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HPTLC METHOD DEVELOPMENT AND VALIDATION FOR STANDARDIZATION OF AYURVEDIC FORMULATION: MAHASHANKH VATI

Vineeta Khanvilkar * and Nishigandha Chalak

Bharati Vidyapeeth's College of Pharmacy, C.B.D Belapur, Navi Mumbai - 400614, Maharashtra, India

Keywords:

Piperine, Umbelliferone, Gallic acid, Standardization, HPTLC, Mahashankhvati

Correspondence to Author:
Vineeta V. Khanvilkar

Associate Professor
Department of Quality Assurance
Bharati Vidyapeeth's College of Pharmacy, Sector 8, C.B.D. Belapur, Navi Mumbai - 400 614, India

Email: trushali.k@gmail.com

ABSTRACT: Ayurveda is the primeval complete serving system in medical field. However, one of the barriers in the acceptance of the Ayurvedic formulation is the paucity of standard quality control outline. World health organization (WHO) in 1999 has given a detail procedure for the standardization of herbal drugs comprising of a single content but not for standardization of polyherbal formulations. Mahashankhvati is official in Ayurvedic Formulary of India and is prescribed for treatment of haemorrhoids, malabsorption syndrome, dyspepsia and indigestion. In the proposed work, attempt has been made for standardization of Mahashankh Vati by developing chromatographic method. Piperine from *Piper longum* and *Piper nigrum*, Umbelliferone from *Ferula asafoetida* and Gallic acid from *Terminaliachebula* present in formulation were selected as marker compounds. A new, rapid, simple, precise, selective HPTLC method was developed for marketed preparation of Mahashankhvati. The separation was performed on TLC aluminium plates precoated with silica gel 60 F_{254}, using toluene: ethyl acetate: methanol: formic acid (7:2:2.5:0.5 v/v/v/v) as mobile phase. The densitometric analysis was carried out at the detection wavelength of 290 nm. The R_f values of piperine, umbelliferone and gallic acid was found to be 0.65, 0.52 and 0.32 respectively.The developed method has been validated as per ICH guidelines.

INTRODUCTION: Being resurrecting of interest in natural drugs, especially plants derived, started in the last few decades mainly because of widespread belief that green medicines are healthier and safer than the synthetic once.[1] Standardization of herbal materials and their formulations is essential in order to assess quality of the drugs.

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The quality assessment of herbal formulations is most important in order to justify their acceptability in modern system of medicine. [2] One of the major problems faced by the herbal industry is the deficit of rigid quality control profiles for herbal materials and their formulations.

The World Health Organization (WHO) has appreciated the importance of medicinal plants for public health care in developing nations and has evolved guidelines to support the member states in their efforts to formulate national policies on traditional medicine and to study their potential usefulness including evaluation, safety and efficacy.[3] Mahashankh Vati is official in Ayurvedic formulary of India. It is a polyherbal formulation;

which is consists of ten ingredients of plant origin chitraka (*Plumbagozeylanica*), maricha (*Piper nigrum*), pippali (*Piper longum* fruit), pippalimoola (*Piper longum* root), ginger (*Zingiber officinale*), hing (*Ferula asafoetida*), dantimool (*Baliosper mummontanum*), hard a (*Terminalia chebula),* Chincha (*Tamarindus indica*), ajwain (*Trachy spermumammi*) shankh bhasma and panchalavana (vida, Sauvarchala, samudra, audbhida, Saindhava). It is widely used for the treatment of irritable bowel syndrome, rheumatoid arthritis and loss of appetite.[4]

Literature and market survey states that the above formulation available in market is product of numerous companies. Modern analytical methods are not yet reported for standardization of Mahashankh Vati. As it is difficult to estimate each and every ingredient for its chemical constituents, piperine (**Fig.1)** from *Piper longum* and *Piper nigrum*; umbelliferone (**Fig.2**) from *Ferula asafoetida* and gallic acid (**Fig.3**) from *Terminalia chebula* present in formulation were selected as marker compounds. Literature survey reveals that few HPTLC, RP-HPLC and UV methods are reported for estimation of piperine [5, 6, 7, 8], umbelliferone [9, 10] and gallic acid [11] individually as well as in combination with other constituents. However, no analytical method has been reported for simultaneous estimation of piperine, umbelliferone and gallic acid; which can be further applied for standardization of Mahashankh Vati.

