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Research Article

A NOVEL REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS ESTIMATION OF ELLAGIC ACID, QUERCETIN, AND PIPERINE IN AYURVEDIC FORMULATIONS

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ABSTRACT

Objective: The objective of the study was to develop a novel, accurate, precise, and linear reverse-phase high-performance liquid chromatographic (RP-HPLC) method for simultaneous qualitative and quantitative estimation of ellagic acid, quercetin, and piperine in different Ayurvedic formulations and validate as per the International Conference on Harmonization guidelines.

Methods: In the present work, a good chromatographic separation was achieved isocratically using a shim-pack HPLC C18 column (4.6×250 mm, 5 µm) and a mobile phase consisting of 0.02 M potassium dihydrogen orthophosphate buffer (pH adjusted to 3.5 with orthophosphoric acid) and acetonitrile in the ratio 60:40, a flow rate of 1.2 ml/min and column temperature maintained at 35°C. The effluents obtained were monitored at 255 nm with ultraviolet-visible detector.

Results: The retention time of ellagic acid, quercetin, and piperine was found to be 1.65 min, 2.94 min, and 14.57 min, respectively. The linearity of ellagic acid, quercetin, and piperine was tested in the range of 6–14 ppm, 3–11 ppm, and 3–13 ppm, respectively. The correlation coefficient for ellagic acid, quercetin, and piperine was found to be 0.997, 0.993, and 0.99, respectively. The high recovery values (98–102%) indicate a satisfactory accuracy. The low percent relative standard deviation values in the precision study reveal that the method is precise.

Conclusion: The developed method is novel, simple, precise, rapid, accurate, and reproducible for simultaneous quantitative estimation of ellagic acid, quercetin, and piperine in Ayurvedic formulations. Hence, the developed method can be used for quantitative analysis and quality control of extracts and commercial samples of other plant species and formulations containing these three markers.

Keywords: Ellagic acid, Quercetin, Piperine, Ayurvedic formulation, Reverse-phase high-performance liquid chromatographic, Validation, International Conference on Harmonization.

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INTRODUCTION

Standardization and analysis of chemical markers in ayurvedic or complex polyherbal formulations are always a challenging task. Quantitative determination of chemical markers of each crude drug in any polyherbal preparation required optimal separation techniques by which these markers are separated with the highest resolution and least interference from each other [1]. Herbal medicine has been enjoying revitalization 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 constituent of plant-based drugs, it is difficult to establish quality control parameters. Modern analytical techniques are increasing to overcome these complications [2]. Separation, identification, and determination of chemical components are very problematic for such complex 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 chromatographic (HPLC) with reversed phase columns are most commonly applied for the analysis of multiple constituents present in medicinal plants and herbal preparations. Hence, herbal drugs or pure active compound needs analytical techniques such as HPLC and high-performance thin layer chromatography to confirm its identity, quality, purity, potency, safety, and efficacy of the plant.

In the present study, we have selected two marketed ayurvedic dental powders, which are used to maintain oral hygiene. It is indicated for various dental problems and makes teeth and gums stronger. The selected ayurvedic formulations contain *Terminalia bellirica* (Combretaceae), *Piper nigrum* (Piperaceae), *Terminalia chebula* (Combretaceae), and other crude drugs. Three chemical markers were selected for quantification, namely, ellagic acid, quercetin, and piperine.

The literature survey reveals that various analytical methods for estimation of ellagic acid, quercetin, and piperine 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 ellagic acid, quercetin, and piperine.

In the present investigation, we have developed a simple, optimized, and validated HPLC method for the standardization of ayurvedic formulations using three chemical markers, namely, ellagic acid, quercetin, and piperine. The method was validated as per the International Conference on Harmonization (ICH) guidelines. This novel validated method has wide applicability in industry as well as in academia.

MATERIALS AND METHODS

HPLC grade ellagic acid, quercetin, and piperine (purity 99%) were procured as gift sample from Yucca Enterprises, Mumbai, India. An Ayurvedic preparation Vithoba dental powder (Vithoba Industries Pvt. Ltd.) and Dabur dental powder **(**Dabur India Ltd) used for analysis were

purchased from local market. HPLC grade solvents were purchased from Thomas Baker. Reverse phase (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 (250×4.6 mm, 5 µm).

