Mumbai, Maharashtra, India. Marketed formulations of three brands (M1, M2, M3) of Kankayan Vati were obtained from the local Ayurvedic shops of Mumbai, Maharashtra, India.

Standards and Reagents

All the chemicals used in experiment were of analytical grade procured from S. D. Fine Chemicals, Mumbai, India. Standard ferulic acid and plumbagin were procured from Sigma Aldrich Pvt. Ltd, Mumbai, India.

Instrumentation

Chromatographic separation was achieved on HPTLC plates using Camag (Muttenz, Switzerland) Linomat V sample applicator equipped with 100μl Hamilton syringe. For detection TLC scanner 3 with winCATS software was used.

Experimental

Preliminary studies

The physicochemical studies like determination of ash value, extractive values, moisture content and phytochemical studies were carried out for Kankayan Vati as well as raw materials.

HPTLC method development

Preparation of standard stock solution and working solution

100 mg of ferulic acid and plumbagin were weighed accurately and transferred to individual volumetric flasks of 100 ml each. Volume was made up to 100 ml using methanol to obtain concentration of 1000μg/ml. Working solution was prepared from this standard solution. 1 ml of each stock solutions were transferred to separate 10 ml volumetric flask and volume was made upto 10 ml with methanol to get concentration in the range of 100μg/ml.

Preparation of In-house formulation

In-house formulation (D) of Kankayan Vati was prepared as per procedure given in the Ayurvedic Pharmacopoeia of India. All the ingredients were mixed in specified ratio to obtain uniform mixture to which Citrus juice (*Citrus limon)* was added and ground to obtain homogenous blend which was then compressed into Vati. Vati were then dried and stored into air tight container for further use.

Extraction of ferulic acid and plumbagin from In-house and marketed formulation

Vati equivalent to 10 gm was triturated and extracted with 50 ml methanol by stirring on magnetic stirrer for 24 hrs, filtered through Whatmann filter paper no. 41 and this procedure was repeated once using fresh 50 ml methanol. The final volume was made upto 100 ml

with methanol. This solution was then used for quantification of ferulic acid and plumbagin.

Chromatographic conditions

Chromatographic separation was achieved on HPTLC plates precoated with silica gel 60 $F_{254.}$ Standard solutions of markers and samples (extracts) were applied to the plates as bands of 6.0 mm wide, 10.0 mm from the bottom edge of the chromatographic plate by use of a Camag (Muttenz, Switzerland) Linomat 5 sample applicator equipped with a 100μl Hamilton syringe. Ascending development to a distance of 80 mm was performed at room temperature (25 ± 2°C), with mobile phase, in a Camag glass twin-trough chamber previously saturated with mobile phase vapour for 30 min. After development, the plates were dried and then scanned at 281 nm with a Camag TLC Scanner 3 using the deuterium lamp with winCATS software.

HPTLC method validation

The developed method was validated as per ICH guidelines Q2(R1) for following parameters[17]:

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. It was obtained by plotting peak area Vs concentration of standard and finding regression coefficient (r^2).

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. The specificity of the method was ascertained by comparing the R_f value and the peak purity was assessed by comparing the spectrum of standard ferulic acid and plumbagin with methanolic extract of Vati.

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. As per the ICH guidelines precision should be performed at three different levels: Lower Quality Control (LQC), Medium Quality Control (MQC), Higher Quality Control (HQC). Repeatability also termed as intra-assay precision expresses precision under the same operating conditions over a short interval of time. It is assessed by using minimum of 9 determinations covering the specified range for the procedure. The intra-day assay precision was performed 3 times on same day, while inter-assay precision was performed on 3 different days.

Limit of detection (LOD) and Limit of quantification (LOQ)

The detection limit (LOD) is the lowest amount of an analyte in the sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. Limit of Quantification (LOQ) is the lowest amount analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. LOD and LOQ were determined by k x SD/s where 'k' is a constant (3.3 for LOD and 10 for LOQ), 'SD' is the standard deviation of the analytical signal and 's' is the slope of the calibration curve.

