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Original Article

DEVELOPMENT AND VALIDATION OF A RP-HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF CURCUMIN, PIPERINE AND CAMPHOR IN AN AYURVEDIC FORMULATION

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ABSTRACT

Objective: To develop a novel, accurate, precise and linear reverse phase high performance liquid chromatographic (RP-HPLC) method for simultaneous qualitative and quantitative estimation of curcumin, piperine and camphor in an ayurvedic formulation and validate as per international conference on harmonization (ICH) guidelines.

Methods: In the present work, good chromatographic separation was achieved isocratically using a shim-pack HPLC C18 column (4.6 x 250 mm, 5µm) and mobile phase consisting of 0.02 M potassium dihydrogen orthophosphate buffer (pH adjusted to 3.5 with orthophosphoric acid) and acetonitrile in the ratio 40:60, at flow rate of 1 ml/min and column temperature maintained at 35 °C. The effluents obtained were monitored at 255 nm with UV-visible detector.

Results: The retention time of curcumin, piperine and camphor was found to be 6.57 min, 7.32 min and 8.57 min respectively. Linearity of curcumin and camphor were found in the range of 4-8 ppm and that of piperine was found to be 5-9 ppm. The correlation coefficient for curcumin, piperine and camphor were 0.998, 0.99 and 0.994 respectively. The high recovery values (98 %-102 %) indicate a satisfactory accuracy. The low percent relative standard deviation (% RSD) values in the precision study reveals that the method is precise.

Conclusion: The developed method is novel, simple, precise, rapid, accurate and reproducible for simultaneous quantitative estimation of curcumin, piperine and camphor in an ayurvedic formulation. Hence the developed method can be used for quantitative analysis and quality control of extracts and commercial samples of other species containing these three markers.

Keywords: Curcumin, Piperine, Camphor, Ayurvedic formulation, RP-HPLC, Validation, ICH

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INTRODUCTION

Standardization and analysis of chemical markers in an ayurvedic or poly herbal formulations is always a difficult task. Quantitative determination of chemical markers of each ingredient in any poly herbal preparation required optimal separation techniques by which these markers are separated with the highest resolution and least interferences from each other [1]. Herbal medicine has been enjoying renaissance among the customers throughout the world. However, one of the impediments in the acceptance of the ayurvedic medicines is the lack of standard quality control profiles. The quality of herbal medicine, i.e. the profile of the constituents in the final product, has implication in efficacy and safety. Due to the complex nature and inherent variability of the chemical constituents of plant-based drugs, it is difficult to establish quality control parameters. Modern analytical techniques are increasing to overcome these problems [2]. Separation, identification and determination of chemical components are very difficult for such polyherbal formulations. The advances in chromatographic separation techniques made it possible to quantify the chemical constituents in a mixture with comparatively little clean-up [3]. Particularly, methods using high performance liquid chromatography (HPLC) with reversed phase columns are most commonly applied for the analysis of multiple constituents present in medicinal plants and herbal preparations.

In the present study we have selected an ayurvedic dental powder, which is used to maintain oral hygiene. It is indicated for various dental problems and makes teeth and gums stronger. The selected ayurvedic formulation contains *curcuma longa* (zingiberaceae), *piper longum* (piperaceae), *cinnamomum camphora* (lauraceae) and other crude drugs. Three chemical makers were selected for quantification, and one was from each medicinal herb used as raw materials, curcumin from *C. longa*, piperine from *P. longum* and camphor from *C. camphora*. These markers are responsible for the physiological action of the respective plants.

The literature survey reveals that various analytical methods for estimation of curcumin, piperine and camphor were reported alone and in combination with other drugs [4-10] but to the best of our knowledge, there is no such reported HPLC analysis method for simultaneous estimation of curcumin, piperine and camphor.

In the present investigation, we have developed a simple, optimized and validated HPLC method for the standardization of an ayurvedic formulation using three chemical markers namely curcumin, piperine and camphor. The method was validated as per the international conference on harmonization (ICH) guidelines. This novel validated method has applicability in industry as well as in academia.