Methods

Selection of wavelength

The suitable wavelength for the HPLC analysis was determined by recording ultraviolet (UV) spectrums in the range of 200–400 nm for individual drug solutions of ellagic acid, quercetin, and piperine 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).

Chromatographic conditions

The method was developed using RP, shim-pack HPLC C18 column (250×4.6 mm, 5 µm). The runtime was of 16 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 60:40 at a flow rate of 1.2 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 a volumetric flask. The solution was then filtered using 0.45 µ membrane filter.

Preparation of standard solution

About 100 mg of ellagic acid, quercetin, and piperine 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 a solvent (1000 ppm). The final concentration of ellagic acid, quercetin, and piperine was made to 10 ppm, 7 ppm, and 8 ppm, respectively, by suitable dilutions.

Sample preparation

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

RESULTS AND DISCUSSION

Method development

A novel RP-HPLC method was developed keeping in mind the system suitability parameters, i.e., resolution factor (R), tailing factor (T), number of theoretical plates (N), runtime, and the cost-effectiveness. The developed optimized method resulted in the elution of ellagic acid at 1.65 min, quercetin at 2.94 min, and piperine at 14.57 min. Figs. 2-4 represent chromatograms of ellagic acid, quercetin, and piperine standard solution, respectively. The total runtime was 16 min. System suitability tests are an essential part of method development and are used to ensure satisfactory performance of the chromatographic system.

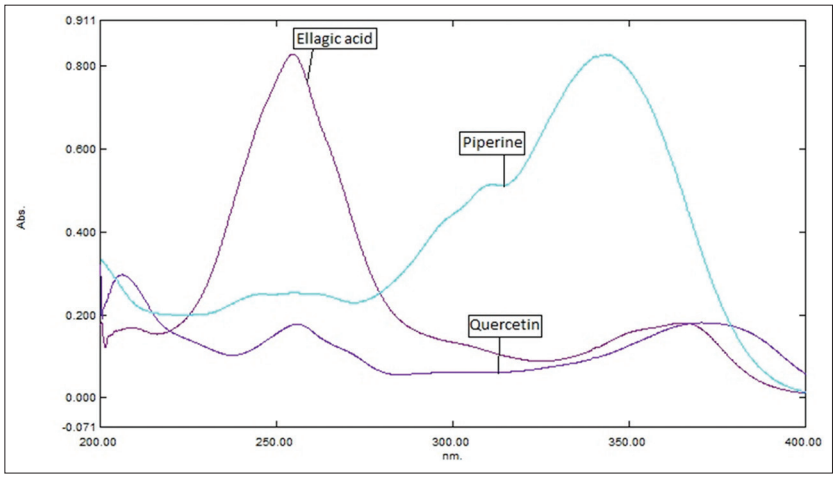

Fig. 1: Ultraviolet overlap spectrum of ellagic acid, quercetin and piperine

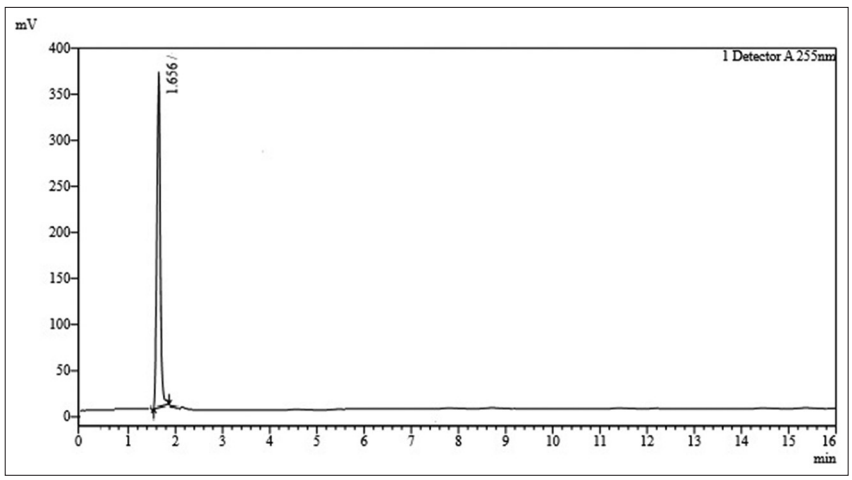

Fig. 2: Typical chromatogram of ellagic acid standard solution

System suitability parameters, i.e., retention time (tR), the number of theoretical plates, and resolution and tailing factor were evaluated for six replicate injections of the standard working concentration. The results given in Table 1 were within the acceptable limits [11,12].

