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Standardization of Chitrakadi Vati: An Ayurvedic Polyherbal Formulation

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ABSTRACT

Standardization of herbal formulation is essential in order to assess the quality, purity, safety and efficacy of drugs. World health organization (WHO) in 1999 has given a detail protocol for the standardization of herbal drugs comprising of a single content but very little literature is available for the standardization of polyherbal formulations. Chitrakadi Vati is official in ayurvedic formulary of India and it is prescribed for the treatment of irritable bowel syndrome, rheumatoid arthritis and loss of appetite. In the present work, attempt has been made to develop a chromatographic method for standardization of Chitrakadi Vati. All raw materials used were standardized by macroscopic, microscopic and physico-chemical parameters. Piperine in *Piper nigrum* and *Piper longum*; Plumbagin in *Plumbago zeylanica* are active components in the formulation and can be considered as marker compounds. A simple, rapid, precise, accurate and reproducible High Performance Thin Layer Chromatography (HPTLC) densitometric method was developed. The separation was performed on TLC aluminium plates precoated with silica gel 60 F_{254}, good separation was achieved in the mobile phase of toluene: ethyl acetate: formic acid (7.5:2.5:0.5 v/v/v) and densitometry determination of piperine (0.39 ± 0.02) and plumbagin (0.70 ± 0.02) was carried out at single wavelength scanning at 280 nm. The developed method has been validated as per ICH (International conference on harmonization) guidelines. The developed and validated HPTLC method was applied for standardization of Chitrakadi Vati by detection and quantification of markers piperine and plumbagin simultaneously from in-house and marketed formulations.

Keywords: Chitrakadi Vati, Piperine, Plumbagin, HPTLC, Polyherbal formulations, ICH.

INTRODUCTION

Herbal formulations have been used by the majority of Indians since ancient times. In recent years, there has been an increased inclination towards the herbal formulations due to the healthy life style and trend towards the natural sources. [1-2] The complexity, side effects and costly treatment associated with allopathic drugs have caused both the health care practitioners and majority of world population to turn towards alternative therapies, more likely towards the herbal medicines. [3-4]

The quality assessment of herbal formulations is important to ensure safety and efficacy of herbal medicines. World Health Organization (WHO) stresses the importance of the qualitative and quantitative methods for characterizing samples, quantification of biomarkers, chemical markers and fingerprint profiles. Chitrakadi Vati is official in Ayurvedic formulary of India. It is a polyherbal formulation, constituting of nine ingredients of plant origin chitraka (*Plumbago zeylanica*), maricha (*Piper nigrum*), pippali (*Piper longum* fruit), pippalimoola (*Piper longum* root), Chavya (*Piper chaba*) ginger (*Zingiber officinale*), hing *(Ferula foetida*),

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ajmoda (*Apium leptophyllum*), yava ksara (*Hordeum vulgare*) and panchalavana (Sauvarchala, Saindhava, vida, samudra, audbhida). It is widely used for the treatment of irritable bowel syndrome, rheumatoid arthritis and loss of appetite. Literature and market survey states that the above formulation available in market is product of numerous companies which might have deviations in quality as well as quantity of ingredients used in this formulation. [5-6]

Standardization is a system to ensure that every packet of medicine that is being sold has the correct amount and will induce its therapeutic effect. Standardization of natural product drugs, is based on chemical entities, "marker compounds" which are chemically defined constituents may or may not have therapeutic activity and are of high significance for control purpose. *Piper nigrum, Piper longum* and *Plumbago zeylanica* are major active ingredients in the formulation. Hence, piperine from *Piper* species and plumbagin from *Plumbago zeylanica* were selected as marker compounds. [7-10]

Piperine is an alkaloid present in *Piper nigrum* and *Piper longum* family Piperaceae and is reported as anti-inflammatory, anticonvulsant and antiulcer agent. Plumbagin is a napthaquinone glycoside present in *Plumbago zeylanica* family Plumbaginaceae. There are reports on extraction of piperine and plumbagin using various extraction techniques and their individual estimation from single herbs and polyherbal formulations. However, no analytical method has been reported for simultaneous estimation of piperine and plumbagin; which can be further applied for standardization of Chitrakadi Vati.