Accuracy (Recovery)

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. As per ICH, Accuracy should be assessed using a minimum of 9 determinations over a minimum of three concentration levels covering the specified range i.e. 3 concentrations levels in triplicate. (e.g., 3 concentrations/ 3 replicates each). The accuracy of the method was examined by performing recovery experiments by the standard addition method. The recovery of the drugs at different levels in the formulations was checked by spotting the test samples of known concentration of ferulic acid and plumbagin simultaneously on the plates. The spots were then spiked in three different concentrations (80%, 100% and 120% w/w) by further adding known amount of standard mixture of ferulic acid and plumbagin. These samples were then analyzed and the results obtained were compared with expected results.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The robustness was studied by evaluating the effect of small but deliberate variations in the chromatographic conditions. Following the introduction of small changes in the mobile phase composition (±0.2 ml for major component), the effect on the results was examined. The amount of mobile phase was varied over the range of ± 5 %. The saturation time of development chamber was varied by ± 5 min. The robustness of the method was determined at two concentration levels (300 and 400 ng/spot) for ferulic acid and (200 and 300 ng/spot) for plumbagin.

Application of method to Formulation

The developed HPTLC method was applied for standardization of in-house and marketed formulations

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of Kankayan Vati by simultaneous estimation and quantification of marker constituents ferulic acid and plumbagin. Standard and sample solutions were applied in triplicate. Standard solutions of 100μg/ml of ferulic acid and plumbagin were applied. Vati extract was directly used for quantification of ferulic acid and plumbagin. The amount of ferulic acid and plumbagin present per gram of formulation was calculated by comparing areas measured from extracts with calibration curve constructed from peak area obtained from standard solution of ferulic acid and plumbagin.

Results and Discussion

Preliminary studies

Preliminary studies were carried out for marketed and in-house formulations of Kankayan Vati as well as raw materials. The results are shown in table 1.

Table 1: Results of preliminary studies for Kankayan Vati

Parameters	Marketed Formulation (M1)	Marketed Formulation (M2)	Marketed Formulation (M3)	In-house Formulation (D)
Loss on drying(% w/w)	10.37	4.91	5.26	6.1
Water soluble extractive value(% w/w)	41.6	25.6	43.2	28
Alcohol soluble extractive value (% w/w)	6.4	1.6	8	6.4
Ash value(% w/w)	17	38.5	25	15

HPTLC method development

In situ HPTLC spectral overlain of ferulic acid and plumbagin were taken. Isoabsorptive point was found at 281nm and was selected as scanning wavelength (Figure 3).

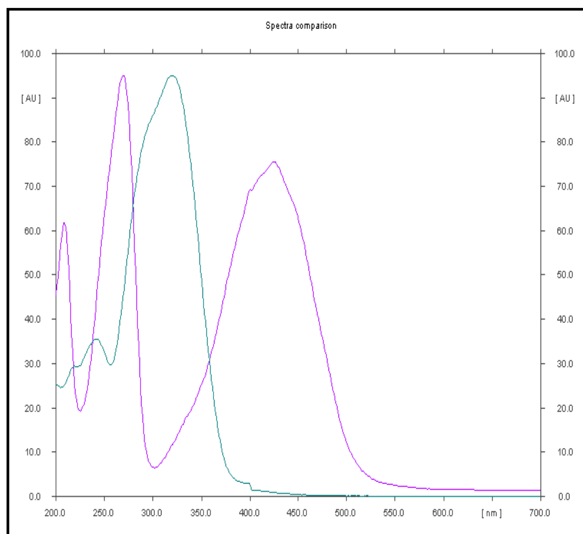


Fig. 3: HPTLC overlain spectra of ferulic acid and plumbagin

The experimental conditions for HPTLC such as wavelength of detection and mobile phase composition were optimized to provide accurate, precise and reproducible results for the determination of ferulic acid and plumbagin. The mixed standard stock solution containing 100μg/ml of ferulic acid and plumbain was spotted on the TLC plate and developed in different solvent systems. Good resolution and sharp peaks were obtained with minimum tailing by using mobile phase consisting of Toluene: Ethyl acetate: Formic acid in the ratio 6.5:3.5:0.1(v/v/v). Ferulic acid and plumbagin were satisfactorily resolved with R_f values at 0.32±0.03 and 0.73±0.03, respectively (Figure 4)

