"QUANTIFICATION OF EUGENOL, CURCUMIN AND CINNAMALDEHYDE FOR HERBAL STANDARDIZATION OF DENTAL PRODUCT BY HPLC METHOD"

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ABSTRACT
The purpose of the present study was to develop a simple, precise, and accurate RP-HPLC method for the simultaneous estimation of Curcumin, Eugenol, and Cinnamaldehyde in their pharmaceutical dental dosage form. Chromatographic separation was performed in an isocratic mode using an ODS C18 column (250 *4.6 mm, 5µm) as the stationary phase. The mobile phase consisted of a mixture of methanol, acetonitrile, and water (35:20:45 v/v/v) delivered at a constant flow rate of 0.8 mL/min, with the column oven temperature maintained at 35°C and an injection volume of 20 µL. Detection was monitored via a Photodiode Array (PDA) detector, and the final analytical wavelength was selected at 254 nm to provide optimum peak intensity for all three target analytes. The method exhibited an excellent linear response with a correlation coefficient (R^2) of 0.995 for Curcumin in the range of 1-4 µg/ml, 0.998 for Eugenol in the range of 50-150 µg/ml, and 0.998 for Cinnamaldehyde in the range of 2-6 µg/ml. The developed method successfully quantified the active marker compounds in the formulation, revealing total contents of 46.5 mg/100 g for Eugenol, 26.0 mg/100 g for Cinnamaldehyde, and 4.0 mg/100 g for Curcumin.The proposed method was validated as per regulatory requirements and proves to be highly reliable for the routine quality control and standardization of herbal dental formulations.

Keywords: Curcumin, Eugenol, Cinnamaldehyde, HPLC, PDA Detection, Validation
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1. INTRODUCTION

Herbal dental products have gained significant therapeutic prominence due to their natural multi-targeted benefits, including antimicrobial, anti-inflammatory, and analgesic properties. However, ensuring the quality, safety, and batch-to-batch uniformity of these complex plant-derived formulations requires stringent quality control.[1-5]. The therapeutic efficacy of these products relies heavily on three key bioactive marker compounds: Eugenol (4-allyl-2-methoxyphenol), a clove-derived phenolic compound traditionally used as a local anaesthetic and analgesic in dental cements; Curcumin [(1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione], a turmeric extract celebrated for its potent anti-inflammatory and plaque-reducing properties in periodontics; and Cinnamaldehyde [(2E)-3-phenylprop-2-enal], a cinnamon constituent valued for its powerful action against cariogenic oral biofilms and its role as a natural flavouring agent[5-15].

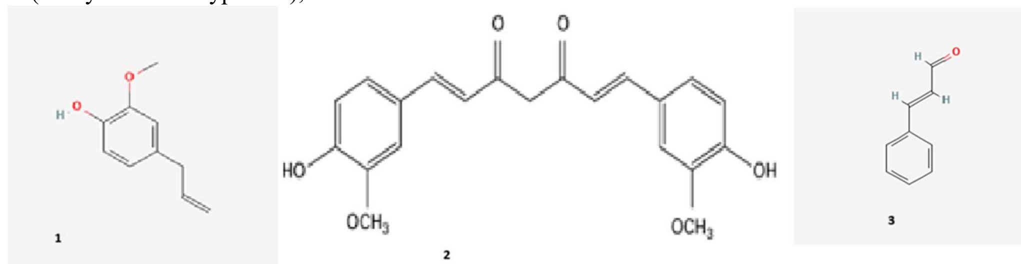

Figure 1 Structure of 1-Eugenol 2-curcumin 3-Cinnamaldehyde

2. MATERIAL AND METHOD

2.1 Chemicals

All API Eugenol, Curcumin and Cinnamaldehyde were obtained from Oxford Lab Fine Chem, Maharashtra, India. Acetonitrile (Hplc grade,99.80%), HPLC- grade water and Methanol (Hplc grade) were purchased from Thermo fisher scientific India Pvt Ltd.

2.2 Instrumentation

The chromatographic analysis was performed using an Agilent Infinity 1200 High-Performance Liquid Chromatography (HPLC) system. The instrument configuration featured a low-pressure quaternary gradient pump for precise mobile phase delivery, an autosampler for automated sample injection, and a UV-VIS detector for analyte monitoring. Chromatographic separation was achieved on a LiChrospher ODS C18 column (250 *4.6 mm), and system control, data acquisition, and processing were managed using OpenLab software.