The present research work deals with development of HPTLC method for standardization of Chitrakadi Vati by detection and quantification of markers piperine and plumbagin simultaneously from in-house and marketed formulations. The proposed method was validated on the basis of its linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and robustness according to ICH guidelines.

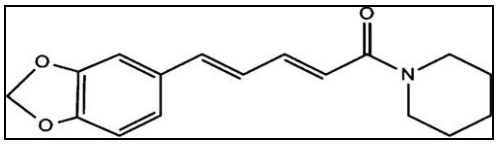

Fig. 1: Chemical structure of piperine

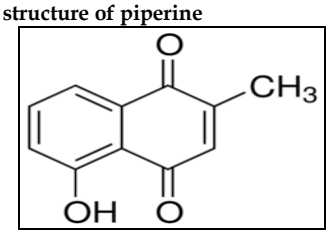

Fig. 2: Chemical structure of plumbagin

MATERIALS AND METHODS

Materials

Raw materials used for the preparation of Chitrakadi Vati and three different marketed brands (M-01, M-02, M-03) of Chitrakadi Vati were procured from Ayurvedic medical shop, Mumbai and stored in air tight containers at room temperature. The stationary phase used was precoated with silica gel 60 F_{254} (20×20 cm) TLC plates of 0.2 mm thickness obtained from E. Merck Ltd. Mumbai, India.

Standards and reagents

The organic solvents and chemicals of analytical grade were procured from S.D Fine chemicals Pvt. Ltd. Mumbai, India. Standard piperine and plumbagin were procured from Sigma Aldrich Pvt. Ltd. Mumbai, India.

Instrumentation

Camag Linomat 5 semiautomatic sample applicator equipped with a 100µl Hamilton syringe (Camag, Switzerland) and winCATS software (CAMAG Ver.1.4.1), Camag TLC Scanner 3, Twin trough chamber.

Experimental

Preliminary studies

The quality of raw materials used in the preparation of Chitrakadi Vati was assessed by determining the proximate parameters like ash value, extractive value and loss on drying using standard pharmacopoeial methods. Tablet extracts were qualitatively evaluated by chemical tests for the presence of various phytoconstituents like alkaloids, glycosides, saponins, phenolic compounds tannins and phytosterols.

HPTLC Method Development

Preparation of standard solution

Stock solutions of piperine and plumbagin (1000µg/ml) were prepared separately by dissolving 10 mg of accurately weighed standard in 10 ml of methanol. From this stock solution 100µg/ml was prepared by transferring 1 ml stock solution to 10 ml volumetric flask and volume was then adjusted with methanol.

Preparation of In-house formulation

All the ingredients were collected, dried and powdered separately, passed through 100 # sieve and then mixed together in specified proportions in a geometrical manner to get uniform mixture. To which citrus juice (*Citrus aurantium*) was added and grounded well to form a homogenous blend and compressed into tablets. The tablets were dried and packed in air tight containers for further analysis.

Extraction of piperine and plumbagin from marketed and in-house formulations

Vati equivalent to 10g were triturated and extracted with 25ml methanol, sonicated for 30 min, filtered through Whatmann filter paper no. 41 and this procedure was repeated consecutively for three times using fresh 25ml of methanol. The final volume was then made up to 100 ml with methanol. This solution was used for quantification of plumbagin. For piperine quantification 1 ml of this solution was further diluted to 10ml with methanol and was used.

Chromatographic conditions

Chromatographic separation was achieved on HPTLC plates (10×10 cm) pre-coated with silica gel 60 F_{254} of 0.2 mm thickness with aluminium sheet support. Standard solutions of markers and extracts were applied to the

plates as bands 6.0 mm wide, 10.0 mm from the bottom edge of the same 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 (24 ± 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 280 nm with a Camag TLC Scanner 3 using the deuterium lamp with winCATS software.

Optimization of Mobile phase

Mobile phase composition was optimized to provide accurate, precise and reproducible results for the determination of piperine and plumbagin. The standard stock solution containing 100µg/ml of piperine and plumbagin was spotted on to TLC plate and developed in different solvent systems. Many preliminary trials were carried out for selection of mobile phase.