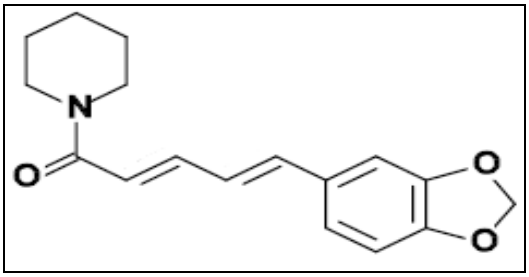

FIG.1: STRUCTURE OF PIPERINE

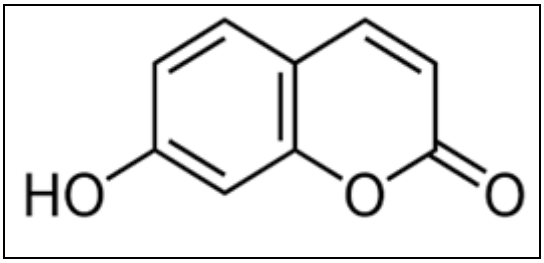

FIG. 2: STRUCTURE OF UMBELLIFERONE

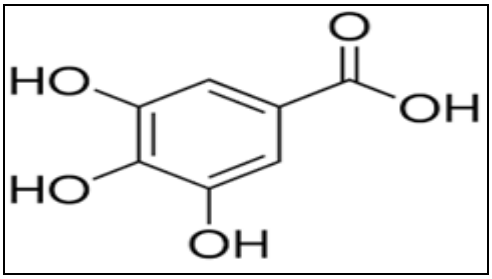

FIG. 3: STRUCTURE OF GALLIC ACID.

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

MATERIALS AND METHODS:

Materials:

Raw materials used for the preparation of MahashankhVati and two different marketed brands (M1, M2) of Mahashankh Vati were procured from Ayurvedic medical shop, Mumbai and stored in air tight containers at room temperature. The stationary phase used was TLC plates precoated with silica gel 60 F_{254} (20×20 cm) 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, umbelliferone and gallic acid 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.

Method:

Preparation of standard solution:

Stock solutions of piperine, umbelliferone and gallic acid (1000μg/ml) were prepared separately

by dissolving 10 mg of accurately weighed standard in 10 ml of methanol. From this stock solution, 100μg/ml solution was prepared by transferring 1 ml stock solution to 10 ml volumetric flask then volume 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 this mixture 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, umbelliferone and gallic acid from marketed and in-house formulations:
Vati equivalent to 5g were triturated and extracted with 25ml methanol, reflux 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 piperine, umbelliferone and gallic acid.

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 using 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 290 nm with a Camag TLC Scanner 3 using the deuterium lamp with win CATS software.

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

Assay:
For assay purpose standard and sample (extract) solutions were applied on TLC plate in triplicates. Standard solutions of piperine, umbelliferone and gallic acid 100μg/ml were applied. Calibration curves constructed from peak areas obtained from standard solutions of piperine, umbelliferone and gallic acid. Sample (extract) solution was used for quantification of markers. The amount of piperine, umbelliferone and gallic acid present per gram of formulation was calculated by comparison of the areas measured for the sample with the calibration curves.

Method validation: [12]
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, umbelliferone and gallic acid 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 was 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, umbelliferone and gallic acid. 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 and the mobile phase saturation time. The final results were examined by calculation of %RSD of concentration.