2.3 Chromatographic Condition

Chromatographic separation was performed in isocratic mode using a mobile phase consisting of a mixture of methanol, acetonitrile, and water in a volume ratio of 35:20:45 v/v/v. The mobile phase was delivered at a constant flow rate of 0.8mL/min through an ODS C18 stationary phase column (250 *4.6 mm, 5 µm particle size), with the column oven temperature maintained at 35 C. The injection volume for all samples and standards was 20 µL. A standard stock solution containing a mixture of curcumin, eugenol, and cinnamaldehyde (each at a concentration of 10 µg/mL) was prepared in acetonitrile. Detection was monitored via a Photodiode Array (PDA) detector. While multi-wavelength scanning was evaluated (221nm, 254 nm, and 281nm), an analytical wavelength of 254nm was selected for final quantification as it provided the optimum peak intensity for all three target analytes.

2.4 Preparation of Stock Solution

Eugenol (EUG) Standard Stock Solution

The standard stock solution of Eugenol (EUG) was prepared by accurately weighing 100 mg of EUG reference standard and transferring it into a 100 ml volumetric flask. The drug was dissolved and the volume was made up to the mark with acetonitrile (ACN) to obtain a master stock solution with a concentration of 1000 µg/ml. From this master stock, an aliquot of 5 ml was transferred to a 50 ml volumetric flask and diluted to volume with ACN to achieve a working standard solution of 100 µg/ml.

Cinnamaldehyde (CIN) Standard Stock Solution

The standard stock solution of Cinnamaldehyde (CIN) was prepared by accurately weighing 10 mg of CIN reference standard and transferring it into a 100 ml volumetric flask. The analyte was dissolved and diluted to volume with acetonitrile (ACN) to yield a master stock concentration of 100 µg/ml. A working standard solution containing 4 µg/ml was subsequently prepared by pipetting 4 ml of the master stock solution into a 100 ml volumetric flask and making up the volume with ACN.

Curcumin (CUR) Standard Stock Solution:

The standard stock solution of Curcumin (CUR) was prepared by accurately weighing 10 mg of CUR reference standard into a 100 ml volumetric flask, dissolving it in acetonitrile, and bringing the total volume to the mark to attain a master stock solution of 100 µg/ml. To prepare the working standard solution at a concentration of 3 µg/ml, a 3 ml aliquot of the master stock solution was transferred into a 100 ml volumetric flask and diluted to the mark with acetonitrile.

Preparation of standard stock solution of mixture

Accurately weighed amounts of 100 mg EUG, 4 mg CIN, and 3 mg CUR were transferred into a single 100 ml volumetric flask and dissolved in acetonitrile, resulting in a stock mixture consisting of 1000 µg/ml EUG, 40 µg/ml CIN, and 30 µg/ml CUR. A 10.0 ml aliquot of this mixed solution was further diluted to 100 ml with acetonitrile to obtain a working standard mixture with final concentrations of 100 µg/ml EUG, 4 µg/ml CIN, and 3 µg/ml. CUR.

2.5 Validation

The method was validated as per ICH Q2 (R2) guideline for parameter like linearity, precision, LOD, LOQ, accuracy, robustness and system suitability Parameters.

2.5.1 System Suitability Parameters

A system suitability test was an integral part of the method development to verify that the system is adequate for the analysis of CUR, EUG and CIN to be performed. The system suitability test of the chromatography system was performed with Six replicate injections of Standard solution of both drug in mixture. Retention time, tailing factor, resolution factor, and theoretical plates were determined.

2.5.2 Linearity

The linearity of an analytical method was carried out to check its ability to give test results within given range. Different concentration of CUR (1-5 µg/ml),EUG (50-150 µg/ml) and CIN (2 -6 µg/ml) were prepared in to 10 ml volumetric flask with mobile phase as a diluent and inject each concentration in mixture form in HPLC system and chromatogram were recorded. A calibration graph was plotted as concentration (µg/ml) verses chromatographic peak area (mV).

2.5.3 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. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

2.5.4 Robustness

The robustness of the method was confirmed by making small deliberate changes in the flow rate, mobile phase, pH, temperature and wavelength. The effect of these changes was recorded and % relative standard deviation was calculated.