Calibration curves of piperine and plumbagin

Serial dilutions were made in the concentration range of 20-70µg/ml and 20-80µg/ml for piperine and plumbagin, respectively. Aliquot of above solutions (10µl) were applied with the band width of 6 mm, in triplicate on TLC plate (10×10 cm) to obtain a concentration range of 200-700 ng/spot for piperine and 200-800 ng/spot for plumbagin. Peak area for each band was recorded. Separate calibration curves were obtained by plotting a graph of peak area vs. concentration of piperine and plumbagin.

Assay

For assay purpose standard and sample (extract) solutions were applied on HPTLC plate in triplicates. Standard solutions of piperine and plumbagin 100µg/ml were applied. Extracted solution was directly used for quantification of plumbagin. For quantification of piperine 1 ml of extracted solution was pipetted out and volume was made up to 10 ml with methanol. The amount of piperine and plumbagin present per gram of formulation was calculated by comparison of the areas measured for the sample with the calibration curves constructed from peak areas obtained from standard solutions of piperine and plumbagin.

Method validation [11]

In accordance with ICH guidelines Q2 (R1) the optimized HPTLC method was validated with respect to following parameters.

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 of analyte in the sample. It was determined by plotting a graph of peak area v/s concentration of standards to obtain correlation coefficient (r^2) and equation of the line.

Specificity

Specificity is the ability to assess the analyte in the presence of components that may be expected to be present in the sample matrix. 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 piperine and plumbagin with sample.

Precision

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the percent relative standard deviation (%RSD) for a statistically significant number of samples. As per the ICH guidelines precision should be performed at three different levels low quality control (LQC), medium quality control (MQC) and high quality control (HQC). Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed as intra-assay precision. 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)

Limit of detection (LOD) is the lowest amount of an analyte in a 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

Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals. Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g. 3 concentrations /3 replicates each of the total analytical procedure). The percent recovery was calculated by performing recovery studies in triplicates of three concentration levels viz. 80%, 100%, 120% by adding known amount of standard mixture of piperine 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. It was studied in triplicate at 300 ng/spot and 400 ng/spot by making small changes in mobile phase composition, the mobile phase saturation time and amount of mobile phase. The final results were examined by calculation of %RSD of concentration.

RESULTS AND DISCUSSION

Preliminary studies

The results of preliminary studies were given in (Table 1).

HPTLC Method Development

In situ HPTLC spectral overlain of piperine and plumbagin were taken. Isoabsorptive point was found at 280 nm and was selected as scanning wavelength (Figure 3).

Good resolution and sharp peaks with minimum tailing were obtained with mobile phase consisting of toluene: ethyl acetate: formic acid 7.5:2.5:0.5 (v/v/v). Piperine and plumbagin were satisfactorily resolved with R_f values at 0.39 ± 0.02 and 0.70 ± 0.02 respectively (Figure 4).

HPTLC Method Validation

Linearity

Linear relationship was observed by plotting drug concentration against peak area for each compound. Piperine and plumbagin showed linear response in the concentration range of 200-700 ng/spot and 200-800 ng/spot, respectively (Figure 5A and 5B). The linearity was validated by the high value of the correlation coefficients. The results are tabulated in (Table 2).

Specificity

When the spectra of standard piperine and plumbagin were overlayed or compared with extracts of Chitrakadi Vati it was observed that constituents present in the extract did not interfere with the peaks of Piperine and Plumbagin. Thus the proposed method was proved to be specific. The spectra of the standard piperine (Figure 6A) and plumbagin (Figure 6B) corresponded with extract of vati.

