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Original Article

VALIDATION, STABILITY STUDIES, AND SIMULTANEOUS ESTIMATION OF CO-ENCAPSULATED CURCUMIN, EPIGALLOCATECHIN GALLATE NANOFORMULATION BY RP-HPLC METHOD

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ABSTRACT

Objective: A new reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed to simultaneously determine curcumin and epigallocatechin gallate (EGCG) in novel nanoformulation.

Methods: The high-performance liquid chromatography (HPLC) method was achieved by using a Thermo Scientific Hypersil Base Deactivated Silica (BDS) C18 column (25 cm X 4.6 mm, 5 µm) at 35 °C column oven temperature. The chromatographic procedure was performed with a mobile phase of acetonitrile and 0.025 M (pH 4.0) potassium dihydrogen phosphate (KH_2PO_4) buffer by gradient mode of elution. The injection volume was 20 µl, and the flow rate was 1.5 ml/min, with ultraviolet (UV) detection using a diode array detector (DAD) at a 268 nm isosbestic wavelength.

Results: Drug entrapment efficiency studies were performed with co-encapsulated EGCG and curcumin nanoformulation, which were found to be 94.35 % and 95.12 %, respectively. This shows that the developed method is highly effective. EGCG and curcumin were eluted at 3.9 min and 10.7 min, respectively. The linearity range was 25-175 µg/ml for EGCG and 12.5-100 µg/ml for curcumin. The correlation coefficient was 0.991 for EGCG and 0.999 for curcumin from the linearity curve, which indicates that the method can produce good sensitivity. Forced degradation studies were conducted in acidic, basic, oxidative, thermal, photolytic, and UV stress conditions, where all the degradation peaks were monitored.

Conclusion: The developed method was linear, simple, rapid, robust, and precise. It could be used to quantify EGCG and curcumin simultaneously in various nanoformulations for *in vivo* and *in vitro* applications.

Keywords: RP-HPLC method, UV detection, EGCG, Curcumin, Nanoformulation

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INTRODUCTION

A compound is considered bioactive if it directly affects living organisms after administration. These effects may be therapeutical or toxicological [1]. Bioactive compounds boost anti-inflammatory and antioxidant properties while lowering platelet aggregation, circulating LDL (low-density lipoprotein), and tumor growth [2]. In epidemiological studies, higher vegetable consumption has been linked to a lower risk of chronic illnesses, including cardiovascular disease, cancer, and age-related impaired functioning [3]. These health benefits are assumed to be attributed to bioactive compounds, micronutrients, and macronutrients, found in vegetables [4]. The therapeutic properties of these compounds, such as curcumin (from turmeric), allicin (from garlic), and catechin (from tea), assist in preventing diseases such as diabetes, cancer, and degenerative neurological disorders [5]. However, limited stability due to susceptibility to light, temperature, poor solubility, oxygen, and low bioavailability are all issues that restrict their use in pharmaceutical formulations [6]. Increasing the solubility and stability of bioactive compounds, as well as their bioactivity and bioavailability, can be done by nanoformulation with hydrophilic carriers [7]. Bioactive compounds can be encapsulated to nanoparticles to improve their targeting ability, efficiency, and specificity. It can also extend the presence of therapeutic substances in the bloodstream and enhance cellular absorption, and provide synergistic effects when multiple drugs are used together [8].

Curcumin is the major phytochemical in the rhizome of *Curcuma longa* L. (Zingiberaceae), generally known as turmeric [9]. Turmeric is a plant that has been used for medical purposes since 4000 BC in India's Vedic civilization. It was utilized as a culinary spice and had religious importance [10]. It has been shown that curcumin has antioxidant, anticancer, wound healing, anti-inflammatory, antimutagenic, antimicrobial, and antiviral properties [11]. Fig. 1 depicts the chemical structure of curcumin.

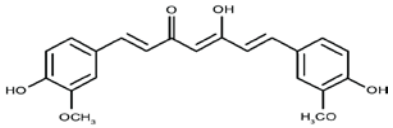

Fig. 1: Chemical structure of curcumin

Green tea is derived from the leaves of the Eastern Asian plant *Camilla sinensis* [12]. Catechin is a significant component of green tea. Catechin is a common term for four different substances: epigallocatechin (EGC), epigallocatechin gallate (EGCG), epicatechin gallate (ECG), and epicatechin (EC) [13]. EGCG is a polyphenol organic molecule in green tea that has been demonstrated to have therapeutic properties such as antioxidative, antifungal, anticancer, antiviral, and antimicrobial activities [14]. Fig. 2 depicts the chemical structure of EGCG.

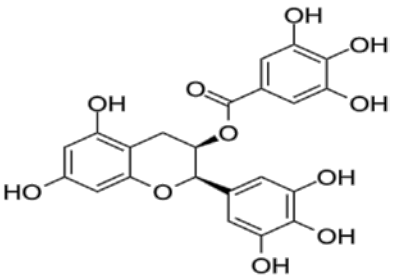

Fig. 2: Chemical structure of EGCG

Curcumin and EGCG have limited therapeutic potential due to low gastrointestinal stability and restricted membrane permeability across the gut [15]. In the research on anticancer activity, the combination of curcumin and EGCG culminated in a synergistic effect [16]. Nanostructure-based drug delivery methods, molecular modification, and simultaneous administration of other bioactive compounds can boost bioavailability. The nanoencapsulation of these drugs on carbohydrate-based, protein-based, and nanostructured lipid carrier (NLC) based nanoparticles enhanced stability, controlled release, and cell membrane penetration, resulting in greater bioavailability [17]. Many studies have found a high association between a medicine's drug loading values and its therapeutic activity-the higher the proportion of drug loading, the greater the therapeutic potential [18].