2.5.5 LOD and LOQ

The LOD is defined as the lowest concentration of an analyte that can reliably be differentiated from background levels and LOQ of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy.

$$LOD = 3.3 \times \sigma/S$$

$$LOQ = 10 \times \sigma/S$$

where σ is the standard deviation of y-intercepts of regression lines and S is the slope of the calibration curve.

2.5.6 Accuracy

Accuracy of the analytical method has been performed by spiking of blank with the standard. Spiking of the sample was performed at 50, 100 and 150 % of the target concentration (15,30,45µg/ml for CUR, 20,40,60 µg/ml for CIN and 500,1000,1500 µg/ml for EUG. The solution was injected and peak area was recorded.

2.5.7 Standardization Process

Sample preparation - (Label Content: 100 gm powder: 33% of raw curcumin, 33% raw cinnamon powder, 33% raw clove powder)10 gm of sample powder is taken in 100 ml volumetric flask and make up the volume of the flask to the mark with acetonitrile subjected by cold maceration for 24 hours day. After 24 hours, the flask is taken out and subjected to sonication for about 15 minutes at 40 C. After successful sonication, the, contents are transferred to 15 ml centrifugation tubes (15 ml X 8) and centrifuged for 5 minutes. After centrifugation, the 1 ml of supernant is collected and diluted to 100 ml with mobile phase.

Test preparation - Withdraw 1.0ml from Master Stock Solution and makeup to 10ml with mobile phase.

3. RESULTS AND DISCUSSION

3.1 Selection of Wavelength

As better intensity was observed at 254 nm for all the components, it was selected as analytical wavelength.

3.2 Optimization of Mobile Phase

The mobile phase consisting of methanol, acetonitrile, and water in a volume ratio of 35:20:45 was found to be optimal for method development, yielding well-resolved, symmetric peaks for all three analytes. Under these optimized chromatographic conditions, eugenol, cinnamaldehyde, and curcumin were successfully separated with excellent resolution. The retention times for eugenol, cinnamaldehyde, and curcumin were found to be 2.886 min, 3.902 min, and 9.976 min, respectively, with all system suitability parameters falling within acceptable limits

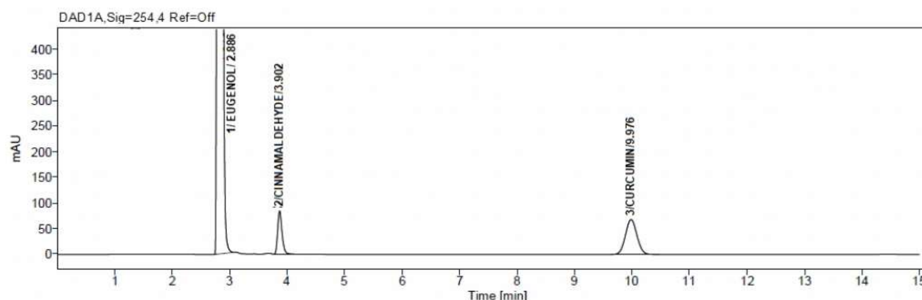

Figure 2 Optimization of chromatographic condition.

3.3 VALIDATION

3.3.1 System Suitability Parameters Result

Table 1 System Suitability Parameters Result

SR. NO	Parameters	Results		
		CUR	CIN	EUG
1	Tailing factor	1.05±0.01	1.24±0.01	1.17±0.01
2	Resolution	7.69±0.02	30.01±0.03	-
3	Theoretical plates	11644±4.22	4945±2.8	1549.9±1.63
4	Retention time	9.97±0.006	3.9±0.002	2.88±0.002

The system suitability test was evaluated to verify the performance and reproducibility of the chromatographic system, and the results are summarized in Table. The tailing factors for curcumin (CUR), cinnamaldehyde (CIN), and eugenol (EUG) were found to be 1.05 ± 0.01, 1.24 ± 0.01, and 1.17 ± 0.01, respectively, indicating excellent peak symmetry. High chromatographic efficiency was observed with high theoretical plate counts, particularly for CUR (11644±4.22) and CIN (4945 ±2.8). Furthermore, the system exhibited robust separation with a resolution of 7.69±0.02 between the adjacent peaks and highly reproducible retention times for all three analytes. These outcomes confirm that the developed method satisfies all regulatory system suitability requirements for simultaneous estimation.