MATERIALS AND METHODS

HPLC grade curcumin, piperine and camphor (purity 99%) were procured as gift sample from Yucca Enterprises, Mumbai, India. An ayurvedic preparation Patanjali divya dant manjan used for analysis was procured from local market. HPLC grade solvents were purchased from Thomas Baker. RP-HPLC shimadzu (LC 2030) model with "Lab Solution" software was employed in this method. Analytical column used for the separation of analytes was shim-pack HPLC C18 (250 X 4.6 mm, 5 µm).

Methods

Selection of wavelength

Suitable wavelength for the HPLC analysis was determined by recording UV spectrums in the range of 200-400 nm for individual

drug solutions of curcumin, piperine and camphor then overlapped. UV overlain spectra of these three markers showed that the drugs absorb appreciably at 255 nm and hence 255 nm was taken as a detection wavelength for HPLC analysis (fig. 1).

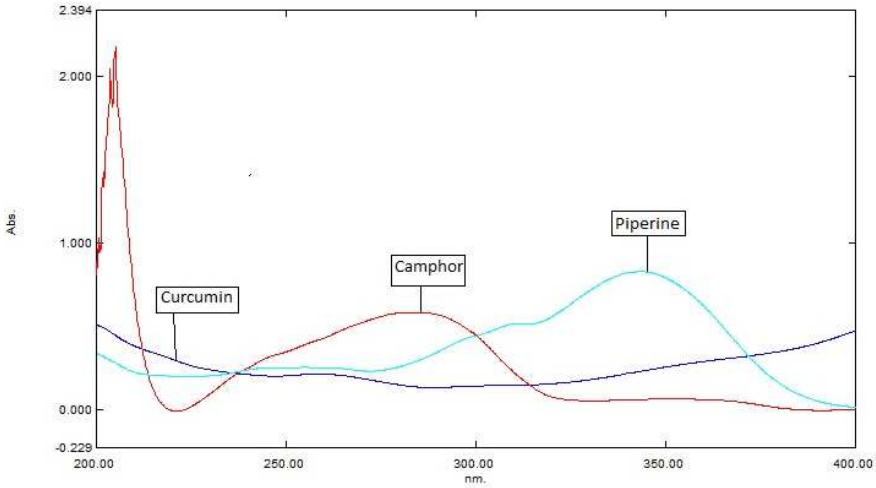

Fig. 1: UV overlap spectrum of curcumin, piperine and camphor

Chromatographic conditions

The method was developed using reverse phase, shim-pack HPLC C18 column (250 X 4.6 mm, 5 µm). The run time was of 10 min. The mobile phase used was 0.02 M potassium dihydrogen orthophosphate buffer (pH adjusted to 3.5 with orthophosphoric acid) and acetonitrile in the ratio 40:60 at a flow rate of 1.0 ml/min, column temperature maintained at 35 °C and a detection wavelength of 255 nm using a UV-visible detector.

Preparation of 0.02 M phosphate buffer (pH 3.5)

About 3.48 g of potassium dihydrogen orthophosphate was accurately weighed and dissolved in 950 ml of water. The pH was adjusted to 3.5 with orthophosphoric acid and the volume was made up to 1000 ml in volumetric flask. The solution was then filtered using 0.45 µ membrane filter.

Preparation of standard solution

100 mg of curcumin, piperine and camphor standard were accurately weighed and transferred into 100 ml volumetric flask respectively. About 70 ml solvent was added, sonicated to dissolve and diluted up to the mark using solvent (1000 ppm). Final concentration of curcumin, piperine and camphor were made to 6 ppm, 7 ppm and 6 ppm respectively by suitable dilutions.

Sample preparation

Accurately about 500 mg of dental powder was extracted with 100 ml methanol. The sample solution was filtered to obtain a clear solution. The stock solution after suitable dilutions was used for further analysis.

RESULTS AND DISCUSSION

Method development

A reverse phase HPLC method was developed keeping in mind the system suitability parameters, i.e. resolution factor between peaks, tailing factor, number of theoretical plates, runtime and the cost effectiveness. The developed optimized method resulted in the elution of curcumin at 6.57 min, piperine at 7.32 min and camphor at 8.57 min. Fig. 2,3 and 4 represent chromatograms of curcumin, piperine and camphor standard solution respectively. The total run time was 10 min. System suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Retention time, number of theoretical plates, peak resolution and peak tailing factor were evaluated for six replicate injections of the standard working concentration. The results given in table 1 were within the acceptable limits [11].