To test the applicability of the developed method to ayurvedic formulations, dental powder extracts were chromatographed, and it is shown in Figs. 5 and 6. The sample peaks were identified by comparing the relative retention times with standard markers (Figs. 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 using a linear regression equation. For the analysis of a sample, extract of 500 ppm of dental powder was injected in triplicate and quantified for three active markers using a linear regression equation. The results of dental powder extract analysis are reported in Table 2.

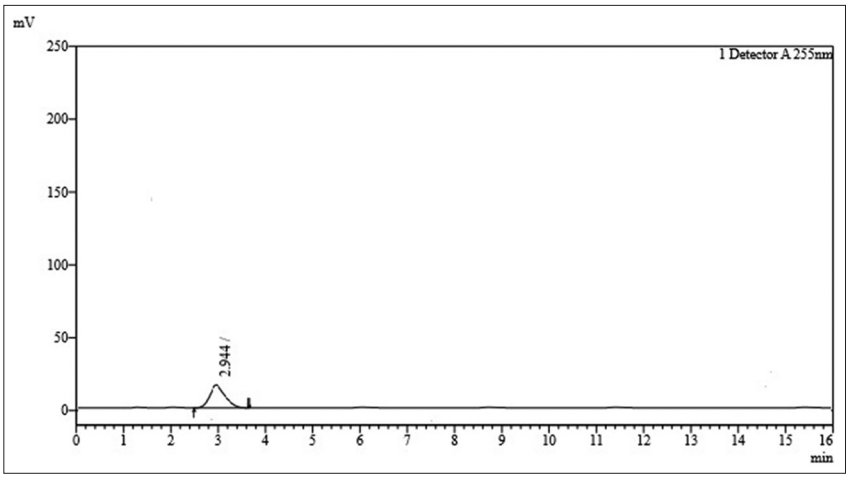

Fig. 3: Typical chromatogram of quercetin standard solution

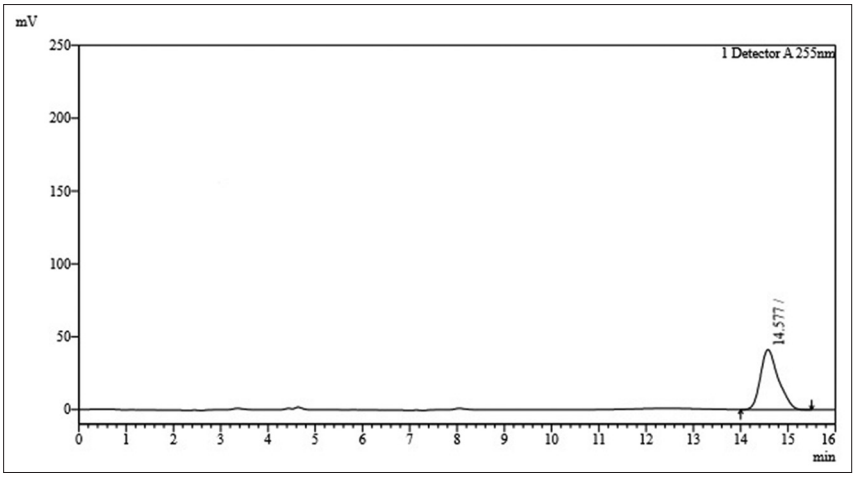

Fig. 4: Typical chromatogram of piperine standard solution

Table 1: Results of system suitability studies

Parameters	Acceptance limits	Ellagic acid	Quercetin	Piperine
Retention time (min)	-	1.65	2.94	14.57
Resolution factor	Not<2	-	8.04	13.00
Number of theoretical plates	Not<2000	3608	3786	7343
Tailing factor	Not more than 2	1.02	1.21	1.26