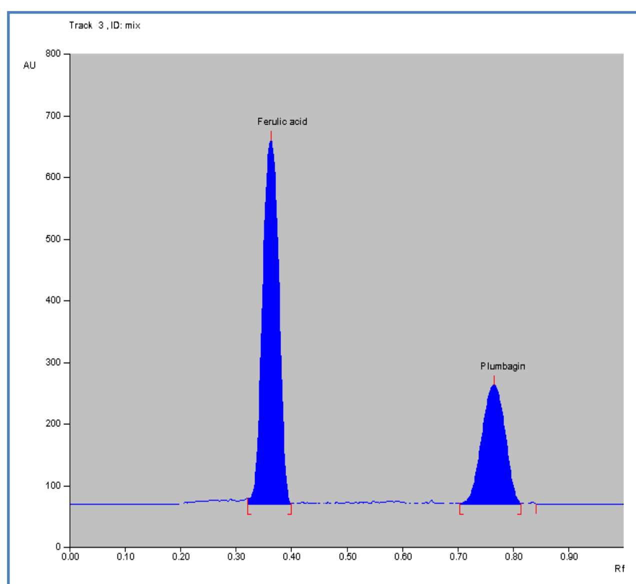


Fig. 4: Chromatogram of standard Ferulic acid [Rf: 0.32±0.03 and Plumbagin [Rf: 73±0.03]

HPTLC method validation

Linearity

Linear relationship was observed by plotting drug concentration against peak area for each compound. Ferulic acid and plumbagin showed linear response in the concentration range of 200-500 ng/spot and 100-400 ng/spot, respectively (Figure 5). The linearity was validated by the high value of the correlation coefficients. The results are tabulated in Table 2.

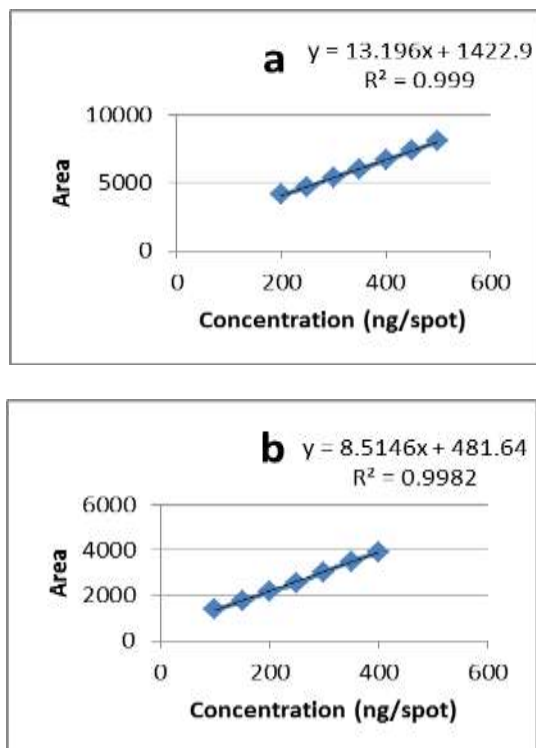


Fig. 5: Calibration curve of Ferulic acid (a) and Plumbagin (b)

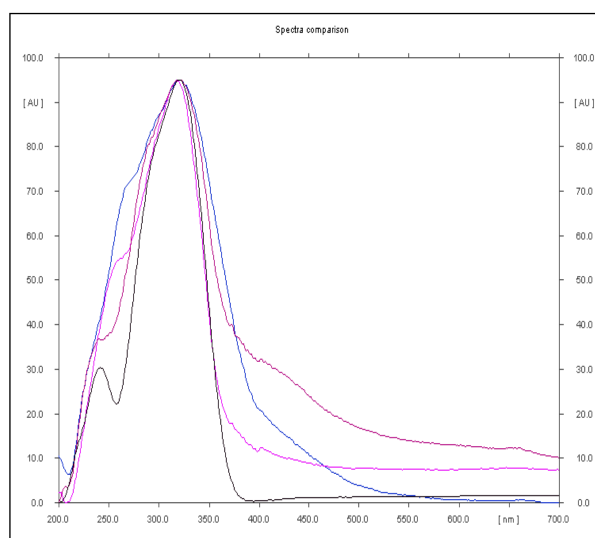
Table 2: Linear regression data for calibration plot for Ferulic Acid and Plumbagin (n=3)

Parameters	Ferulic acid	Plumbagin
Linearity range (ng)	200-500	100-400
Regression equation	y=13.19x+1422	y=8.514x+481.6
Correlation coefficient (r^2±S.D.)	0.999±0.001	0.998
Slope (mean± S.D.)	13.19± 0.5294	8.514±0.3256
Intercept (mean± S.D.)	1422±145.0956	481.6±15.5457