3.3.2 Linearity

The linearity of the proposed analytical method was evaluated by constructing calibration curves over a specific concentration range for each analyte. The method exhibited an excellent linear response, with calibration curves found to be linear in the range of 1-5µg/mL for curcumin, 50-150µg/mL for eugenol, and 2-6 µg/mL for cinnamaldehyde. The corresponding correlation coefficients (R^2) were found to be 0.995, 0.998, and 0.998, respectively, confirming a highly reliable relationship between peak area response and concentration across all evaluated ranges.

Sr. no	EUG		CIN		CUR	
	Concentration	Area	Concentration	Area	Concentration	Area
1	50	224.35	2	2.148	1	3.558
2	75	347.106	3	3.136	2	6.554
3	100	463.352	4	4.073	3	9.278
4	125	552.885	5	4.967	4	11.695
5	150	676.493	6	6.114	5	14.273
Regression Equation	y = 4.4906x + 3.1469		y = 1.0024x + 0.065		y = 2.8143x + 0.524	
R^2	0.998		0.998		0.995	

Table 2 Concentration and Area for linearity

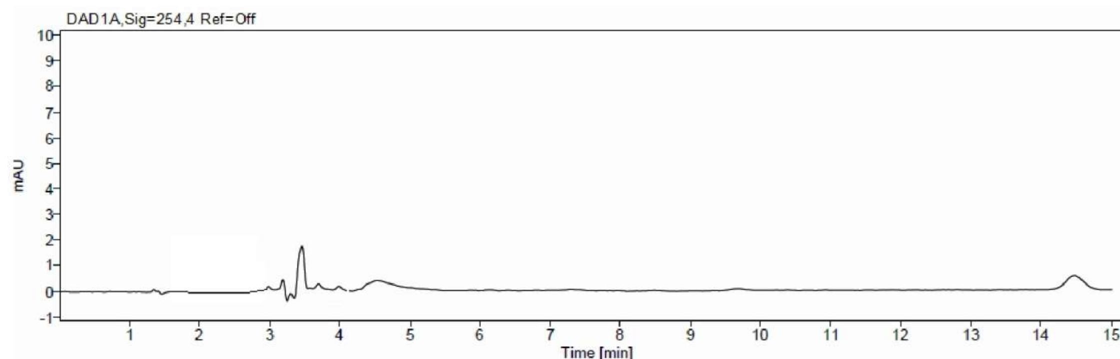

Figure 3 Linearity chromatogram (Blank)

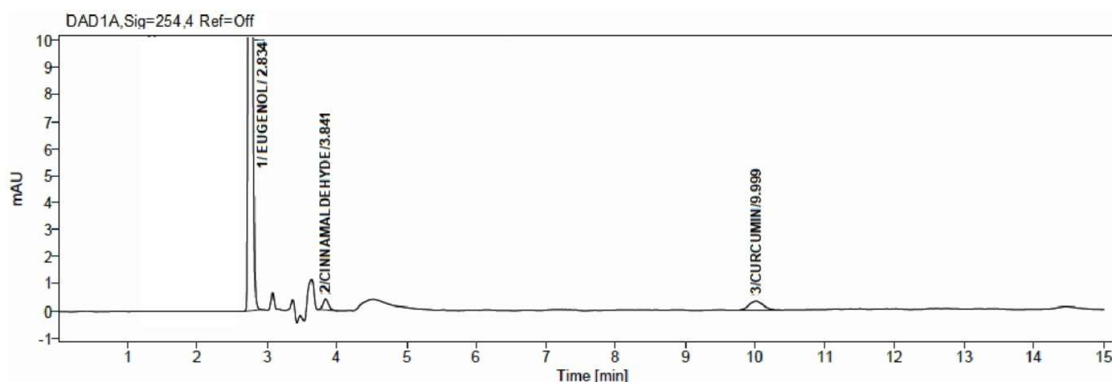

Figure 4 Linearity chromatogram (Mix-1)