An analytical approach for estimating drug concentration in nanoformulations is required for designing a co-drug delivery system of bioactive compounds. Several studies of the literature indicate techniques for singular estimation of curcumin and EGCG, including high-performance thin-layer chromatography (HPTLC), liquid chromatography-mass spectrometry (LC-MS), high-performance liquid chromatography (HPLC), and ultra-high-performance liquid chromatography (UHPLC) [19-26]. However, no technique for determining and quantifying co-encapsulated curcumin and EGCG in a carbohydrate polymer system has been disclosed thus far. The present research aims to create an HPLC technique for rapidly identifying and estimating curcumin and EGCG in a co-encapsulated nanoformulation system in a carbohydrate matrix.

MATERIALS AND METHODS

Materials

Curcumin (purity>99 %) and EGCG (purity>99 %) were obtained from Sigma-Aldrich, USA. HPLC grade acetonitrile and methanol from Rankem, India, were utilized in the experiment. AR grade chemicals and reagents from Merck were utilized throughout. Ascorbyl palmitate was purchased from Gattefosse India Pvt. Ltd. (Mumbai, India). Locust bean gum (LBG), hydrolyzed shellac, hydroxypropyl methylcellulose (HPMC), rat plasma, and Aerosil 200 were all procured from Sigma-Aldrich, USA. Ethanol and diethylene glycol was purchased from SRL Pvt. Ltd., India. For the preparation of the mobile phase buffer, MilliQ water was used. Filters of 0.45 µm Millex syringe were used to filter the sample solutions.

UV wavelength selection

EGCG and curcumin were separately dissolved in methanol, and both solutions were scanned in a UV-Visible spectrophotometer in the 200-400 nm range. EGCG shows absorption maxima at 275 nm, whereas curcumin at 262 nm. Both spectra cross at 268 nm, which was the isosbestic wavelength. So, 268 nm was considered for UV detection. The overlay UV spectrum of curcumin and EGCG is shown in fig. 3.

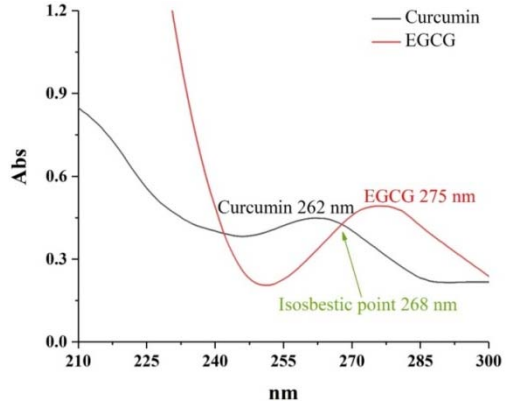

Fig. 3: UV spectrum overlay of curcumin and EGCG

Apparatus and chromatographic conditions

HPLC analyses were carried out on the Agilent HPLC system (Agilent 1260 series). A Shimadzu UV 1800 UV-visible spectrophotometer fitted with UV probe software was used for recording the UV spectrum. The HPLC system consisted of a quaternary pump, a diode array detector (DAD), a column oven compartment, and an autosampler. Chromatographic separations were carried out using a gradient elution mode on a Thermo Scientific Hypersil BDS C18 column, with a flow rate of 1.5 ml/min and a column oven temperature of 35 °C. The mobile phase consisted of (A) 0.025 M KH_2PO_4 buffer pH 4.0 and (B) acetonitrile. The gradient programming was 80 % A: 20 % B for the first 5 min, 40 % A: 60 % B for the next 5-13 min, and 80 % A: 20 % B for the last 13-16 min. The injection volume was 20 µl, and the UV wavelength of 268 nm was chosen for detection. The chromatographic conditions are given in table 1.

Table 1: Chromatographic conditions of the validated analytical method

Parameters	Conditions
Column	Thermo Scientific Hypersil BDS, C18 column (250 mm × 4.6 mm, 5 μm)
Mobile phase	Buffer (0.025 M KH_2PO_4, pH 4.0) and acetonitrile; Gradient programming
Diluent	Mobile phase
Flow rate	1.5 ml/min
Column oven tempt	35 °C
Detection wavelength	268 nm
Injection volume	20 µl
Run Time	16 min

Data processing

UV data were recorded and processed by UV probe software (Shimadzu). EZ Chrome Elite software (Aligent) was used to collect and process all HPLC data. The statistical calculation was performed by Origin software.

Preparation of stock and sample solution

EGCG and curcumin standard stock solutions are prepared in methanol at 500 µg/ml concentration. A mixed standard solution was prepared from individual standard stock solutions.

To determine the drug loading and entrapment efficiency, the necessary amount of nanoformulations was dissolved in methanol, and subsequent dilution was carried out using a diluent [27].

Method validation

International Conference on Harmonization (ICH) standards for system suitability, robustness, linearity, specificity, accuracy, precision, the limit of detection (LOD), and the limit of quantification (LOQ) were used to validate the method [28].

Specificity

Specificity refers to the capacity of the suggested method to distinguish the primary peaks from any additional impurities or degradation products. The method specificity was evaluated by using the HPLC technique to evaluate blank nanoparticle (drug-free) solutions and stress degradation solutions under various conditions. The obtained peaks were compared to the peaks of standard drug solutions. Apart from the peak of EGCG and curcumin, no interference peak was seen.

Linearity

Linearity studies were developed from a standard stock solution of curcumin (500 µg/ml) and EGCG (500 µg/ml). 12.5, 25, 50, 75, and 100 µg/ml concentrations of curcumin, and 25, 50, 100, 150, and 175 µg/ml concentrations of EGCG were prepared from standard stock solutions for linearity. Peak area as the Y-axis was plotted against linearity concentration as the X-axis to create the calibration curve.

Accuracy

The accuracy of the developed method was determined by adding known standards (at 75 %, 100 %, and 125 % levels) of EGCG and

curcumin to the nanoformulation. The percent recovery of the standards was determined for every level.

Precision

Three different concentration levels were prepared from each standard stock solution to determine the precision of the developed method, and percent relative standard deviation (RSD) was measured. Lower, middle and higher concentration levels were prepared at 12.5, 50, and 100 µg/ml for curcumin and 25, 100, and 175 µg/ml for EGCG. Every solution was injected on the same day in replicate (n=3) for the intraday precision study. For the interday precision study, every solution was kept in freezer and injected (n=3) on a different day (next day) under similar chromatographic conditions.

