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Research Article

Curcuminoids -RP-HPLC Development

Method Development and Validation of Curcuminoids in *Curcuma Angustifolia* Roxb. By RP-HPLC and Its Antifungal Activity

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Abstract: *Curcuma angustifolia* Roxb., a rhizomatous herb, belongs to the Zingiberaceae family of plants. The species is rich in nutrients and is used mostly as a source of starch for Indian dishes and medicines. The published research articles for the measurable estimation of components in *Curcuma angustifolia* Roxb. rhizomes are very scarce. The present study aimed to develop a quantitative analysis method for estimating the constituents in the methanolic extract of *Curcuma angustifolia* Roxb. rhizome and to investigate its biological activity. Within the context of this challenge, the current work is a unique effort that recommends a high-performance liquid chromatography (HPLC) method for estimating curcuminoids, namely curcumin, demethoxycurcumin, and bis-demethoxycurcumin, present in the methanolic extract of *Curcuma angustifolia* Roxb. rhizome. A reverse-phase HPLC method was developed to estimate curcuminoids in the extract. The method was validated and found to be precise, sensitive, specific, linear, accurate, and robust. The method validation study's findings demonstrated the method's suitability for routine use. The HPLC method was successfully applied to a marketed formulation containing rhizome powder from *Curcuma angustifolia* Roxb. as one of its constituents. This study investigates the antifungal activity of the methanolic extract *in vitro* using an agar well diffusion assay method. A minimum inhibitory concentration (MIC) was established, and the antifungal activity was evaluated against *Candida albicans* and *Candida glabrata* with fluconazole as the standard reference. The development and validation of an HPLC method for testing curcuminoids in *Curcuma angustifolia* Roxb. rhizome and the evaluation of rhizome extract antifungal activity are excellent examples of herbal medicine quality control in its entirety. The *in vitro* antifungal activity results reaffirm the usage of *Curcuma angustifolia* Roxb. as a source of antifungal agents. They can lead to the developing of new herbal medicines in the coming days.

Keywords: *Curcuma angustifolia* Roxb., Rhizome, HPLC method, curcuminoids, antifungal activity

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1. INTRODUCTION

About 110 species of the highly significant genus Curcuma, belonging to the Zingiberaceae family, are found throughout tropical Asia and Asia-Pacific. In addition to Korea, China, Australia, and the South Pacific, the genus has the highest level of diversity in India, Myanmar, and Thailand.[1]Herbs that are either annuals or perennials can be included in the Curcuma genus, which is known for its rhizomatous plants. The species can be found in broad-leaved evergreen woods and tropical forests in their natural habitats. Applications in a wide variety of fields, as well as significant repercussions for the economy, can be found in the Curcuma genus. Because of the diverse applications that can be found for plants belonging to the genus Curcuma, the importance of these plants on a global scale is steadily growing. This is because these plants have a wide range of potential uses. Several research initiatives and exploratory endeavors have focused on these plants directly over the past few years due to the reasons above.[2] *Curcuma angustifolia* Roxb. an herb species from the Curcuma genus, commonly known as Wild or East Indian arrowroot, narrow-leaved turmeric, is innate to the Asian subcontinent, distributed in India, Laos, Myanmar, Nepal, and Pakistan. It is found in the wild in India's Bengal, northeastern areas, and western coastal plains and hills. It is found plentiful in Madhya Pradesh, Chhattisgarh, Orissa, and Andhra Pradesh, as well as in the uplands of Tamil Nadu and Kerala. It is cited in ancient history as a source of arrowroot.[1]Tubers, the principal resource of *C. angustifolia,* is regarded as an item of food. According to local healers, they enhance vigor and fatten the body.[3] The species has a high nutritional value, particularly when used as a source of starch for traditional Indian cuisine and traditional Indian medicine.[4] *C. angustifolia* is known to have a variety of therapeutic qualities ascribed to bioactive substances present in various plant components. Diphenylheptanoids, also called curcuminoids, are an extensive class of constituents inherent in nature. In the Curcuma genus and the Zingiberaceae family, aryl–C7–aryl diarylheptanoids are prevalent. Curcumin, demethoxycurcumin (DMC), and bis-demethoxycurcumin (BDMC) are the three most abundant diphenylheptanoids within the genus Curcuma.[1] Curcuminoids, which have several curative properties, have been reported as being present in the rhizomes of *C. angustifolia* in published research.[5,6]The review of the literature shows that there are quite a few HPLC methods for estimating curcuminoids. However, almost all these methods are for *Curcuma longa* (turmeric).[7–13] It has been demonstrated that the methanolic extract of the rhizomes of the *C. angustifolia* plant possesses several bioactivities, some of which are anti-cancer, anti-bacterial, and antioxidant properties.[14] The leaf essential oil has been shown to have antibacterial and antioxidant properties. [15,16] The rhizome essential oil is reported to have antibacterial properties.[17] Only a few antifungal medications are effective against Candida species, opportunistic human fungal pathogens that cause acute and chronic infections.[18] Many infections, both minor and serious, have been linked to the presence of Candida species. Even though *Candida albicans* remains the most frequent species recovered from clinical samples, the occurrence of non-albicans Candida infections triggered by species such as *Candida glabrata, Candida auris, Candida krus*ei, and others has rapidly grown.[19] As per the literature survey, no methods are recorded for assessing markers in *C. angustifolia* rhizomes. The current work presents an accurate, specific, precise, and validated HPLC method for quantitatively estimating curcuminoids in the rhizome extract of *C. angustifolia*. The developed method is being used to test a commercial product: Kerakhand Granules, which treat internal ulcers and painful conditions in rheumatoid arthritis and contain *C. angustifolia* rhizome powder as one of their constituents. Significant antibacterial activity was shown by the methanolic extract of *Curcuma angustifolia* Roxb. against pathogenic bacteria such as *Escherichia coli, Staphylococcus aureus, Shigella flexne*ri, and *Pseudomonas aeruginosa.*[15] The antifungal activity of *Curcuma angustifolia* Roxb. rhizomes has never been reported. This study demonstrates the *in vitro* antifungal activity of methanolic extracts of *C. angustifolia* rhizomes.