Table 2: Analysis of ayurvedic dental powder extracts

Formulation	Marker	Amount found (ppm) n=3	Content (%)
Ayurvedic dental powder extract (500 ppm) Vithoba	Ellagic acid	7.00	1.40
	Quercetin	3.28	0.65
	Piperine	4.46	0.89
Ayurvedic dental powder extract (500 ppm) Dabur	Ellagic acid	3.58	0.71
	Quercetin	2.65	0.53
	Piperine	4.86	0.97

[#]n: Number of injections

Method validation

Analytical method validation is the process to substantiate that the analytical procedure used for a specific examination is appropriate for its designated purpose. Food and Drug Administration regulations quality standards such as ISO17025 necessitate analytical methods to be validated before and during routine use. The developed HPLC method was validated according to the ICH guidelines [13] for validation of analytical procedures. The method was validated for the parameters such as linearity, accuracy, system precision, method precision, robustness, limit of detection (LOD), and limit of quantitation (LOQ).

Specificity

Figs. 2-6 for standard marker solutions and sample chromatograms reveals that the peaks obtained in the standard solutions and sample solution at working concentrations are just because of the drugs and blank has no peak at the tR of ellagic acid, quercetin, and piperine. Accordingly, it can be concluded that the method developed is said to be specific [14,15].

Precision

System precision

Six replicate injections of the standard marker solutions at working concentration presented percent relative standard deviation (SD) (% RSD) <2 concerning the peak area for each marker, which indicates the satisfactory reproducibility and thereby the precision of the system [16,17]. 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. Three injections of the sample from the same homogeneous mixture