Sensitivity

The limit of detection (LOD) along with the limit of quantification (LOQ) was measured for the sensitivity of the analytical method. LOD means detecting the smallest concentration of the substance, and LOQ means quantifying the smallest concentration of the substance that can be detected within the acceptable parameters.

Robustness

The robustness of the proposed method was investigated by observing the effect of small intentional changes in chromatographic method parameters. The results were checked accordingly.

System suitability

One mixed standard solution of EGCG (100 µg/ml) and curcumin (50 µg/ml) was prepared and injected (20 µg/ml) into the HPLC system under refined chromatographic method parameters. The resolution, tailing factor, the number of theoretical plates, retention time, and other system suitability parameters were considered [29].

Forced degradation studies

5 ml was taken from each standard stock solution of EGCG (500 µg/ml) and curcumin (500 µg/ml) for various stress degradation studies such as acidic, basic, peroxide, photo light, thermal, and UV light. The solutions were diluted, having a concentration of 50 µg/ml for curcumin and 100 µg/ml for EGCG. Curcumin and EGCG were quantified against the known concentration of curcumin (50 µg/ml) and EGCG (100 µg/ml) [30]. Different forced degradation conditions of EGCG and curcumin are given in table 2.

Table 2: Optimized condition for forced degradation study of EGCG and curcumin

Stress type	Stress condition
Acid	5 ml of 0.1 (M) hydrochloric acid for 5 h at 80 °C
Base	5 ml of 0.1 (M) sodium hydroxide for 5 h at 80 °C
Peroxide	5 ml of 10 % v/v hydrogen peroxide for 5 h at 80 °C
Photo light	Kept in daylight for 5 h
Heat	Kept at 80 °C for 5 h
UV light	Kept in UV light at 254 nm for 5 h

Purposed method for application

The developed method was applied to determine the drug loading and drug entrapment efficiency study of curcumin and EGCG-loaded nanoparticles prepared in a carbohydrate matrix system [31].

Nanoparticle preparation by carbohydrate polymer-based system

For lipid phase preparation, curcumin, EGCG, and ascorbyl palmitate were dissolved in a beaker with diethylene glycol and ethanol at 40 °C and constantly stirred until the solution became clear. Next, the aqueous phase was obtained by dissolving hydrolyzed shellac, locust bean gum (LBG), and hydroxypropyl methylcellulose (HPMC) in MilliQ water at 70 °C, in a separate beaker. The lipid phase was gently added to the aqueous phase drop by drop, and then homogenization was conducted at 20,000 rpm for 10 min at 50 °C. Next, Aerosil 200 was added to the homogenized mixture and triturated for another 10 min at room temperature. Finally, the obtained nanoparticles were vacuum dried and kept at 4 °C until further analysis.

Nanoparticles characterization

Dynamic light scattering (Malvern Instruments, Model: ZEN 1600) was used to analyze the polydispersity index (PDI), average particle size, and the zeta potential values of co-encapsulated drug-loaded nanoparticles. Before instrumental analysis, sample solutions were made in MilliQ water (1 mg/ml).

Determination of drug entrapment efficiency and drug loading

To determine the drug loading and entrapment efficiency of curcumin and EGCG nanoparticles, 30 mg nanoparticles were dissolved in 10 ml methanol. Then, the centrifugation of the solution was performed at 5000 rpm for 15 min, and the 2 ml supernatant solution was diluted to 10 ml diluent. Next, a 0.45 µm syringe filter was used to filter the solution, and a volume of 20 µl was injected into the HPLC system. Finally, curcumin and EGCG were quantified against a known concentration of the mixed standard solution of curcumin (500 µg/ml) and EGCG (100 µg/ml).

Preparation of calibration standards spiked with plasma and quality control (QC) sample

Curcumin and EGCG individual standards (100 µg/ml) were prepared in methanol, and the mixed standard was prepared at a concentration of 50 µg/ml from the mother solution. Curcumin, EGCG calibration standard, and QC samples were prepared by spiking rat blank plasma with the mixed standard at a final concentration of 2, 4, 8, 10, and 12 µg/ml. Lower quality control (LQC), middle-quality control (MQC), and higher quality control (HQC) concentrations were 3, 7, and 13 µg/ml [32].

Curcumin and EGCG extraction from plasma

Plasma samples were kept at room temperature before the experiment. Each 100 µl plasma sample was added to ice-cooled 100 µl methanol and vortexed for 5 min to extract curcumin and EGCG. Plasma protein was precipitated, and the solution was centrifuged for 10 min. The supernatant was collected and dried under a vacuum oven. Finally, 100 µl diluent was mixed with the drugs in the tube and sonicated for 1 min. 20 µl sample was injected into the HPLC system to quantify drugs [33].

RESULTS AND DISCUSSION

Evolution of the method

The HPLC method was optimized during method development by changing different chromatographic parameters. Different columns like cyano, phenyl and various columns were used. The change of mobile phase composition in gradient elution mode, change of flow rate, and change of mobile phase pH were used for method optimization. EGCG and curcumin UV spectrum was recorded, and 268 nm isosbestic wavelength was selected for UV detection. After variation of different parameters, the proposed method was fixed to quantity EGCG, and curcumin was eluted at 3.9 min and 10.7 min, respectively. Quercetin was used as an internal standard (IS) and was eluted at 7.8 min between two peaks of EGCG and curcumin. All the system suitability parameters were found within the limit. HPLC chromatograms of curcumin, EGCG, and quercetin (IS) in single and mixed are represented in fig. 4, 5, 6, and 7, respectively.

Method validation

The RP-HPLC method was validated per ICH guidelines for system suitability, robustness, linearity, specificity, accuracy, precision, sensitivity, and robustness [35].