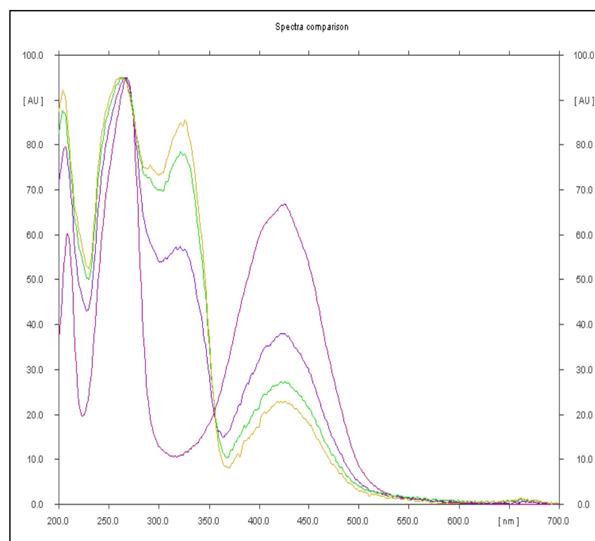
S.D. = Standard deviation

Specificity

When the spectra of standard ferulic acid and plumbagin were overlayed with the spectra of Kankayan Vati extracts it was observed that the other constituents present in extract did not interfere with the peaks of ferulic acid and plumbagin as shown in Figure 6a and 6b respectively. Therefore the method is specific.



6(a)



6(b)

Fig. 6: Spectra of standard ferulic acid and ferulic acid from methanolic extract (a), standard plumbagin and plumbagin from methanolic extract (b)

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Precision

The % RSD values depicted in Table 3 shows that the proposed method provides acceptable intra-day and inter-day variation in concentrations of ferulic acid and plumbagin, which indicate good precision for the developed method.

Table 3: **Intra-day and inter-day precision results for Ferulic acid and Plumbagin**

	Concentration (ng/spot)	INTRADAY			INTERDAY		
		Mean area	S.D.	%RSD	Mean area	S.D.	%RSD
Ferulic acid	250	4655.1	35.52	0.76	4661.1	51.27	1.09
	350	6075.9	0.54	0.83	6072.1	40.45	0.66
	450	7293.4	105.08	1.44	7291.1	74.51	1.02
Plumbagin	150	1846.8	26.68	1.44	1778.13	8.01	0.45
	250	2646.47	46.61	1.76	2633.47	49.02	1.86
	350	3615.14	41.28	1.14	3551.07	48.08	1.35

RSD= Relative Standard Deviation, Each result is an average of six measurements

Limit of detection (LOD) and Limit of quantification (LOQ)

The LOD and LOQ were found to be 36.25, 109.77 ng/spot for ferulic acid and 29.02, 87.96 ng/spot for plumbagin, respectively.

Accuracy (Recovery)

The recovery of ferulic acid from marketed formulations (M1, M2, M3) and in-house formulation (D) was found to be 101.61%, 99.85%, 100.6% and 100.36 %, respectively while that of plumbagin from the same four formulations was found to be 101.05%, 98.57%, 100.92% and 100.01% respectively as shown in the data of table 4 and 5 respectively.

Table 4: Recovery data for ferulic acid (n=3)

Formulation	Level of % recovery	Amount of marker present (ng) A	Amount of marker added (ng) B	Total amount of marker (ng) A+B	Amount of marker found	Recovery (%)	Average recovery (%)
M1	80	280	230	510	524.02	102.74	101.61
	100	280	280	560	558.30	99.69	
	120	280	340	620	635.01	102.42	
M2	80	260	210	470	465.87	99.12	99.85
	100	260	260	520	528.64	101.66	
	120	260	310	570	563.07	98.78	
M3	80	230	180	410	406.48	99.14	100.6
	100	230	230	460	464.92	101.06	
	120	230	280	510	518.21	101.60	
D	80	250	200	450	446.8	99.28	100.36
	100	250	250	500	503.06	100.61	
	120	250	300	550	556.67	101.21	

Table 5: Recovery data for plumbagin (n=3)

Formulation	Level of % recovery	Amount of marker present (ng) A	Amount of marker added (ng) B	Total amount of marker (ng) A+B	Amount of marker found	Recovery (%)	Average recovery (%)
M1	80	260	210	470	477.84	101.66	101.05
	100	260	260	520	525.67	101.09	
	120	260	310	570	572.32	100.40	
M2	80	190	150	340	335.21	98.59	98.57
	100	190	190	380	373.77	98.36	
	120	190	230	420	414.84	98.77	
M3	80	290	230	520	525.10	100.98	100.92
	100	290	290	580	585.23	100.90	
	120	290	350	640	645.65	100.88	
D	80	180	140	320	31916	99.73	100.01
	100	180	180	360	356.98	99.16	
	120	180	210	390	394.51	101.15	