RESULTS AND DISCUSSION:

In situ HPTLC spectral overlain of piperine, umbelliferone and gallic acid were taken. Isoabsorptive point was found at 290 nm and was selected as scanning wavelength **(Fig. 4).**

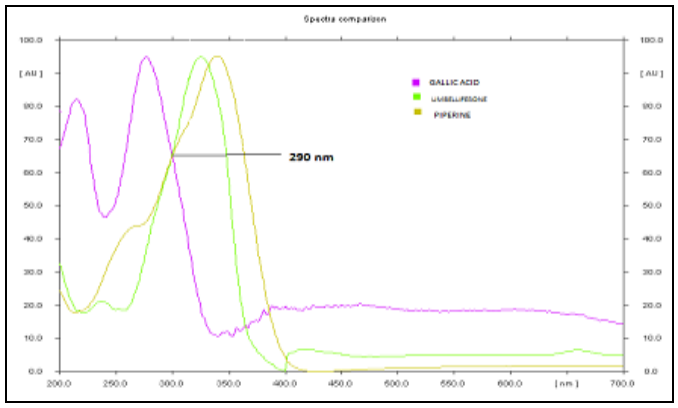

FIG. 4: HPTLC IN SITU OVERLAIN SPECTRA OF PIPERINE, UMBELLIFERONE AND GALLIC ACID.

Good resolution and sharp peaks with minimum tailing were obtained with mobile phase consist of toluene: ethyl acetate: methanol: formic acid 7:2:2.5:0.5 (v/v/v/v). Piperine, umbelliferone and gallic acid were satisfactorily resolved with R_f values at 0.65 ± 0.02, 0.52 ± 0.02 and 0.32 ± 0.02 respectively (**Fig. 5).**

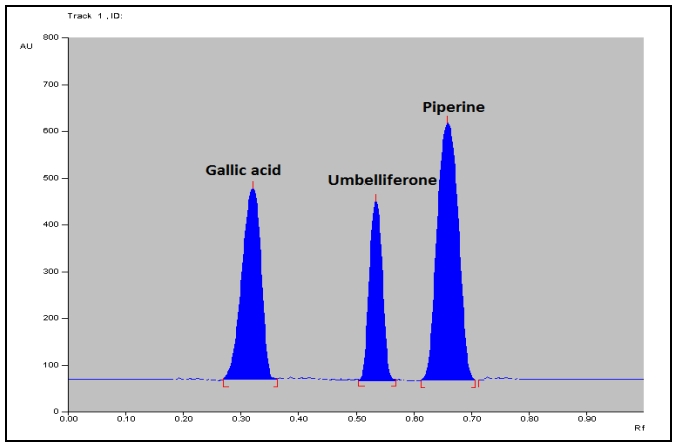

FIG. 5: CHROMATOGRAM OF STANDARD PIPERINE [R_f: 0.65 ± 0.02], UMBELLIFERONE [R_f: 0.52 ± 0.02] AND GALLIC ACID [R_f: 0.32 ± 0.02]

HPTLC Method Validation:

Linearity:

Linear relationship was observed by plotting drug concentration against peak area for each compound. Piperine, umbelliferone and gallic acid showed linear response in the concentration range of 200-800 ng/spot, 200-900 ng/spot and 300-900 ng/spot, respectively **(Fig.6A, 6B** and **6C)**. The linearity was validated by the high value of the correlation coefficients. The results are tabulated in (**Table 1).**

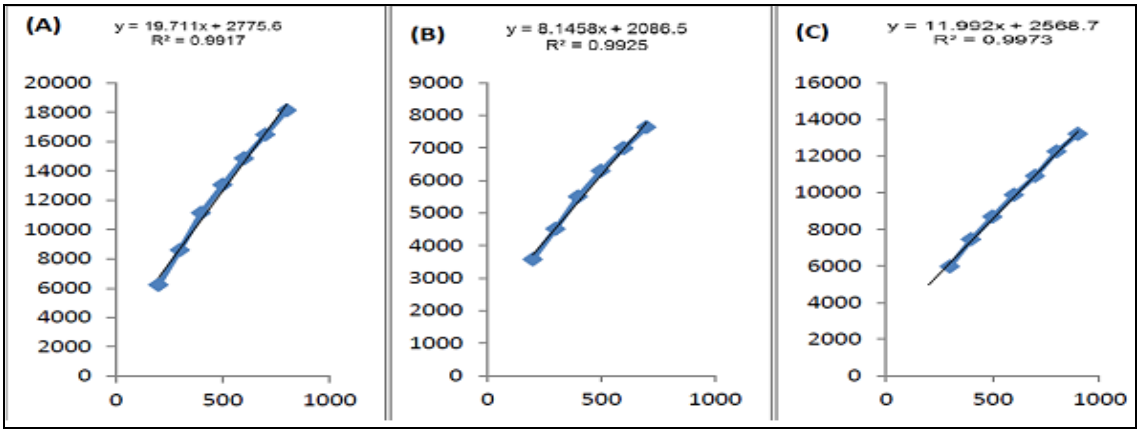

FIG.6: CALIBRATION CURVE OF (A) PIPERINE, (B) UMBELLIFERONE, (C) GALLIC ACID.