Specificity

EGCG and curcumin peaks were found at a retention time of 3.9 min and 10.7 min, and the internal standard quercetin peak was found at 7.8 min. The absence of overlapping peaks in the nanoparticle sample demonstrates the specificity of the method. Furthermore, after various stress degradation studies, the peaks of EGCG and curcumin were clearly distinguished from the degradation products [28].

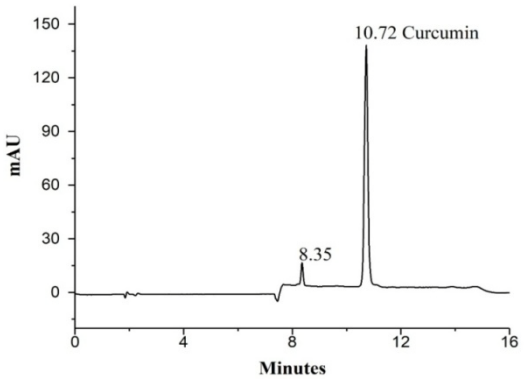

Fig. 4: Chromatogram of curcumin standard

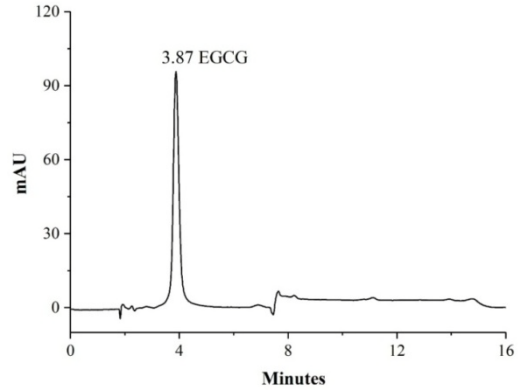

Fig. 5: Chromatogram of EGCG standard

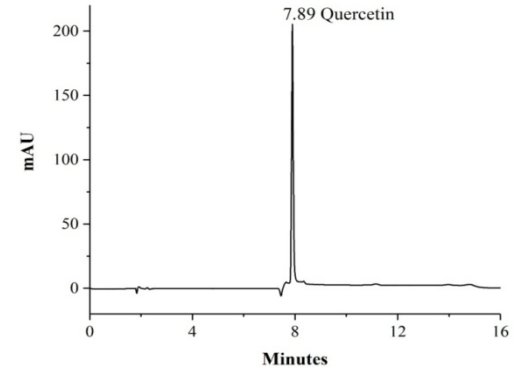

Fig. 6: Chromatogram of quercetin standard (Internal standard)

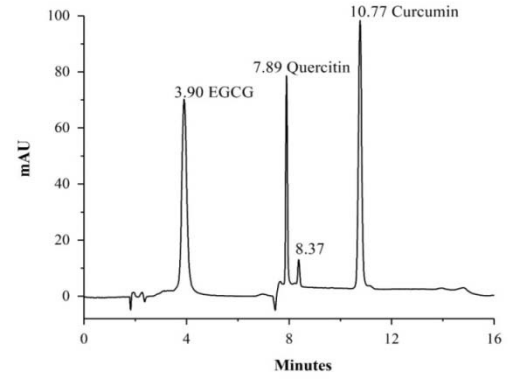

Fig. 7: Optimized chromatogram of curcumin, EGCG, and quercetin in mixed standard

Linearity

The linearity concentration of EGCG and curcumin was 25-175 µg/ml and 12.5-100 µg/ml, respectively. The regression equation for EGCG was found y = 46092 x+20019 whereas, for curcumin, y = 51643 x–17550. The linearity curve's correlation coefficient for EGCG was 0.991, and for curcumin, it was 0.999, both of which were within the acceptable range. The method fulfills the acceptance requirements for linearity [36]. Thus the method is linear for determining EGCG and curcumin. The calibration curves for curcumin and EGCG are represented separately in fig. 8 and 9, respectively.

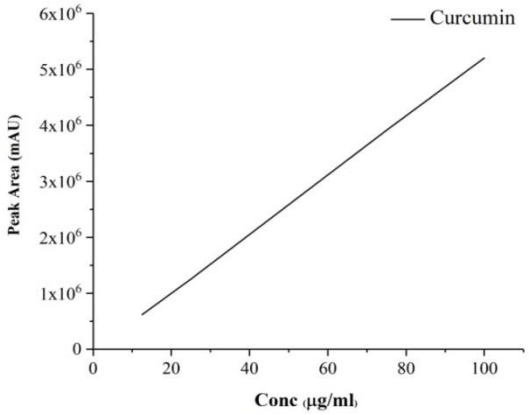

Fig. 8: Calibration curve of standard curcumin

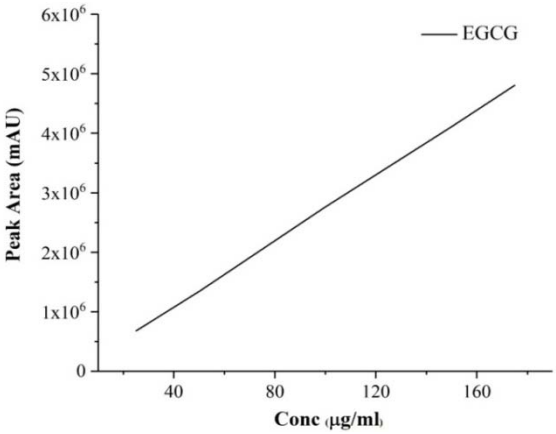

Fig. 9: Calibration curve of standard EGCG

Accuracy

The concentration of EGCG and curcumin were 100 µg/ml and 50 µg/ml in the mixed standard. The average recovery of EGCG and curcumin was 97.90-98.63 %, within the acceptable range [37]. Thus the method was highly accurate for quantifying EGCG and curcumin. Accuracy data are given in table 3.

Precision

The RSD % value for intraday and interday precision was found to be 0.068-0.321 for EGCG and 0.129-0.421 for curcumin. Thus, it has been observed that the current method shows very precise results, with RSD values less than 2 % (acceptable range) [38]. From the data, it means the method was very precise. The precision data are tabulated in tables 4 and 5.