2. MATERIALS AND METHODS

2.1. Plant Materials

Curcuma angustifolia Roxb. rhizome was acquired from Jorhat, Assam, and Dr. L. B. Chaudhary verified the plant from the CSIR National Botanical Research Institute; Lucknow, with the Accession No. It was provided by CSIR-NBRI Herbarium (LWG) 109910.The rhizome was cleaned under moving water, expurgated into miniature pieces, and kept for drying for five days undercover. Further, it was powdered in a mixer grinder and strained to achieve an excellent powder. Future experiments employed the powdered rhizomes, which were stored in closed jars.

2.2. Chemicals and standards

Methanol and acetonitrile (ACN) used for the study were HPLC grade (Merck); formic acid was purchased from Merck; and HPLC grade water from Oxford Lab Fine Chemicals was used throughout the analysis. The standards for curcumin, demethoxycurcumin (DMC), and bis-demethoxycurcumin (BDMC) (purity > 98%) were purchased from Zeta Scientific LLP, Mumbai, India. Whatman Filter Paper 41 and MDI 0.45 µm nylon syringe filters were used to filtrate extracts. For the antifungal study, two organisms were used: *Candida albicans* ATCC 10231 and *Candida glabrata* ATCC 48435. Fluconazole was used as a positive control for the study. Sterile Sabourauds dextrose broths (1X and 2X) from Himedia, INT dye, Resazurin dye, Mueller- Hinton broth from Himedia, and absolute ethanol from Changshu Hongsheng were used for the study.

2.3. HPLC Analysis

2.3.1. Instrumentation

A Shimadzu ultrafast liquid chromatography system was used to carry out the analysis. Separations were conducted on a Phenomenex Luna 5 µm C8 100A liquid chromatography (LC) Column (250 mm x 4.6 mm).

Table 1: HPLC conditions for separation and estimation of curcuminoids in the methanolic extract *of C. angustifolia* rhizomes

Parameters	Chromatographic conditions
Mobile Phase	0.1% formic acid: acetonitrile (52:48)
Diluent	Methanol
Flow	1.2 mL/min
Column oven temperature	40 °C
Injection volume	10 µL
Wavelength	420 nm
Run time	Standard -12 min Sample - 20 min

Table 1 details the parameters and chromatographic conditions of the HPLC method. Isocratic elution was utilized with the mobile phase of 0.1% formic acid: ACN (52:48 v/v) mixture, a 1.2 mL per min flow rate, and the column temperature at 40°C. The injection volume was 10 µL, and a PDA detector was used for the analysis with chromatographic detection at 420 nm. Utilizing a retention time comparison, the peak identities were verified.

2.3.2. Standard preparation

Individual stock solutions of curcumin, DMC, and BDMC were prepared by dissolving the standards using methanol as a diluent. The stock solutions are further diluted with methanol to achieve an approximate 100 µg/mL concentration of each curcuminoid. The details of the standard preparation are tabulated in Table 2.