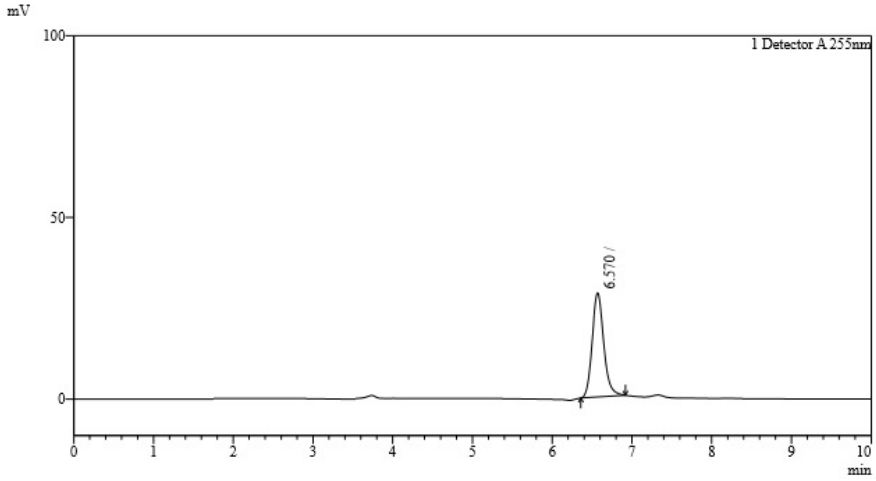

Fig. 2: Typical chromatogram of curcumin standard solution

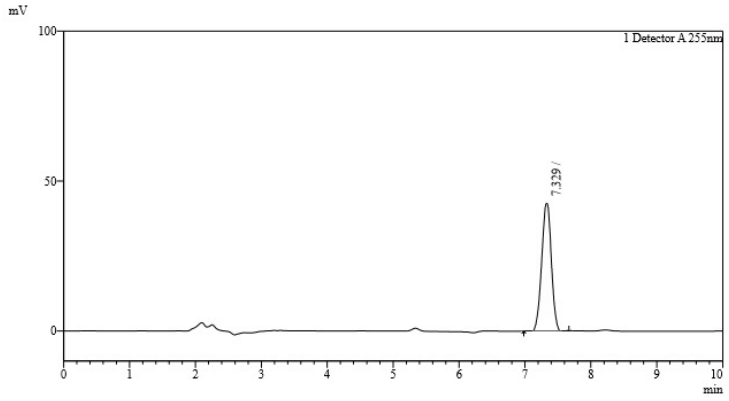

Fig. 3: Typical chromatogram of piperine standard solution

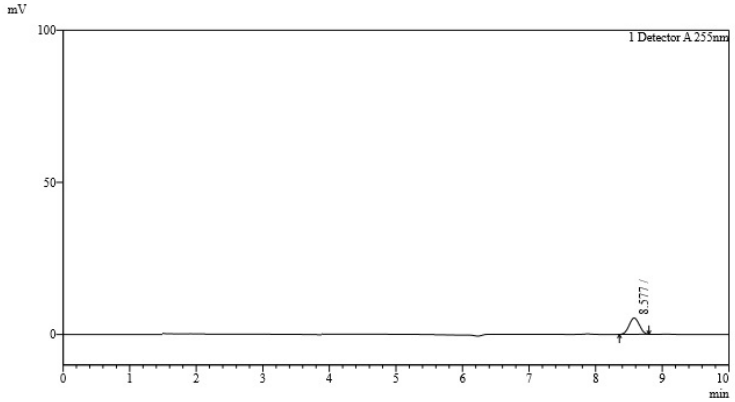

Fig. 4: Typical chromatogram of camphor standard solution

Table 1: Results of system suitability studies

Parameters	Acceptance limits	Curcumin	Piperine	Camphor
Retention time (min)	-	6.57	7.32	8.57
Resolution factor	Not less than 2	-	5.82	7.50
Number of theoretical plate	Not less than 2000	9472	8495	6654
Tailing factor	Not more than 2	1.17	0.984	1.03

In order to test the applicability of the developed method to an ayurvedic formulation, dental powder extract was chromatographed and it is shown in fig. 5. The sample peaks were identified by comparing the relative retention times with standard markers (fig. 2-4). System suitability parameters were within the acceptable limits, ideal for the chromatographed sample. Integration of the separated peak area was done and each marker concentration was determined by using the peak area concentration relationship obtained in the standardization step. For the analysis of sample, extract of 500 ppm of dental powder was injected in triplicate and quantified for three active markers using linear regression equation. The results of dental powder extract analysis are reported in table 2.