Sensitivity

The LOD value was 2.51 µg/ml for EGCG and 1.02 µg/ml for curcumin. The LOQ value were 7.53 µg/ml for EGCG and 3.05 µg/ml for curcumin. These low values demonstrated that the developed method was very sensitive to quantifying EGCG and curcumin [39].

Table 3: Accuracy data by the proposed HPLC method

Drugs	Spiked level (%)	Spiked amount (mg)*	Recovered amount (mg)*	Recovery (%)	RSD (%)
EGCG	75	7.52±0.16	7.39±0.12	98.27	0.67
	100	11.92±0.35	11.67±0.35	97.90	0.58
	125	16.13±0.28	15.91±0.21	98.63	0.54
Curcumin	75	7.46±0.25	7.31±0.17	97.98	0.52
	100	12.13±0.19	11.92±0.38	98.27	0.37
	125	15.93±0.33	15.69±0.25	98.49	0.45

RSD–Relative Standard Deviation; *All the values are presented as mean±SD, n=3

Table 4: Intraday precision data by the proposed HPLC method

Drugs	Nominal concentration (µg/ml)	Recovered concentration (µg/ml)*	Recovery (%)*	Precision (Repeatability) % RSD
EGCG	50	49.31±0.56	98.62±1.21	0.210
	100	99.22±0.35	99.22±0.68	0.068
	150	149.29±0.89	99.52±0.56	0.071
Curcumin	25	24.33±0.55	97.32±0.71	0.421
	50	49.41±0.62	98.82±0.36	0.318
	75	74.34±0.48	99.12±0.65	0.212

RSD–Relative Standard Deviation; *All the values are presented as mean±SD, n=6

Table 5: Interday precision data by the proposed HPLC method

Drugs	Nominal concentration (µg/ml)	Recovered concentration (µg/ml)*	Recovery (%)*	Precision (Repeatability) % RSD
EGCG	50	48.73±0.63	97.46±0.58	0.321
	100	98.31±0.41	98.31±1.32	0.225
	150	147.87±0.58	98.58±0.87	0.105
Curcumin	25	24.18±0.81	96.72±0.72	0.412
	50	48.54±0.72	97.08±0.53	0.129
	75	73.76±0.54	98.34±0.25	0.165

RSD–Relative Standard Deviation; *All the values are presented as mean±SD, n=6

Robustness

The robustness of the developed method was evaluated by various chromatographic parameters that were intentionally altered, like flow rate, column temperature, buffer pH, and wavelength for UV detection. Low RSD values were obtained from the robustness study, which indicated that the established method was robust [40]. The robustness data for EGCG and curcumin are summarized in table 6.

Table 6: Robustness parameter of the proposed method

Parameters	% RSD of the area of EGCG	% RSD of the area of curcumin
A. Change in buffer pH of mobile phase		
3.4	0.061	0.083
3.5	0.081	0.673
3.6	0.072	0.731
B. Change in UV detector wavelength (nm)		
266	0.131	0.157
268	0.152	0.235
270	0.126	0.321
C. Change in flow rate (ml/min) of mobile phase		
1.4	0.089	0.053
1.5	0.132	0.139
1.6	0.087	0.158
D. Change in column oven temp(°C) of mobile phase		
29	0.113	0.105
30	0.136	0.162
31	0.083	0.087

RSD–Relative Standard Deviation; n=3

Table 7: System suitability parameters of the validated analytical method

Parameters	EGCG	Curcumin
Retention time	3.9±0.03	10.77±0.04
Theoretical plates/meter	3414±131	44128±121
Tailing factor	1.36±0.03	1.29±0.04
Resolution	-	17.86±0.05
Injection precision	0.073±0.02	0.05±0.03

SD-Standard Deviation; All the values are presented as mean±SD, n=6

System suitability

The system suitability studies were carried out to ensure the appropriate working of the established system. The resolution between EGCG and curcumin peak was 22.9±0.03, which indicates that both drugs were well separated. The peak asymmetry values were 1.04±0.06 for EGCG and 1.08±0.03 for curcumin. The theoretical plate number per meter values were 3414±131 for EGCG and 44128±121 for curcumin. The results obtained satisfied the criteria as per ICH Q2R1 guidelines [32, 34]. The system suitability values are given in table 7.

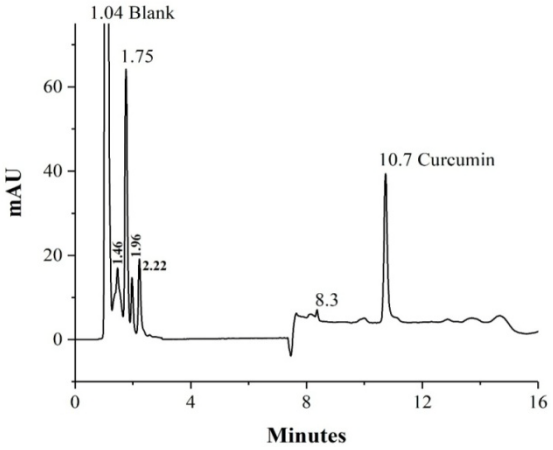

Fig. 10: Forced degradation chromatogram of standard curcumin in 0.1 (M) sodium hydroxide

Forced degradation studies

The EGCG and curcumin standards were subjected to various forced degradation conditions like acidic, basic, oxidative, thermal, photolytic, and UV to induce partial degradation of the compound. Forced degradation studies have been conducted to determine if the developed method is appropriate for the degradation products [38, 40]. Furthermore, the studies provide information on unstable drug conditions so that precautions can be taken during formulation to avoid any instabilities [41].

Curcumin was degraded when exposed to 0.1 (M) NaOH, 10 % H_2O_2 solution, and 0.1 (M) HCl solution. The recovered amount of curcumin was found at 20.48 %, 30.75 %, and 55.88 % for alkaline, peroxide, and acidic degradation. On the other hand, EGCG was also degraded when exposed to 0.1 (M) NaOH, 10 % H_2O_2 solution, and 0.1 (M) HCl. The recovered amount of EGCG was found at 24.32 %, 49.42 %, and 84.97 % for alkaline, peroxide, and acidic degradation.