TABLE 1: LINEAR REGRESSION DATA FOR CALIBRATION PLOT FOR PIPERINE, UMBELLIFERONE AND GALLIC ACID

Parameters	Piperine	Umbelliferone	Gallic Acid
Linearity (*ng*/spot)	200-800	200-700	300-900
Equation	y=19.71x+2775	y=8.15x+2086	y=11.99x+2568
Correlation coefficient (r^2 ± SD)	0.9917 ± 0.003493	0.9925 ± 0.004903	0.9973 ± 0.002601
Slope ± SD	19.71 ± 0.3080	8.15 ± 0.0918	11.99 ± 0.004002
Intercept ± SD	2775 ± 175.5055	2086± 61.3137	2568 ± 0.006152
	SD = Standard Deviation		

Specificity:

When the spectra of standard piperine, umbelliferone and gallic acid were overlayed **(Fig. 7A, 7B and 7C)** or compared with extracts of Mahashankh Vati it was observed that constituents present in the extract did not interfere with the peaks of piperine, umbelliferone and gallic acid. Thus the proposed method was proved to be specific.

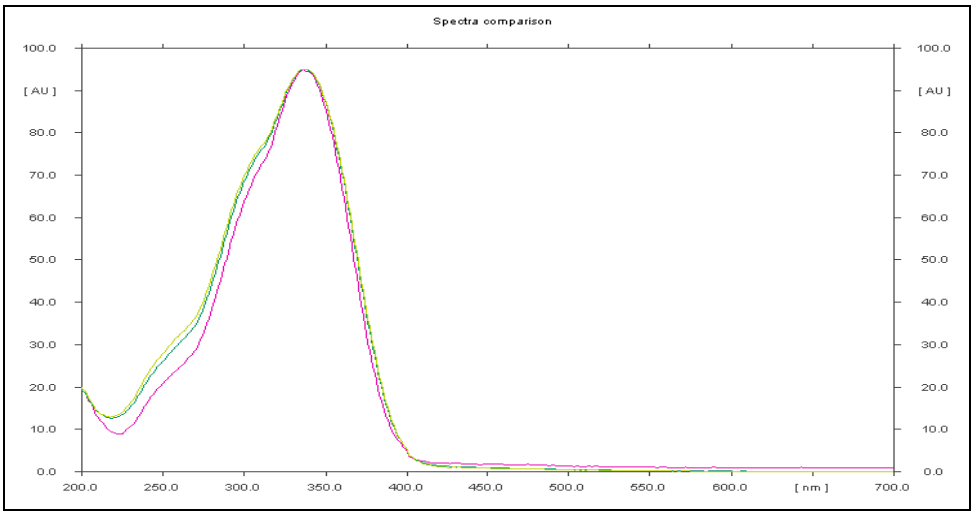

FIG.7A: OVERLAY SPECTRA OF STANDARD PIPERINE AND PIPERINE FROM EXTRACT.