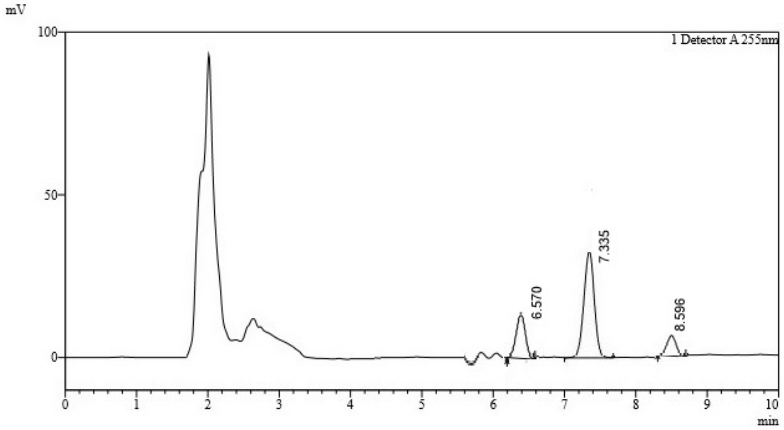

Fig. 5: Typical chromatogram of marketed ayurvedic formulation

Table 2: Analysis of dental powder extract

Formulation	Marker	Amount found (ppm) n=3	Content (%)
Dental powder extract	Curcumin	4.675	0.935
(500 ppm)	Piperine	5.693	1.138
	Camphor	4.261	0.852

#n: number of injections

Method validation

Validation of the analytical method is the process that establishes by laboratory studies in which the performance characteristics of the method meet the requirements for the intended analytical application. The developed HPLC method was validated according to ICH guidelines [12] for validation of analytical procedures. The method was validated for the parameters like linearity, accuracy, system precision, method precision, robustness, limit of detection and limit of quantitation.

Specificity

Fig. 2-5 for standard drug solutions and sample chromatogram reveals that the peaks obtained in the standard solutions and sample solution at working concentrations are only because of the drugs as blank has no peak at the retention time of curcumin, piperine and camphor. Accordingly, it can be concluded that the method developed is said to be specific [13, 14].

Precision

System precision

Six replicate injections of the standard solutions at working concentration showed percent relative standard deviation (% RSD) less than 2 concerning peak area for each marker, which indicates the acceptable reproducibility and thereby the precision of the system [15, 16]. System precision results are tabulated in table 3.

Method precision

Method precision was determined by performing the analysis of the sample under the test of repeatability at working concentration. Six injections of the sample from the same homogeneous mixture at working concentration showed % RSD less than 2 concerning content of three markers indicate that the method developed is precise by the test of repeatability [15,16] and hence can be understood that the method gives consistently reproducible results (table 4).

Table 3: System precision results

S. No.	Peak area of curcumin (6 ppm)	Peak area of piperine (7 ppm)	Peak area of camphor (6 ppm)
1	290208	415799	57236
2	299752	417624	57843
3	289557	416542	56874
4	297654	416547	57632
5	287654	426457	57438
6	296746	417542	56421
Average	293595	418419	57241
SD	5047	3998	522
%RSD	1.72	0.96	0.91

SD: Standard deviation, # %RSD: Percent relative standard deviation

Table 4: Method precision results

Marker	Intra-day		Inter-day	
	Content (ppm) n=3	% RSD	Content (ppm) n=3	%RSD
Curcumin	4.673	0.21	4.676	0.08
Piperine	5.688	0.22	5.649	0.69
Camphor	4.258	0.16	4.259	0.03

n: number of injections, # %RSD: Percent relative standard deviation

Linearity

Standard solutions of curcumin, piperine and camphor at different concentration level were prepared in triplicates. Calibration curves were constructed by plotting the concentration level versus corresponding peak areas for each marker. The results show an excellent correlation between peak areas and concentrations level within the tested concentration range of 5-9 ppm for piperine and that of 4-8 ppm for curcumin and camphor (table 5). The correlation coefficients were greater than 0.99 for each marker, which meet the method validation acceptance criteria [15, 16] and hence the method is said to be linear (fig. 6-8).