Curcumin produces about four degradation products after base degradation, one after peroxide degradation, and no degradation product after thermal, photolytic, and UV degradation. Upon base degradation, EGCG produces one degradation product, peroxide degradation produces one degradation product, acid degradation produces one degradation product, and thermal, photolytic, and UV degradation produces no degradation product. All data are represented in table 8.

The proposed method for application

Nanoparticles characterization

Nanoparticles prepared with a carbohydrate polymer-based system had a mean particle size of 104 nm, a PDI value of 0.21, and an average zeta potential of+8.75 mV. Table 9 and fig. 22 show the particle size distribution of the produced nanoparticles.

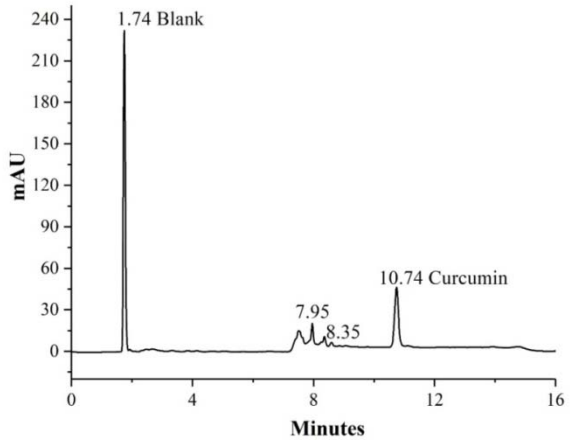

Fig. 11: Forced degradation chromatogram of standard curcumin in 10 % v/v hydrogen peroxide

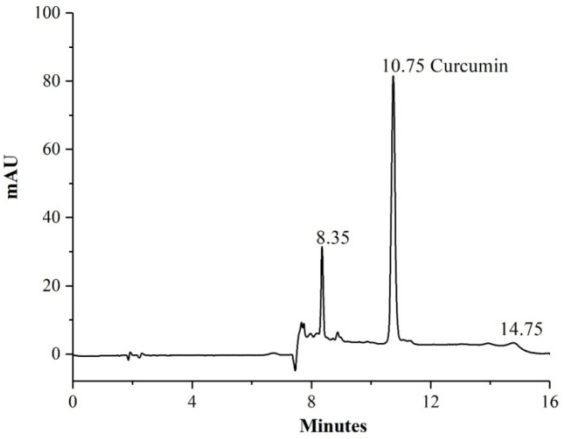

Fig. 12: Forced degradation chromatogram of standard curcumin in 0.1 (M)HCl

Table 8: % Recovery and degradation of EGCG and curcumin after stress conditions

Stress conditions	% Recovery (±SD)		% Degradation (±SD)	
	EGCG	Curcumin	EGCG	Curcumin
Alkali	24.32±0.68	20.48±0.57	75.68±0.87	79.52±1.13
Peroxide	49.42±0.58	30.75±1.21	50.58±1.75	69.25±0.78
Acidic	84.97±0.87	55.88±1.32	15.03±1.83	44.12±0.27
Thermal	99.95±0.28	89.25±0.53	0.05±0.23	10.75±0.83
Photolytic	100.13±0.39	100.18±0.59	0±0.38	0±0.36
UV light	100.21±0.49	99.53±0.37	0±0.53	0.47±0.63

SD-Standard Deviation; All the values are presented as mean±SD, n=3, The HPLC chromatograms after each degradation are represented in fig. 10-21, respectively.

Table 9: Characterization of EGCG and curcumin entrapped nanoparticle

Nanoparticle formulation	Particle size diameter (nm)*	PDI	Zeta potential value (mV)	%EE*	
				EGCG	Curcumin
EGCG and curcumin-loaded nanoparticles	104±2.4	0.21	8.75	94.35±2.6	95.12±1.8

PDI-Polydispersity Index; EE-Entrapment Efficiency. *All the values are presented as mean±SD, n=3