Table 1: Results of preliminary studies of Chitrakadi Vati

Parameters	Marketed formulation (M-01)	Marketed formulation (M-02)	Marketed formulation (M-03)	In-house formulation
Alcohol soluble extractive value (% w/w)	4.8	3.2	5.6	10.4
Water soluble extractive value (% w/w)	104	55.2	30.4	126.4
Loss on drying (% w/w)	18.2	4.4	6.6	5
Ash value (% w/w)	43	53	25	53.5

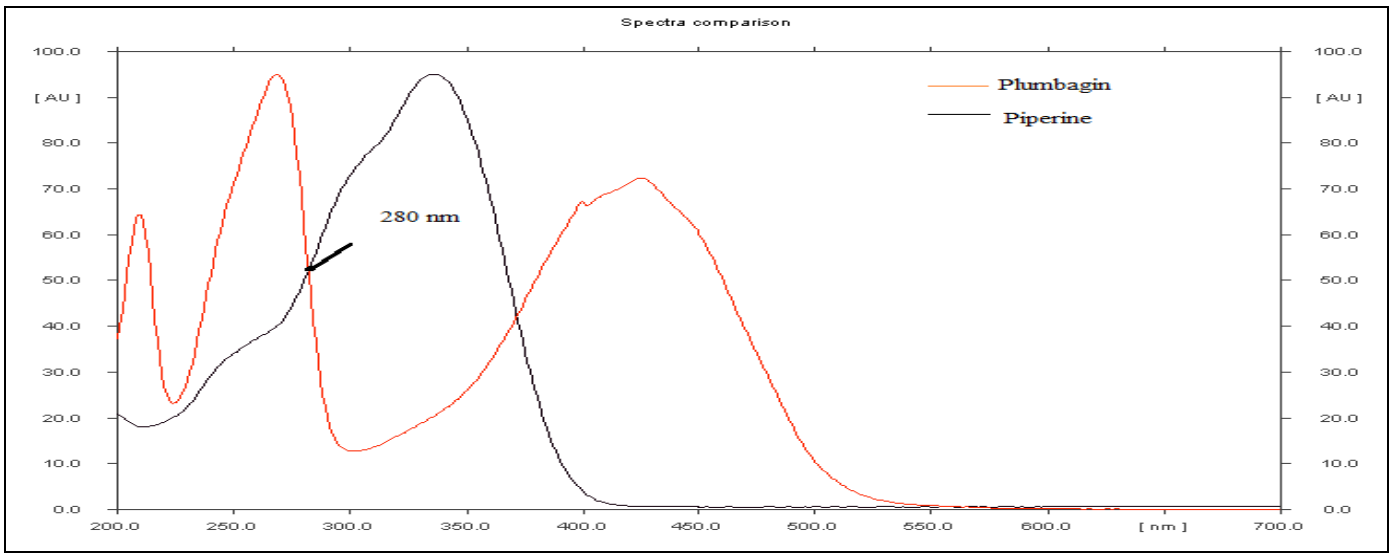

Fig. 3: HPTLC in situ overlain spectra of piperine and plumbagin

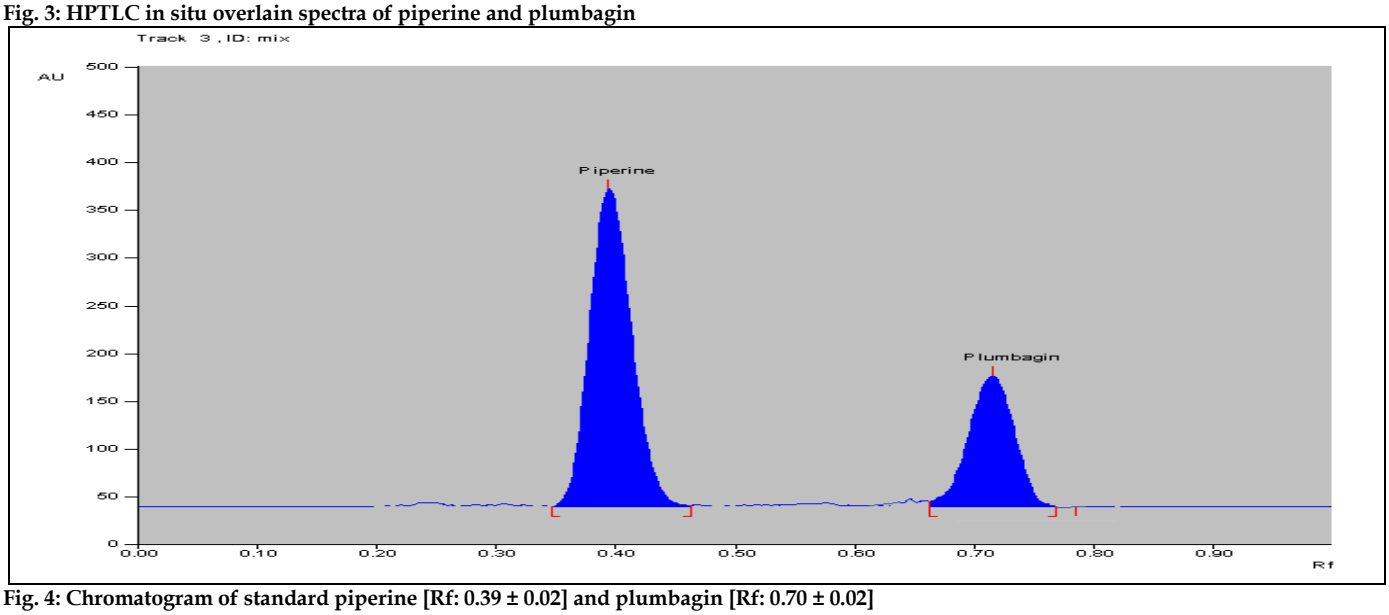