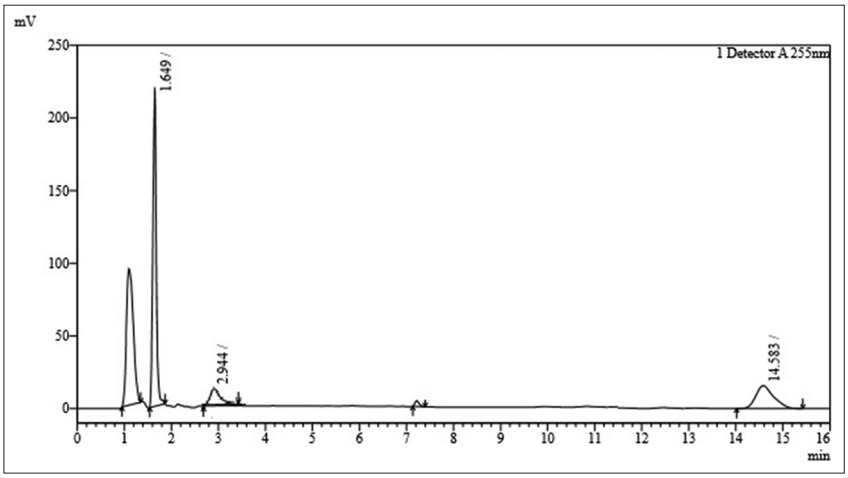

Fig. 5: Typical chromatogram of Vithoba ayurvedic formulation

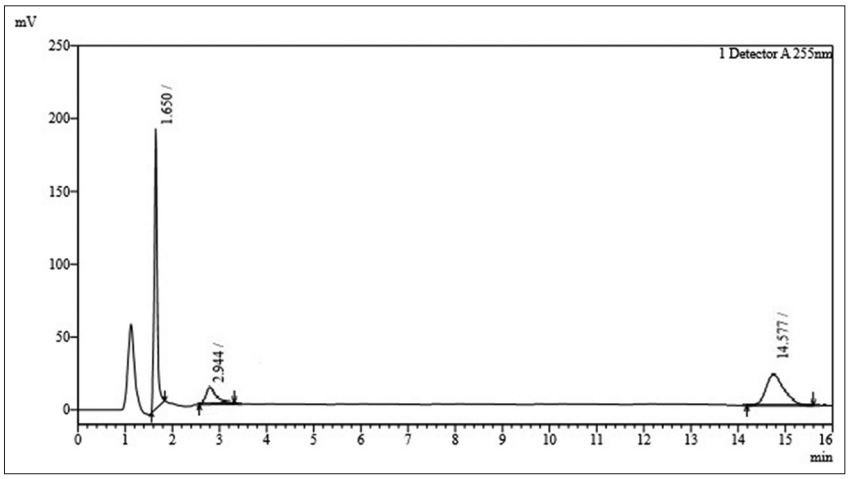

Fig. 6: Typical chromatogram of Dabur ayurvedic formulation

Table 3: System precision results

S. No	Peak area of ellagic acid (10 ppm)	Peak area of quercetin (7 ppm)	Peak area of piperine (8 ppm)
1	1356692	405178	1085821
2	1397586	409767	1087489
3	1386532	402459	1097347
4	1386582	409876	1076584
5	1386239	403625	1097657
6	1397528	402562	1086534
Mean±SD	1385193±14986	405578±3430	1088572±7952
% RSD	1.08	0.85	0.73

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

at working concentration showed % RSD <2 concerning the content of three markers indicate that the method developed is precise by the test of repeatability [16,17], and hence can be understood that the method gives consistently reproducible results (Table 4).

Linearity

Standard solutions of ellagic acid, quercetin, and piperine 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 6–14 ppm for ellagic acid, 3–11 ppm for quercetin, and as that of 3–13 ppm for piperine. The correlation coefficients were >0.99 for each marker, which meets the method validation acceptance criteria [16,17]; hence, the method is said to be linear (Figs. 7-9).

Accuracy

Accuracy was determined by means of recovery experiments, by the determination of % mean recovery of each compound in the formulation at three distinct levels (80%, 100%, and 120%). At each level, three determinations were performed. Percent mean recovery was calculated as shown in Table 5. The accepted limits of mean recovery are 98–102% and all observed data were within the range, which specifies good recovery values, supporting the accuracy of the method developed [16,17].

Robustness

To determine the robustness of the developed method, experimental conditions were purposely altered, and the system suitability parameter T and peak area were evaluated. The solution was prepared as per the test method described earlier and injected at different variable conditions such as column temperature (33°C and 37°C), flow rate (1.1 ml/min and 1.3 ml/min), and detection wavelength (254 nm and 256 nm). Robustness data clearly show that the proposed method is robust at small but deliberate change [16,17]. Robustness data are given in Table 6.

Sensitivity

The sensitivity of measurement of ellagic acid, quercetin, and piperine by use of the proposed method was estimated in terms of the LOQ and LOD. LOQ and LOD were calculated using the equations LOD=3.3 σ/S and LOQ=10 σ/S where σ is the SD of intercepts of calibration plots, and S is the average of the slopes of the corresponding calibration plot (Table 7).

The results obtained from the above set of observations prove that the method is useful in routine qualitative and quantitative analysis of the markers from the complex herbal mixture formulation. Moreover, various analytical methods for estimation of ellagic acid, quercetin, and piperine were reported alone and in combination with other drugs [4-10], but there is no reported HPLC analysis method for simultaneous estimation of ellagic acid, quercetin, and piperine combination and the novel method developed in this report is the first of its kind. The developed method is based on the use of a 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. This novel developed HPLC method for simultaneous determination of ellagic

Table 4: Method precision results (Vithoba Ayurvedic dental powder)

Marker	Intraday		Interday	
	Average content (ppm) n=3 Mean±SD	% RSD	Average content (ppm) n=3 Mean±SD	% RSD
Ellagic acid	7.03±0.07	1.06	7.09±0.07	1.11
Quercetin	3.27±0.02	0.63	3.24±0.04	1.38
Piperine	4.45±0.01	0.22	4.41±0.05	1.26

#n: Number of injections, #SD: Standard deviation, #% RSD: Percent relative standard deviation

Table 5: Recovery study for three markers in dental powder

Compounds	Sample content (ppm)	Standard added (ppm)	Actual amount (ppm)	Total average area found (n=3)	Amount recovered (ppm)	% Recovery
Ellagic acid	3.50	2.80	6.30	1049566	6.46	102
		3.50	7.00	1099009	7.07	101.06
		4.20	7.70	1168448	7.90	102
Quercetin	1.64	1.31	2.95	386810	2.91	98.86
		1.64	3.28	387645	3.26	99.64
		1.96	3.60	389989	3.56	98.92
Piperine	2.23	1.78	4.01	354328	4.09	102
		2.23	4.46	416708	4.46	100.18
		2.67	4.90	476763	4.82	98.46