Robustness

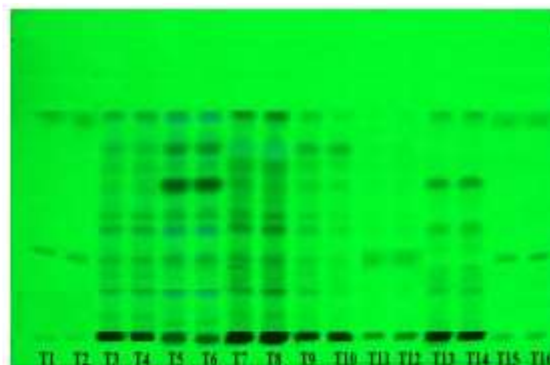
The % RSD of the peak area was calculated in triplicate for changes in mobile phase composition, duration of saturation time and volume of mobile phase for 300 and 400 ng/spot for ferulic acid and 200 and 300 ng/ spot for plumbagin. The values of % RSD as shown in table 6 were less than 2% which indicated that the developed method is robust.

Table 6: Robustness results of ferulic acid and plumbagin

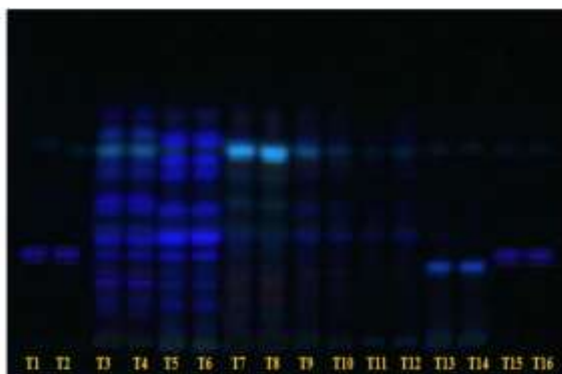
Parameters	Ferulic acid		Plumbagin	
	300 ng/spot	400 ng/spot	200 ng/spot	300 ng/spot
	% RSD	% RSD	% RSD	% RSD
Mobile phase composition				
6.3: 3.7:0.1 (v/v/v)	0.54	0.67	0.81	1.02
6.7: 3.3: 0.1 (v/v/v)	0.37	0.71	1.06	0.36
Saturation time				
25 minutes	1.1	0.59	0.26	0.75
35 minutes	0.91	0.33	0.58	0.66
Mobile phase volume				
+5%	0.78	0.91	1.27	0.52
-5%	1.8	0.61	0.39	1.09

Analysis of marketed and in-house formulations

The developed method was applied for the detection and quantification of ferulic acid and plumbagin in Kankayan Vati from different marketed formulations as well as in-house formulation. The peaks for ferulic acid and plumbagin were observed at Rf 0.32±0.03 and 0.73±0.03, respectively in the densitogram of Vati extract. There was no interference from other compounds present in the Vati. The total content of ferulic acid and plumbagin in marketed formulations M1, M2, M3 and in-house formulation (D) is as shown in table 7.



7(a)



7(b)

Fig. 7: HPTLC fingerprinting profile of standard ferulic acid and plumbagin as well as methanolic extracts of Kankayan Vati at 254 nm (a) and 366 nm (b)

T1, T2, T15 and T16: Standard ferulic acid and plumbagin T3 and T4: Marketed formulation M1, T5 and T6: Marketed formulatiom M2, T7 and T8: Marketed formulation M3, T9 and T10: In-house formulation D

Table 7: Content of ferulic acid and plumbagin in Kankayan Vati

Formulation	Ferulic acid content (% w/w)	Plumbagin content (% w/w)
M1	0.028	0.026
M2	0.023	0.019
M3	0.026	0.029

Conclusion

The HPTLC method was developed for standardization of Kankayan Vati using ferulic acid and plumbagin as marker constituents. The HPTLC method was found to be simple, precise, accurate, specific and reproducible for standardization of Kankayan Vati. The method based on simultaneous estimation of ferulic acid and plumbagin could be applied for both marketed and in-house formulation as well as for routine quality control to check quality and batch-batch variations.

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