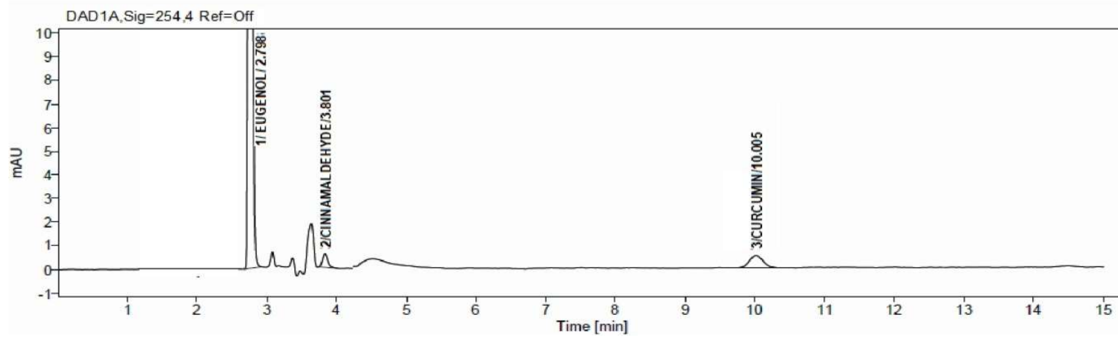

Figure 5 Linearity chromatogram (Mix-2)

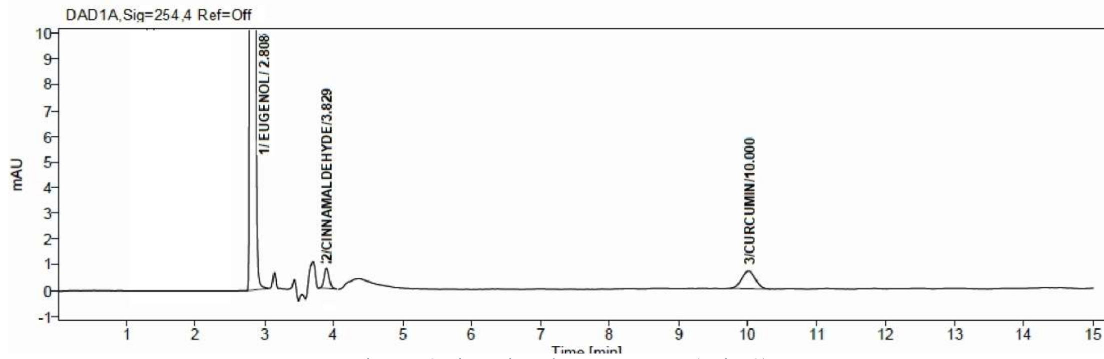

Figure 6 Linearity chromatogram (Mix-3)

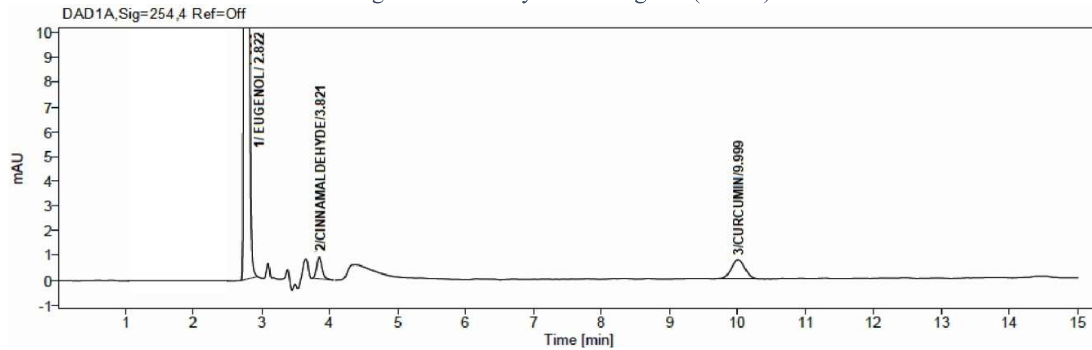

Figure 7 Linearity chromatogram (Mix-4)