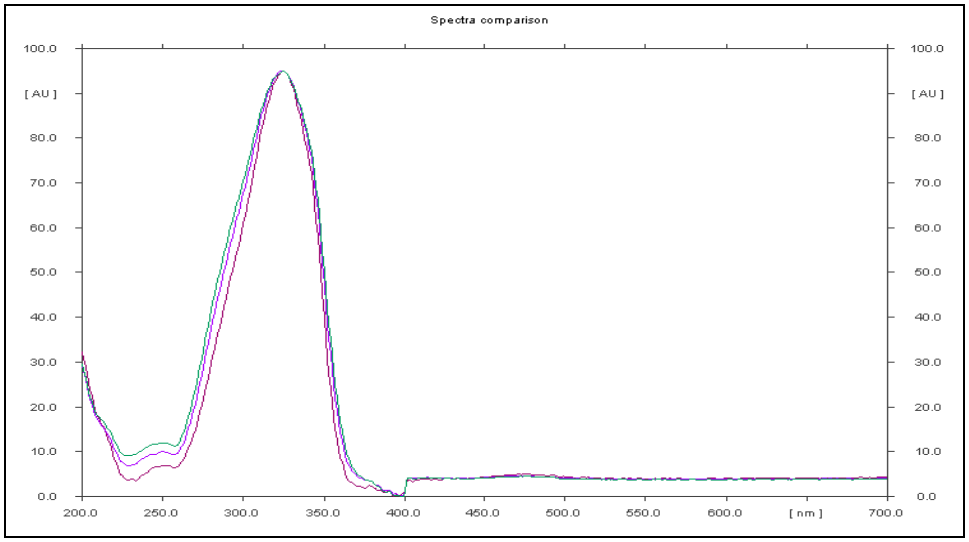

FIG.7B: OVERLAY SPECTRA OF STANDARD UMBELLIFERONE AND UMBELLIFERONE FROM VATI EXTRACT.

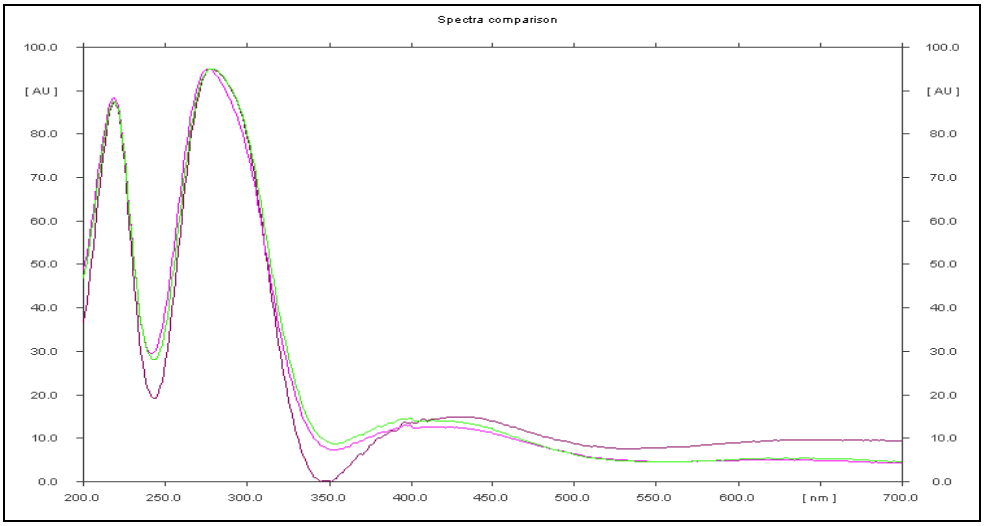

FIG. 7C: OVERLAY SPECTRA OF STANDARD GALLIC ACID AND GALLIC ACID FROM VATI EXTRACT.

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 2**.

TABLE 2: INTRA-DAY AND INTER-DAY PRECISION RESULTS OF PIPERINE, UMBELLIFERONE AND GALLIC ACID.

	Concentration (ng/spot)	Interday			Intraday		
		Mean Area	S.D.	%RSD	Mean Area	S.D.	%RSD
Piperine	300	8650.3	61.89	0.53	8610.37	98.79	0.71
	500	13039.3	250.79	1.24	13033.9	117.6	1.92
	700	16083.2	31.79	1.86	16093.2	55.43	1.44
Umbelliferone	300	4382.8	80.83	1.50	4429.97	79.99	1.82
	500	6261.3	39.10	1.76	6250.42	88.93	1.96
	600	7019.52	105.4	0.71	7079.39	134.88	0.67
Gallic Acid	400	5446.5	55.32	0.45	6446.57	35.33	0.84
	600	10065.5	67.99	0.88	10016.5	8.01	0.55
	800	13244.4	43.89	1.38	12254.1	46.61	0.49

Limit of detection (LOD) and Limit of quantification (LOQ): LOD and LOQ results of piperine, umbelliferone and gallic acid are as shown in **Table 3.**

TABLE 3: LOD AND LOQ RESULTS OF PIPERINE, UMBELLIFERONE AND GALLIC ACID.