Fig. 4: Chromatogram of standard piperine [Rf: 0.39 ± 0.02] and plumbagin [Rf: 0.70 ± 0.02]

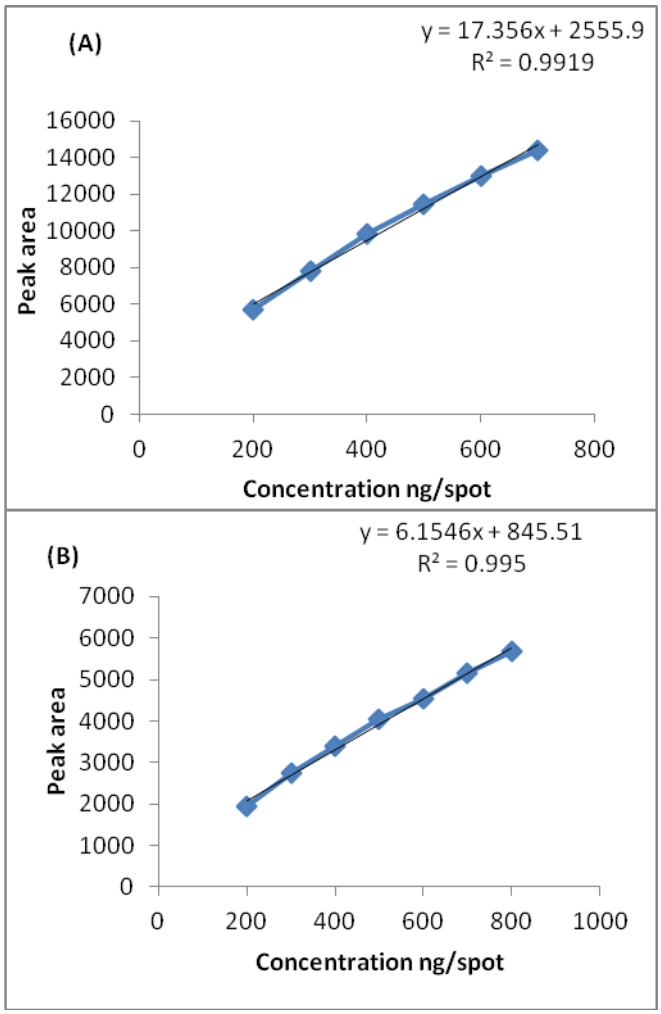

Fig. 5: Calibration curve of (A) Piperine and (B) Plumbagin

Table 2: Linear regression data for calibration plot for piperine and plumbagin

Parameters	Piperine	Plumbagin
Linearity range (ng/spot)	200-700	200-800
Equation	Y= 17.356X + 2555.9	Y= 6.1546X + 845.51
Correlation coefficient (r^2 ± SD)	0.9919 ± 0.003493	0.995 ± 0.004903
Slope ± SD	17.356 ± 0.3080	6.1546 ± 0.0918
Intercept ± SD	2555.9 ± 175.5055	845.51 ± 61.3137