Table 5: Data for linearity studies

Marker	Concentration range (ppm)	Regression equation	R^2
Curcumin	4-8	y=90946x-261113	0.998
Piperine	5-9	y=190503x-890249	0.990
Camphor	4-8	y=9816.8x-2405.6	0.994

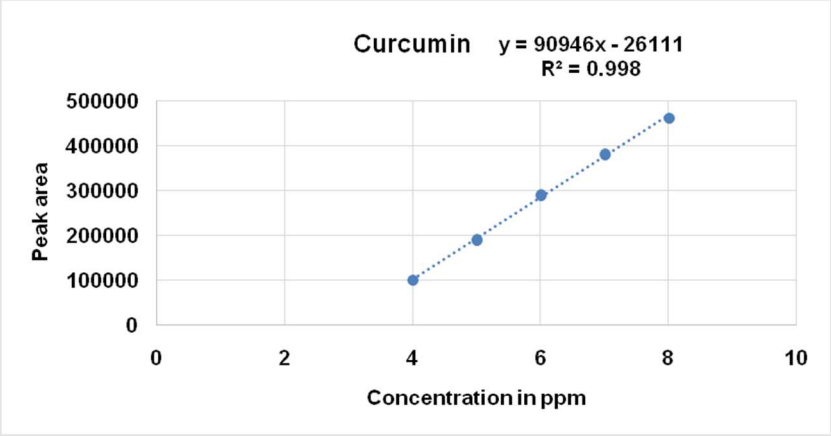

Fig. 6: Calibration curve of curcumin

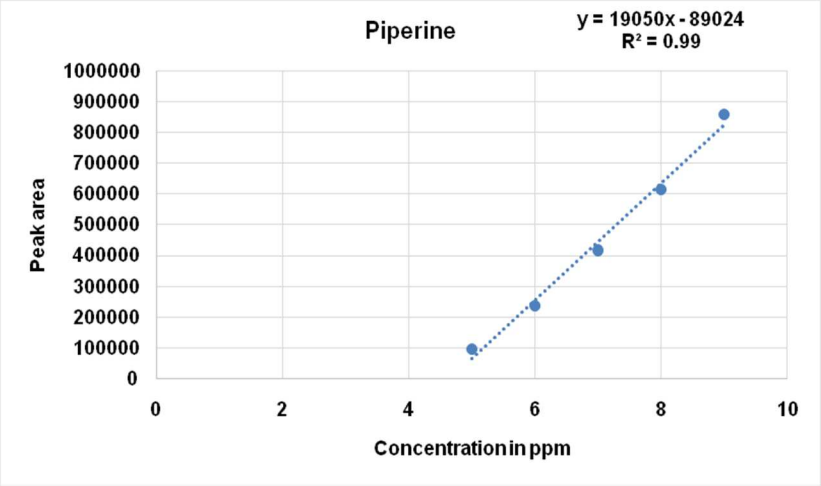

Fig. 7: Calibration curve of piperine

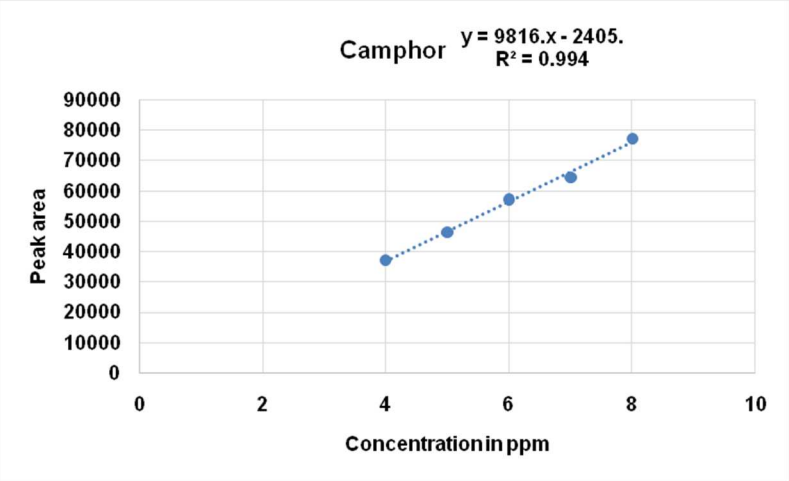

Fig. 8: Calibration curve of camphor

Accuracy

Accuracy was determined by means of recovery experiments, by the determination of % mean recovery of each compound in the formulation at three different levels (80%, 100% and120%). At each level, three determinations were performed. Percent mean recovery was calculated as shown in table 6. The accepted limits of mean recovery are 98%-102% and all observed data were within the required range, which indicates good recovery values, affirming the accuracy of the method developed [15, 16].

Table 6: Recovery study for three markers in dental powder

Compounds	Sample content [ppm]	Standard added [ppm]	Actual amount [ppm]	Total area found [n=3]	Amount recovered [ppm]	% Recovery
Curcumin	2.33	1.86	4.19	115032	4.135	98.70
		2.33	4.66	164123	4.675	100.32
		2.79	5.12	205132	5.126	100.12
Piperine	2.84	2.27	5.11	110256	5.25	102.77
		2.84	5.68	195846	5.70	100.37
		3.40	6.24	275541	6.12	98.16
Camphor	2.13	1.70	3.83	34518	3.76	98.20
		2.13	4.26	38815	4.19	98.56
		2.55	4.68	43412	4.66	99.72

n: Number of injections

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered, and the system suitability parameter tailing factor and peak area were evaluated. The solution was prepared as per the test method described earlier and injected at different variable conditions like column temperature (33 ℃ and 37 °C) and detection wavelength (254 nm and 256 nm). Robustness data clearly shows that the proposed method is robust at small but deliberate change [15, 16]. Robustness data are given in table 7.

Table 7: Robustness data for curcumin, piperine and camphor

Parameters	Curcumin (6 ppm)		Piperine (7 ppm)		Camphor (6 ppm)	
	Peak area	Tailing factor	Peak area	Tailing factor	Peak area	Tailing factor
Minus temp [33 ℃]	290201	1.17	415799	0.98	57236	1.03
Plus temp [37 ℃]	290152	1.09	413444	0.97	56356	0.99
Minus wavelength [254 nm]	280642	0.99	413942	0.93	57136	1.17