	LOD (ng/spot)	LOQ (ng/spot)
Piperine	19.97	60.52
Umbelliferone	31.24	94.68
Gallic Acid	37.82	114.61

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, umbelliferone and gallic acid. Results obtained were given in **Table.4, 5, 6.**

TABLE 4: ACCURACY DATA FOR PIPERINE.

Compound	Level of % Recovery	Amount added (ng)	Measured amount (ng)	Recovery (%)	RSD (%)	Mean Recovery (%)
Marketed	80	1370	1354.7	98.916	1.26	99.40
Formulation	100	1520	1525.2	103.6	0.94	
(M1)	120	1670	1666.8	99.47	1.39	
Marketed	80	1300	1298.1	99.80	0.27	99.16
Formulation	100	1440	1426.5	98.67	0.25	
(M2)	120	1590	1588.2	99.12	0.38	
In-house	80	1340	1338.9	99.15	0.61	98.84
Formulation	100	1480	1466.81	98.63	0.52	
	120	1630	1610.6	98.74	0.68	

TABLE 5: ACCURACY DATA FOR UMBELLIFERONE.

Compound	Level of %recovery	Amount added(ng)	Measured amount(ng)	Recovery (%)	RSD (%)	Mean Recovery (%)
Marketed	80	666	657.3	98.10	0.44	98.87
Formulation	100	740	731.6	98.75	0.99	
(M1)	120	810	808.1	99.75	0.43	
Marketed	80	580	569.9	98.25	0.61	99.15
Formulation	100	640	636.2	99.3	0.63	
(M2)	120	700	678.8	99.82	0.36	
In-house	80	660	653.3	98.98	0.63	99.02
Formulation	100	700	688.8	98.4	0.92	
	120	770	768.1	99.78	0.53	

TABLE 6: ACCURACY DATA FOR GALLIC ACID.

Compound	Level of % recovery	Amount added (ng)	Measured amount (ng)	Recovery (%)	RSD (%)	Mean Recovery (%)
Marketed	80	130	128.99	99.10	0.82	99.45
Formulation	100	140	138.94	99.03	1.83	
(M1)	120	160	160.64	100.41	0.62	
Marketed	80	120	135	136.69	0.29	99.79
Formulation	100	130	150	149.88	0.49	
(M2)	120	150	165	161.73	0.87	
In-house	80	140	139.41	99.59	0.61	99.39
Formulation	100	160	159.61	99.75	0.52	
	120	180	177.94	98.84	0.68	

Robustness:

The % RSD of the peak area was calculated in triplicate for changes in mobile phase composition and duration of saturation time 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 7: ROBUSTNESS RESULTS OF PIPERINE, UMBELLIFERONE AND GALLIC ACID.

Parameters	Piperine		Umbelliferone		gallic acid	
	300ng/spot %RSD	400ng/spot %RSD	300ng/spot %RSD	400ng/spot %RSD	300ng/spot %RSD	400ng/spot %RSD
Mobile phase composition						
toluene: ethyl acetate: methanol: formic acid 6.8:2:2.5:0.5 (v/v/v/v)	0.89	0.22	0.93	1.72	0.34	1.53
toluene: ethyl acetate: methanol: formic acid 7:3:1.5:0.5 (v/v/v/v)	0.92	1.55	0.63	0.85	1.23	0.67
Saturation time						
+ 5 min	0.33	1.33	0.18	0.27	0.5	0.37
- 5 min	0.46	0.72	0.55	1.51	0.98	0.25

Estimation of piperine, umbelliferone and gallic acid in marketed and In-house formulations:

The developed method was applied for the detection and quantification of piperine, umbelliferone and gallic acid from marketed and in-house formulations of Mahashankh Vati. The peaks for piperine, umbelliferone and gallic acid were observed at R_f0.65 ±0.02, 0.52 ±0.02 and 0.32 ±0.02 respectively in the densitogram of extracts. The test samples of marketed formulations and in-house formulation were compared with the ingredients **Fig. 8.**