SD = Standard Deviation

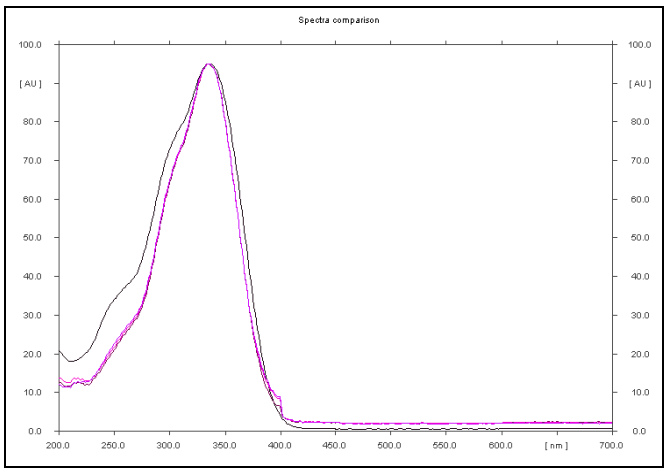

Fig. 6: (A) Overlay spectra of piperine and piperine from Vati extract

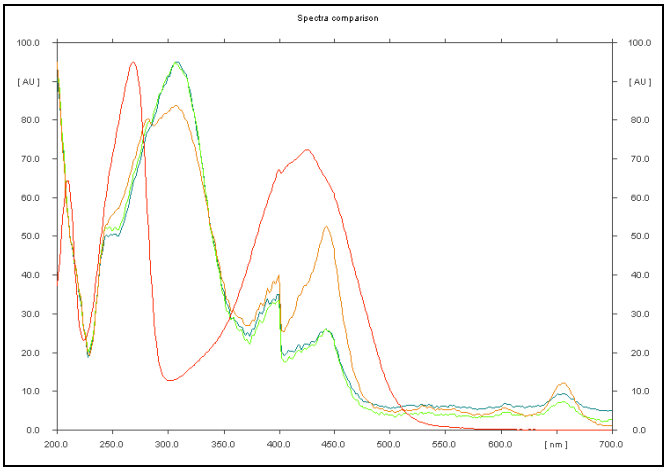

Fig. 6: (B) Overlay spectra of plumbagin and plumbagin from Vati extract

Precision

Intraday precision is used to describe the variation of the method, at three different concentration levels within the same day while interday precision is for variation between different days. The % RSD values for both intraday and interday precision were found within acceptable limit as shown in Table 3 and 4 respectively.

Limit of detection (LOD) and Limit of quantification (LOQ): The LOD and LOQ were found to be 33.39, 101.19ng/spot for piperine and 36.10, 109.4ng/spot for plumbagin respectively.

Accuracy

Accuracy of the method is reported as percent recovery of known added amount of analyte in the sample. The percent recovery was calculated by performing recovery studies in triplicates of three concentration levels viz. 80%, 100%, 120% by adding known amount of standard mixture of piperine and plumbagin. Results obtained were given in Table 5 and 6.

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. The values of % RSD were less than 2% which indicated that the developed method is robust as shown in (Table 7).

Table 3: Intraday precision results

		Piperine			Plumbagin		
Levels		**LQC**	**MQC**	**HQC**	**LQC**	**MQC**	**HQC**
Concentration (ng/spot)		**300**	**400**	**600**	**300**	**500**	**700**
Peak area	Session 1	7913.4	9919.1	13025.7	2750.067	4023.433	5130.733
	Session 2	7931.3	9925	12933.8	2768.033	4031.733	5176.500
	Session 3	7930.9	9914.3	13056.8	2771.6	4014.700	5115.800
Average peak area		7925.2	9919.5	13005.4	2763.233	4023.289	5141.011
S.D		41.09	35.04	88.56	31.466	39.223	51.582
% RSD		0.5175	0.3532	0.6809	1.138	0.974	1.003