#n: Number of injections

Table 6: Robustness data for ellagic acid, quercetin, and piperine

Parameters	Ellagic acid (10 ppm)		Quercetin (7 ppm)		Piperine (8 ppm)	
	Peak area	Tailing factor	Peak area	Tailing factor	Peak area	Tailing factor
Minus temperature (33°C)	1356692	1.02	405178	0.98	1085821	1.03
Plus temperature (37°C)	1356786	1.00	408768	0.97	1086763	0.99
Minus flow rate (1.1 ml/min)	1356825	1.08	404863	1.00	1088648	1.26
Plus flow rate (1.3 ml/min)	1357687	1.09	405283	1.21	1086746	1.00
Minus wavelength (254 nm)	1357358	0.99	405834	0.93	1086476	1.17
Plus wavelength (256 nm)	1367846	1.37	406834	0.82	1086547	1.09

Table 7: LOD and LOQ for ellagic acid, quercetin, and piperine

Marker	LOD (ppm)	LOQ (ppm)
Ellagic acid	0.60	1.84
Quercetin	0.45	1.60
Piperine	0.15	0.47

[#]LOD: Limit of detection, [#]LOQ: Limit of quantitation

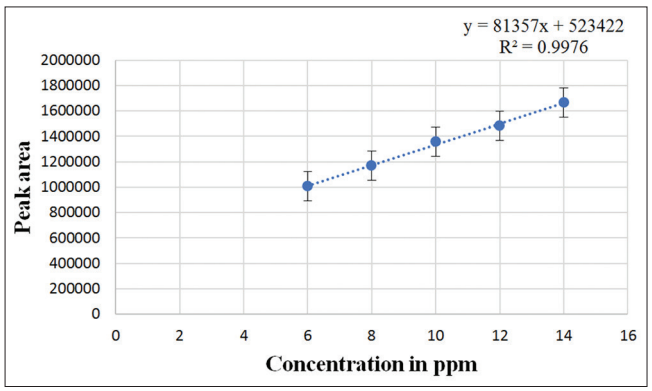

Fig. 7: Calibration curve of ellagic acid

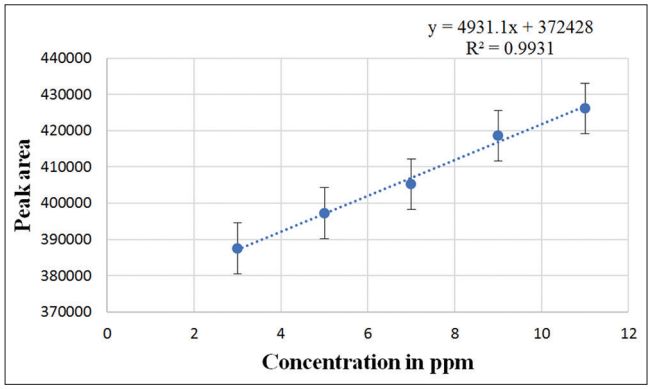

Fig. 8: Calibration curve of quercetin

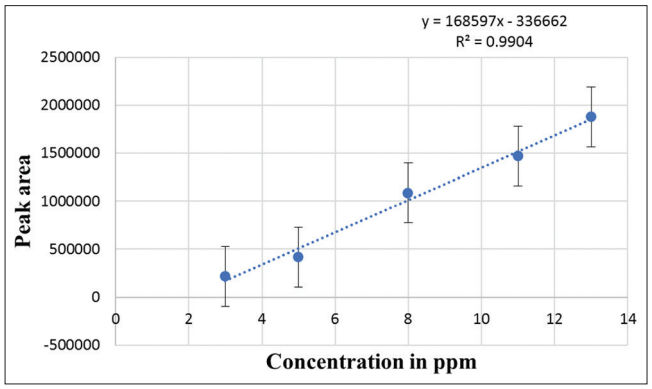

Fig. 9: Calibration curve of piperine

acid, quercetin, and piperine from ayurvedic dental powder is accurate, precise, reproducible, and repeatable. These ayurvedic dental powders also contain 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 use 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 INTEREST

Declared none.

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