Plus wavelength [256 nm]	290212	1.37	405672	0.82	57431	1.09

Sensitivity

The sensitivity of measurement of curcumin, piperine and camphor by use of the proposed method was estimated in terms of the limit of quantitation (LOQ) and limit of detection (LOD). LOQ and LOD were calculated by the use of the equations LOD = 3.3σ/S and LOQ = 10σ/S where σ is the standard deviation of intercepts of calibration plots and S is the average of the slopes of the corresponding calibration plot (table 8).

Table 8: LOD and LOQ for curcumin, piperine and camphor

Compound	LOD (ppm)	LOQ (ppm)
Curcumin	0.183	0.550
Piperine	0.069	0.209
Camphor	0.175	0.531

LOD: limit of detection, # LOQ: limit of quantitation

The results obtained from above set of observations prove that the method is useful in qualitative and quantitative analysis of the markers from the complex herbal mixture formulation. Moreover, various analytical methods for estimation of curcumin, piperine and camphor were reported alone and in combination with other drugs [4-10] but as yet there is no reported HPLC analysis method for simultaneous estimation of curcumin, piperine and camphor combination and the novel method developed in this report is the first of its kind. The developed method is based on the use of very economical solvent, had short chromatographic time and hence can be performed with ease.

CONCLUSION

The results indicate that selected ayurvedic dental powder contains a number of markers that may be responsible for its therapeutic activity. The developed HPLC method will assist in the standardization of dental powder using biologically active chemical markers. The developed HPLC method for simultaneous determination of curcumin, piperine and camphor from ayurvedic dental powder is accurate, precise, reproducible and repeatable. Patanjali divya dental powder also contains a number of other constituents, which are currently the subject of further investigation, apart from those standards studied. With the growing demand for herbal drugs and increased belief in the usage of herbal medicine, the development of a standardization tool will help in maintaining the quality of this important ayurvedic preparation.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally

CONFLICT OF INTERESTS

Declared none

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