Table 4: Interday precision results

Levels		Piperine LQC	Piperine MQC	Piperine HQC	Plumbagin LQC	Plumbagin MQC	Plumbagin HQC
Concentration (ng/spot)		300	400	600	300	500	700
Peak area	Day 1	7925.8	9912.1	12768.5	2769.733	3989.733	5162.500
	Day 2	7940.0	9940.23	12848.6	2766.233	4023.533	5204.600
	Day 3	7917.1	9891.63	12915.8	2778.267	4031.600	5133.633
Average peak area		7927.6	9914.65	12844.3	2771.411	4014.956	5166.911
S.D		27.97	48.62	190.04	28.370	36.031	48.218
%RSD		0.352	0.490	1.47	1.023	0.897	0.933

Table 5: Accuracy: recovery data for piperine

Formulations	Level of percentage recovery	Amount of marker present (ng) (A)	Amount of marker added (ng) (B)	Total amount of marker (ng) (A+B)	Amount of marker found (ng)	Recovery (%)	%RSD	Mean Recovery (%)
Marketed Formulation (M-01)	80	280	230	510	511.069	100.209	0.419	99.722
	100	280	280	560	556.233	99.327	0.838	
	120	280	340	620	617.711	99.630	0.433	
Marketed Formulation (M-02)	80	370	290	660	661.776	100.269	0.270	100.217
	100	370	370	740	749.521	101.286	0.256	
	120	370	480	850	842.320	99.0965	0.388	
Marketed Formulation (M-03)	80	340	270	610	608.416	99.740	0.446	99.287
	100	340	340	680	674.031	99.122	0.995	
	120	340	400	740	732.591	98.998	0.437	
In-house Formulation (4)	80	210	170	380	380.487	100.128	0.616	99.738
	100	210	210	420	419.149	99.797	0.630	
	120	210	250	460	456.735	99.290	0.364	

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

Table 6: Accuracy: recovery data for plumbagin

Formulations	Level of percentage recovery	Amount of marker present (ng) (A)	Amount of marker added (ng) (B)	Total amount of marker (ng) (A+B)	Amount of marker found (ng)	Recovery (%)	%RSD	Mean Recovery (%)
Marketed Formulation (M-01)	80	600	480	1081	1098.174	101.682	0.073	100.920
	100	600	600	1200	1199.911	99.992	0.968	
	120	600	720	1320	1334.344	101.086	0.089	
Marketed Formulation (M-02)	80	570	460	1030	1017.49	98.785	0.590	99.227
	100	570	570	1140	1137.51	99.781	0.321	
	120	570	690	1260	1248.855	99.115	0.003	
Marketed Formulation (M-03)	80	280	220	500	499.597	99.919	0.279	100.103
	100	280	280	560	562.352	100.420	0.148	
	120	280	340	620	619.822	99.971	0..048	
In-house Formulation (4)	80	290	230	520	518.9	99.78	0.986	99.940
	100	290	290	580	575.6	99.241	1.252	
	120	290	350	640	645.3	100.8	0.785	

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

Table 7: Robustness results for piperine and plumbagin

Parameters	Piperine % RSD		Plumbagin % RSD	
	300 ng/spot	400 ng/spot	300 ng/spot	400 ng/spot
Mobile phase composition				
Toulene: Ethyl acetate : Formic acid (7.3:2.7:0.5 v/v/v)	0.02	0.37	0.34	0.40
Toulene: Ethyl acetate : Formic acid (7.5:2.5:0.5 v/v/v)	0.03	0.78	0.67	0.94
Toulene: Ethyl acetate : Formic acid (7.7:2.3:0.5 v/v/v)	0.15	0.70	0.56	0.31
Saturation time (Minutes)				
+ 5 Min	1.01	0.80	1.54	1.26
- 5 Min	0.98	1.45	1.13	0.91
Mobile phase volume				
+ 5%	1.45	0.47	1.43	0.75
-5%	1.29	1.19	0.94	1.21

Estimation of piperine and plumbagin in marketed and In-house formulations

The developed method was applied for the detection and quantification of piperine and plumbagin from marketed and in-house formulations of Chitrakadi Vati. The peaks for piperine and plumbagin were observed at R_f 0.39 ±0.02 and 0.70 ±0.02 respectively in the densitogram of extracts. The test samples of marketed

formulations and in-house formulation were compared with the ingredients (Figure 7). There was no interference from other compounds present in the Vati. The total piperine content in different marketed and in-house formulations of Chitrakadi Vati was found to be satisfactory as shown in (Table 8).