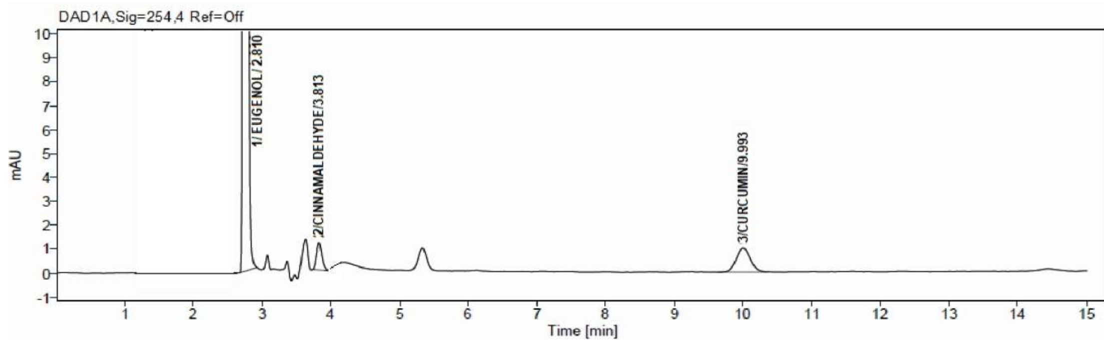

Figure 8 Linearity chromatogram (Mix-5)

3.3.3 Precision

3.3.3.1 Repeatability

Table 3 Result Table for Repeatability

Concentration	50µg/ml	75µg/ml	100µg/ml	125µg/ml	150µg/ml
EUG (Mean±SD)	224.37±0.16	347.8±1.29	463.36±2.63	552.90±0.64	679±1.22

	2µg/ml	3µg/ml	4µg/ml	5µg/ml	6µg/ml
CIN (Mean±SD)	2.15±0.01	3.16±0.02	4.08±0.03	4.96±0.03	6.11±0.02
	1µg/ml	2µg/ml	3µg/ml	4µg/ml	5µg/ml
CUR (Mean±SD)	3.55±0.01	6.55±0.02	9.48±0.06	11.69±0.04	14.27±0.06

The repeatability (injection precision) of the method was evaluated by analyzing six replicates (n = 6) of sample solutions across five different concentration levels for each analyte. The relative standard deviation (%RSD) values were found to be between 0.07%–0.57% for eugenol, 0.25%–0.83% for cinnamaldehyde, and 0.33%–0.64% for curcumin. All the calculated %RSD values were well below the stringent regulatory threshold of 2.0%, demonstrating an exceptionally high degree of instrument precision and measurement repeatability.

3.3.3.2 Intraday and Interday Precision

The precision of the developed method was verified by determining intraday and interday variations at three different concentration levels for each analyte. The relative standard deviation (%RSD) values for intraday precision ranged from 1.16% to 1.63% for eugenol, 1.47% to 1.76% for cinnamaldehyde, and 1.05% to 1.50% for curcumin. For interday precision, the %RSD values were found between 0.92%–1.81%, 1.11%–1.36%, and 1.19%–1.37% for eugenol, cinnamaldehyde, and curcumin, respectively. All obtained %RSD values were well within the acceptable regulatory limit of less than 2.0%, confirming the excellent reproducibility and ruggedness of the method.

3.3.4 Robustness

Following parameters were altered one by one for determination of robustness of the method and their effect was observed by comparing with the standard preparation. Mobile phase flow rate(±0.1mL/min), optimized flow rate was 0.8 mL/min. Mobile phase composition(±2mL), in optimized ratio. Determinations of CUR+CIN+EUG=3+4+100µg/mL for each alteration were carried out and RSD was measured.

Table 4Result table for robustness

Parameter	Level	Amount of drug added	Amount of drug recovered	% Recovery
Flow Rate of Mobile Phase	0.7 ml/min	9.40	4.00	460.40
	0.8 ml/min	9.48	4.07	463.35
	0.9 ml/min	9.56	4.14	466.20
	Mean	9.48	4.07	463.32
	SD	0.08	0.07	2.90
	RSD	**0.84**	**1.72**	**0.63**
Composition of mobile phase	37:20:43	9.42	4.02	461.15
	35:20:45	9.51	4.09	464.20
	33:22:45	9.54	4.12	465.85
	Mean	9.49	4.08	463.73
	SD	0.06	0.05	2.38
	RSD	**0.66**	**1.26**	**0.51**

3.3.5 LOD and LOQ

The sensitivity of the developed method was established by determining the Limit of Detection (LOD) and Limit of Quantification (LOQ) based on the standard deviation of the intercept (sigma) and the mean slope (S) of the calibration curves. The LOD values were found to be 0.16 µg/ml for curcumin, 1.26 µg/ml for cinnamaldehyde, and 20.78 µg/ml for eugenol. The corresponding LOQ values were determined as 0.48 µg/ml 3.81 µg/ml, and 62.98 µg/ml for curcumin, cinnamaldehyde, and eugenol, respectively. These low detection and quantification limits demonstrate that the proposed RP-HPLC method possesses high sensitivity for the simultaneous tracking of all three compounds.