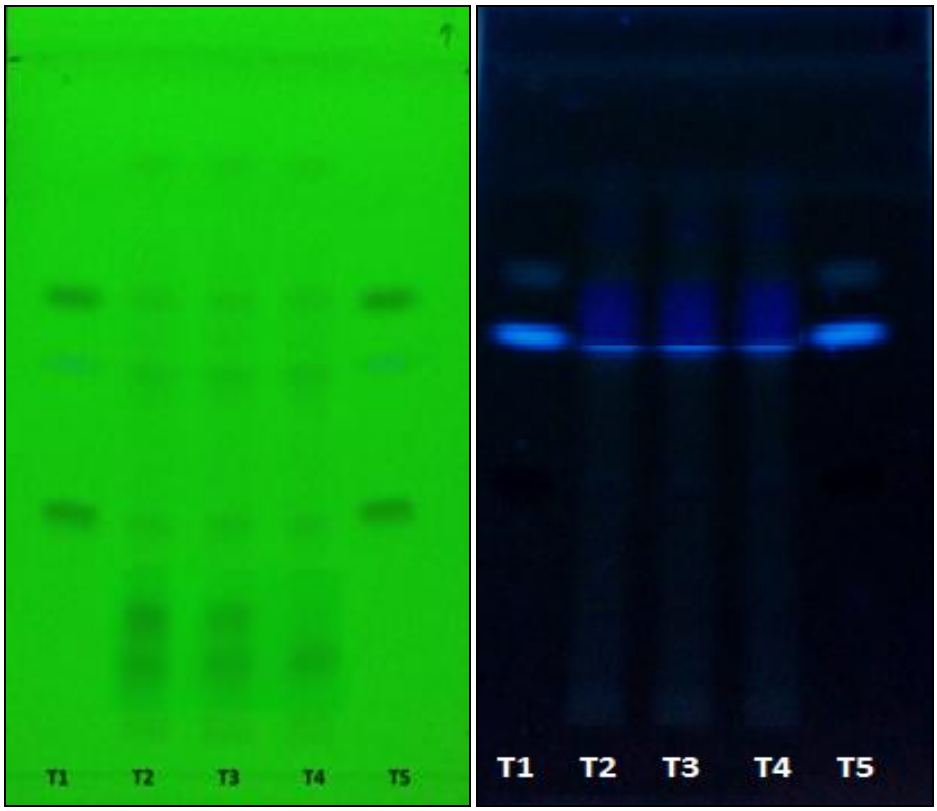

FIG. 8: HPTLC FINGERPRINTING PROFILE OF EXTRACT OF MAHASHANKH VATI AND ITS INGREDIENTS AT 254 nm AND 366 nm RESPECTIVELY.T1, T5-STANDARD PIPERINE, UMBELLIFERONE AND GALLIC ACID; T2-MARKETED FORMULATION (M1); T3-MARKETED FORMULATION (M2); T4-INHOUSE FORMULATION.

There was no interference from other compounds present in the Vati. The total content of piperine, umbelliferone and gallic acid in marketed formulations M1, M2 and in-house formulation is as shown in **Table 8**.

TABLE 8: PIPERINE, UMBELLIFERONE AND GALLIC ACID CONTENT IN POLYHERBAL FORMULATIONS.

Formulation	Piperine content (% w/w)	umbelliferone content (% w/w)	gallic acid content (% w/w)
Marketed Formulation (M1)	0.20	0.079	0.015
Marketed Formulation (M2)	0.15	0.067	0.018
In-house Formulation	0.18	0.071	0.02

CONCLUSION: The HPTLC method was developed for standardization of Mahashankh Vati using piperine, umbelliferone and gallic acid as marker constituents. The HPTLC method was found to be simple, precise, accurate, specific and reproducible for standardization of Mahashankh Vati. The method based on simultaneous estimation of piperine, umbelliferone and gallic acid 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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CONFLICT OF INTEREST: The authors do not have any conflict of interest.

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