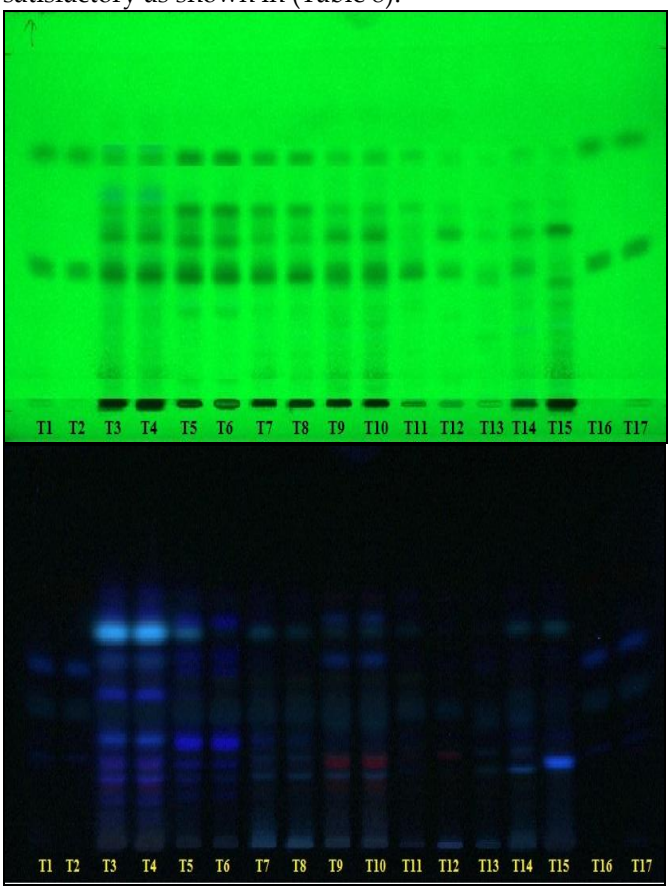

Fig. 7: HPTLC fingerprinting profile of extract of Chitrakadi Vati and its ingredients at 254 and 366 nm respectively. T_1,T_2- Standard piperine and plumbagin, T_3,T_4 –Marketed formulation (M-01), T_5,T_6 - Marketed formulation (M-02), T_7,T_8 – Marketed formulation (M-03), T_9,T_{10}- In-house Formulation, T_{11} –*Piper nigrum* extract, T_{12}- *Piper longum* fruit extract, T_{13}- *Piper longum* root extract, T_{14}- *Piper chaba* extract, T_{15}- *Plumbago zeylanica* extract, T_{16},T_{17}- Standard piperine and plumbagin.

Table 8: Piperine and Plumbagin content in polyherbal formulations

Formulation	Piperine content (% w/w)	Plumbagin content (% w/w)
Marketed Formulation (M-01)	0.280	0.06
Marketed Formulation (M-02)	0.370	0.057
Marketed Formulation (M-03)	0.340	0.028
In-house Formulation	0.210	0.029

Chitrakadi Vati contains *Piper nigrum*, *Piper longum* and *Plumbago zeylanica* as important major ingredients containing marker compounds piperine and plumbagin. These marker compounds are responsible for the therapeutic activity. The HPTLC method was developed for standardization of Chitrakadi Vati using piperine and plumbagin as biologically active chemical markers. HPTLC method was found to be rapid, simple, linear, precise and accurate for quantitative estimation of piperine and plumbagin from Chitrakadi Vati. The proposed method was found to be useful, to evaluate various formulations available in the market containing both the drugs and also useful for determining the adulteration or substitution encountered in commercial market.

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