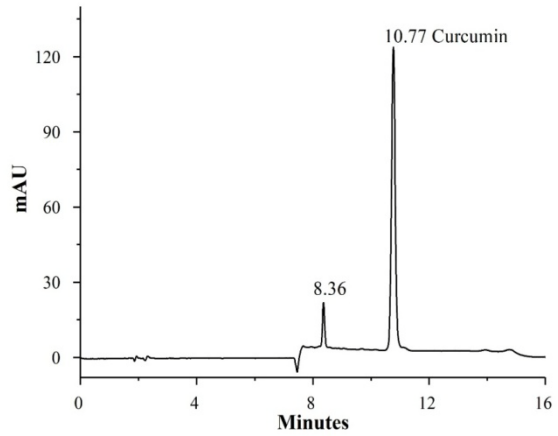

Fig. 13: Forced degradation chromatogram of standard curcumin in heat

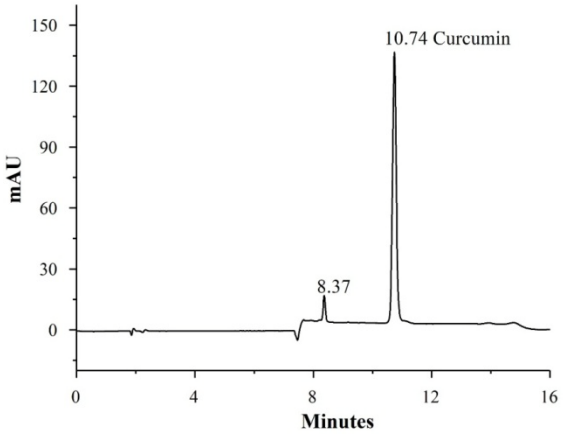

Fig. 14: Forced degradation chromatogram of standard curcumin in photolytic

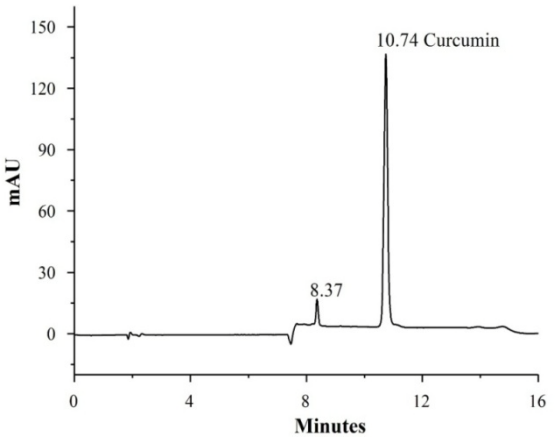

Fig. 15: Forced degradation chromatogram of standard curcumin in UV light

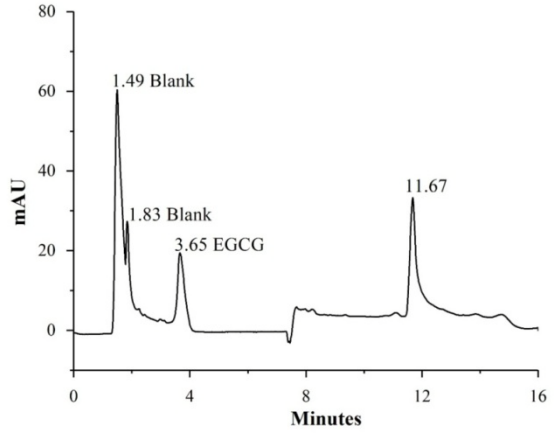

Fig. 16: Forced degradation chromatogram of standard EGCG in 0.1 (M) sodium hydroxide

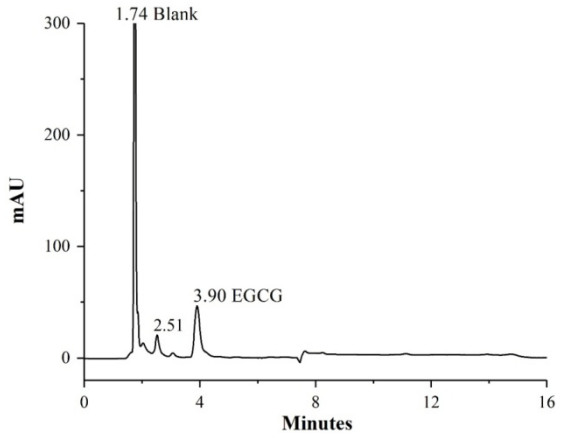

Fig. 17: Forced degradation chromatogram of standard EGCG in 10 % v/v hydrogen peroxide