3.3.6 Accuracy

The accuracy of the method was determined by calculating % recovery. Known amount of standard spike at three different level (50%, 100%, 150 %) to a pre quantified sample solution and percentage recovery was found within range at 98%-102 %. Calculated the percentage recovery.

Drug	Level	Amount of drug added	Amount of drug recovered	% Recovery

Table 5 Result table for Accuracy

EUG (n=3)	50%	50	48.51	97.02
	100%	100	97.47	97.47
	150%	150	146.59	97.73
CIN (n=3)	50%	2.0	1.94	97.17
	100%	4.0	3.90	97.42
	150%	6.0	5.84	97.39
CUR (n=3)	50%	1.5	1.46	97.11
	100%	3.0	2.94	98.11
	150%	4.5	4.38	97.26

3.3.7 Standardization

Table 6 Result table for standardization

Marker Compound	Chromatogram Area	Concentration per injection (ppm)	Total content found (mg/100 g)
Eugenol	13.588	2.3	46.5
Cinnamaldehyde	1.368	1.30	26.0
Curcumin	1.087	0.20	4.0

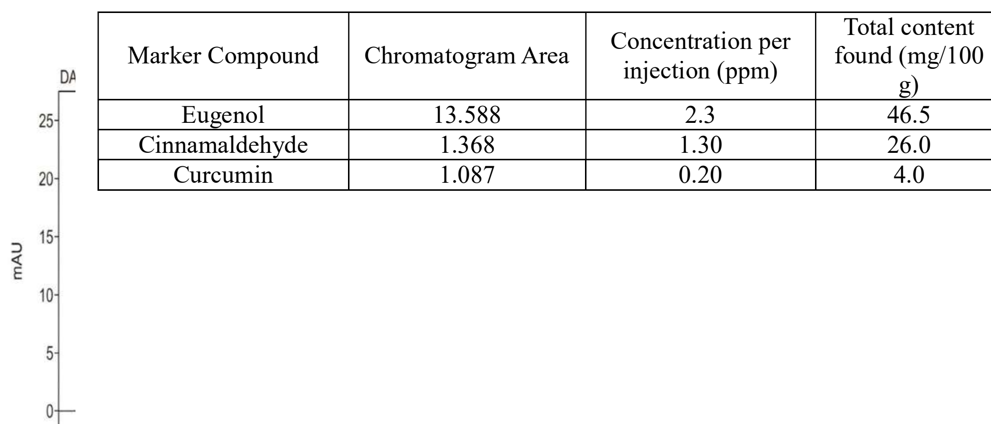

Figure 9 Chromatogram of Standardization

The standardization of the herbal formulation/extract was evaluated using specific marker compounds, with the quantitative results summarized in Table 6. Quantitative estimation showed a significant variance among the active constituents. Eugenol emerged as the predominant marker compound, showcasing the highest total content at 46.5 mg/100 g, supported by a prominent peak area of 13.588. In comparison, Cinnamaldehyde showed a moderate presence with a total content of 26.0 mg/100 g. Curcumin was also successfully quantified, though it was present in a notably lower amount (4.0 mg/100 g) relative to the other two markers. These quantitative profiles serve as critical quality control parameters for ensuring the consistency and efficacy of the sample.

4. CONCLUSION

From this study, it can be concluded that a simple, rapid, accurate, and precise RP-HPLC method was successfully developed for the simultaneous estimation of curcumin, cinnamaldehyde, and eugenol. The statistical parameters, system suitability, and assay results confirm the high efficiency and reproducibility of the method. The proposed method was fully validated in accordance with the ICH Q2(R2) guidelines, with all evaluated parameters falling well within the established acceptance criteria. Consequently, this method proves to be highly reliable and well-suited for the routine quality control analysis of these three active components in pharmaceutical and herbal formulation.

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