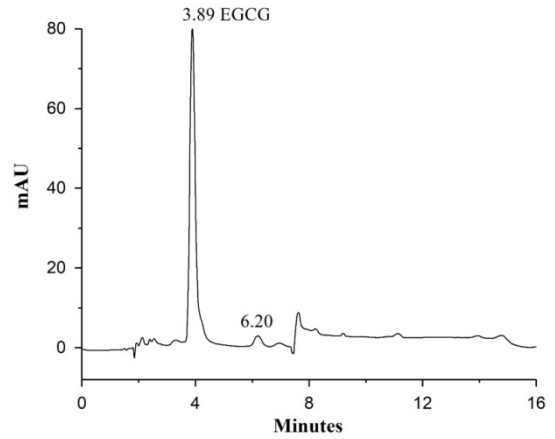

Fig. 18: Forced degradation chromatogram of standard EGCG in 0.1 (M)HCl

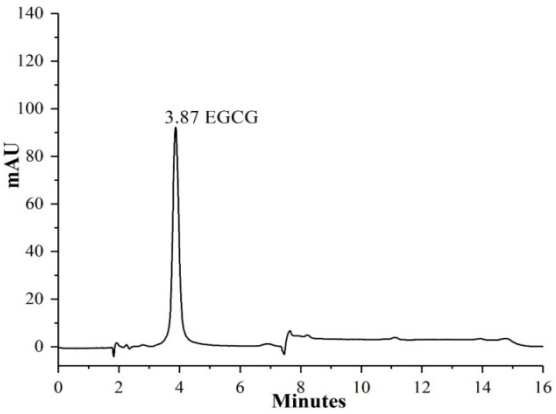

Fig. 19: Forced degradation chromatogram of standard EGCG in heat

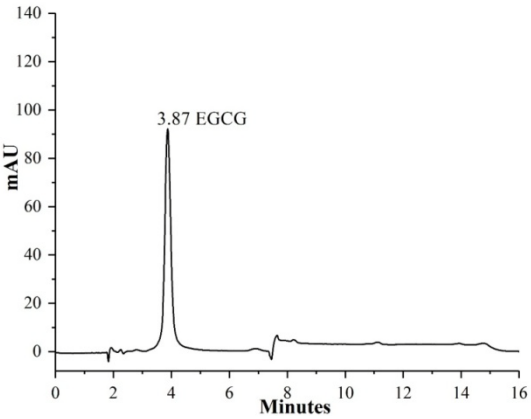

Fig. 20: Forced degradation chromatogram of standard EGCG in photolytic

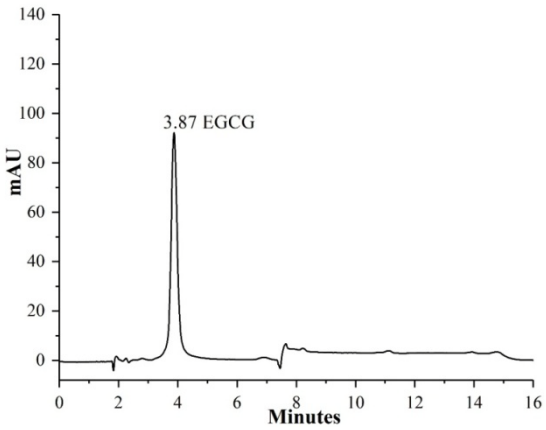

Fig. 21: Forced degradation chromatogram of standard EGCG in UV light

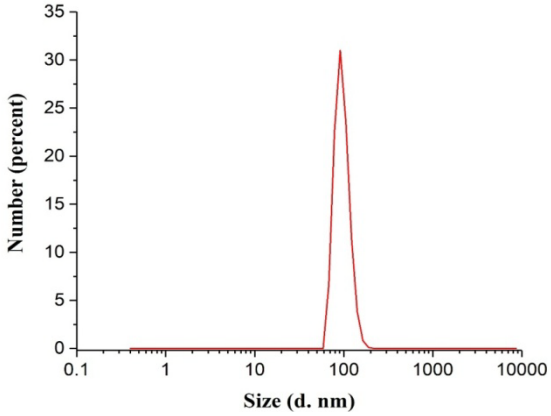

Fig. 22: Particle size distribution plot of curcumin and EGCG nanoparticles

Determination of drug entrapment efficiency and drug loading

The drug loading and drug entrapment efficiency were determined against a known amount of mixed standards. The drug loading was found to be 17.56±0.83 for EGCG and 16.69±0.68 for curcumin, and the drug entrapment efficiency was found to be 94.35±2.6 for EGCG and 95.12±1.8 for curcumin. The standard internal quercetin was spiked in both sample and standard solution, and no peak deviation of the internal standard was observed. The result of drug loading and drug entrapment efficiency is given in table 9. The HPLC chromatogram of drug-loaded nanoparticles is shown in fig. 23.

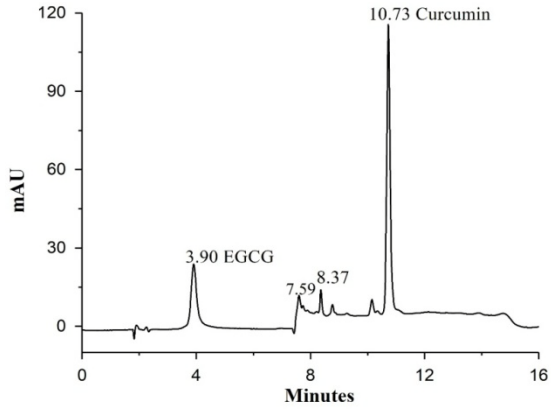

Fig. 23: HPLC chromatogram of curcumin and EGCG in drug-loaded nanoparticles

EGCG and curcumin extraction from plasma

The LQC, MQC, and HQC values after extraction from plasma were 91.23 %, 90.89 %, 91.76 % for EGCG, and the extraction values from plasma were 90.56 %, 91.81 %, 92.7 % for curcumin. This result indicates that the proposed HPLC method may be used for various *in vivo* pharmacokinetic studies [28]. The chromatogram of blank plasma and mixed standard spiked with internal standard and rat plasma are given in table 10 and fig. 24 and 25, respectively.

Table 10: Recovery studies of the spiked EGCG and curcumin from rat plasma

Drugs	LQC recovery (%)	MQC recovery (%)	HQC recovery (%)
EGCG	91.23±0.36	90.89±0.87	91.76±0.83
Curcumin	90.56±0.67	91.81±0.73	92.7±0.85

LQC-Lower Quality Control; MQC-Middle-Quality Control; HQC-Higher Quality Control, All the values are presented as mean±SD, n=3

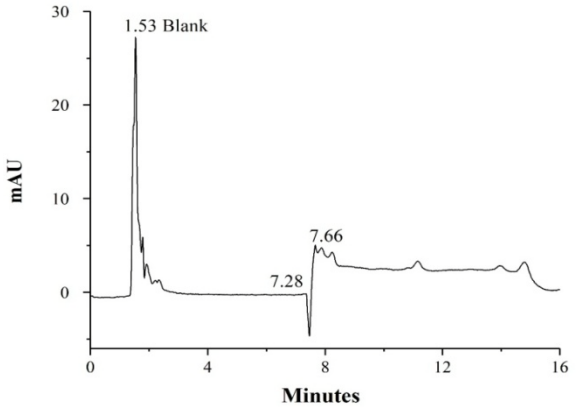

Fig. 24: HPLC chromatogram of blank plasma

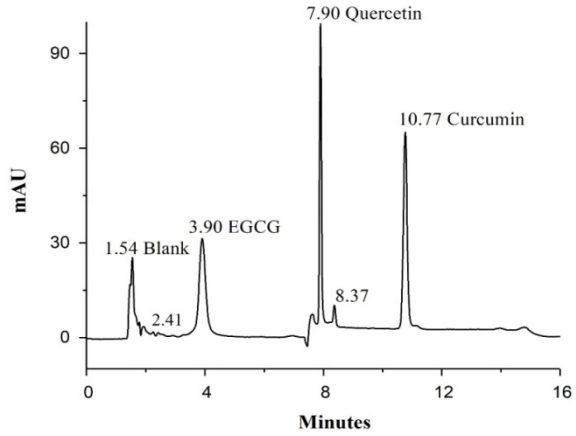

Fig. 25: HPLC chromatogram of curcumin and EGCG in the plasma sample

CONCLUSION

The developed RP-HPLC technique was simple and suitable for estimating curcumin and EGCG simultaneously in the novel nanoformulation and rat plasma. The developed method was satisfied for precision, specificity, sensitivity, linearity, accuracy, and robustness studies. In the degradation study, all the degradation peaks were well separated from the principle peaks of EGCG and curcumin. The system suitability parameters were all within the acceptable range. The mobile phase composition and sample preparation were simple and easy to prepare. The mobile gradient phase easily separated two peaks, and the resolution between the two peaks was excellent. The developed method was very sensitive to detecting and quantifying the minimum amount of curcumin and EGCG in the sample. The developed method may help to quantify drugs for bioequivalence or bioavailability studies.

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AUTHORS CONTRIBUTIONS

All authors have contributed equally.

CONFLICTS OF INTERESTS

The authors have declared no conflict of interest.

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