Table 2: Standard preparation details

Analyte	weight of standard consumed (mg)	dilution (mL)	standard stock solution concentration (µg/mL)	standard stock solution volume (mL)	dilution (mL)	final the concentration of the standard working solution (µg/mL)
BDMC	26.7	25	1046.640	1.0	10	104.664
DMC	11.6	10	1137.148	0.9		102.343
Curcumin	10.6	10	1056.608	1.0		105.661

2.3.3. Sample preparation

Approximately 3 g of plant rhizome powder was placed in a round-bottom flask and refluxed[20] for 20 minutes with 50 mL of methanol using a water condenser. The flask's contents were filtered through Whatman filter paper 41 after cooling. A clear filtrate was obtained. The obtained solution was filtered using a 0.45 µm nylon syringe filter before being injected into the HPLC apparatus. The sample concentration was approximately 60000 µg/mL.

2.3.4. Method validation

System suitability: Under optimized method conditions, the system suitability was assessed by injecting five replicate analyses of the standard working solution, which contained about 100 ppm of each curcuminoid. Specificity: The specificity was shown by injecting the blank (diluent) into the HPLC system and ensuring no peaks at the same retention time as the analytes (BDMC, DMC, and curcumin). Precision: Six sample preparations were injected into the HPLC system to evaluate the method's repeatability as part of precision. Quantitation Limit (QL) and Detection Limit (DL) (sensitivity): Since the method estimates curcuminoids (assay), QL was set at 0.05% of the standard working concentration, and the DL was one-third of the QL concentration. Linearity: The linearity experiment used nine levels. By diluting standard stock solutions, standard solutions for curcumin, DMC, and BDMC were created to evaluate linearity from QL to the standard working solution level. Accuracy: The accuracy of the powdered sample was demonstrated by creating and evaluating samples at 50% and 150% of the sample range utilized in precision experiments in triplicate. Robustness: Flow, column temperature, and mobile phase composition were changed to test the method's robustness. This information would help assess the method's performance and dependability under modified operational conditions.

2.3.5. Method applicability to the Marketed Finished Product (MFP)

The suitability of the HPLC method for detecting curcuminoids in *Curcuma angustifolia* Roxb. listed as an ingredient in a marketed finished product (MFP) was assessed.

2.3.5.1. Sample preparation for marketed finished product (MFP)

In a mortar and pestle, crush the finished product until it is powdery. Transfer 15 g of the powdered sample into a round-bottom flask and reflux it with 25 mL of methanol using a water condenser for 20 minutes. After cooling, the flask contents were filtered through the Whatman filter paper 41. The filtrate was filtered again through a 0.45 µm nylon syringe filter before being injected into the HPLC system. A sample spiked with curcuminoids was also injected into the system, wherein 15 g of the powdered sample was transferred into a round-bottom flask and added 1.0 mL, 0.9 mL, and 1.0 mL of Curcumin, DMC, and BDMC standard stock solutions, which were then refluxed with 20 mL of methanol in a water condenser for 20 minutes. After cooling, the mixture was filtered through Whatman filter paper 41, and the filtrate was passed through a 0.45µ nylon syringe filter and injected into the HPLC system.

2.4. Antifungal Activity

The antifungal activity was performed on methanolic extracts of *Curcuma angustifolia* Roxb. rhizome.

2.4.1. Preparation of methanol extract

3 g of rhizome powder was refluxed for 20 minutes with 50 mL of methanol in a round-bottom flask. After cooling, the flask's contents were filtered with Whatman paper 41 to obtain a clean filtrate. The filtrate was evaporated to dryness, and the residue was diluted and used for antifungal activity.

2.4.2. Determination of Minimum Inhibitory Concentration (MIC)

The MIC was determined using a microtiter plate assay. This assay used 96-well clear flat-bottom microtiter plates. The first row of each column was filled with 100 µL Mueller-Hinton broth with antibiotics, and the second row with 100 µL media. Next, 100 µL of 2X media and samples were added to the third row. From the fourth row onward, each well contained 1X media. From the 3rd to the 12th row, a 2-fold serial dilution was done. Next, 10 µL of culture was added to each well and mixed thoroughly with 1 x 10^6 CFU/mL. The final concentrations in the 3rd to 12th rows ranged from 50% dilutions to 0.1% dilution. The cultured microplate was sealed with the lid and incubated at room temperature for 24 hours. The MIC of samples was detected following the addition (5µL) of 0.1 mg/mL indicator (INT dye for *Candida albicans* and resazurin dye for *Candida glabrata*) in all the wells and incubation at 37°C for 30 min. The smallest sample concentration that exhibits full suppression of microbial growth and no color change (clear) was established as the MIC.

2.4.3. Determination of antifungal activity

2.4.3.1. Mechanism of Curcuma angustifolia Roxb. extract against C. albicans and C. glabrata

Fungal inoculums were prepared from overnight-grown cultures (24 hours) in Sabouraud dextrose agar (SDA). The positive control for antifungal activity was fluconazole (50 ppm). Saline was taken as a negative control to ascertain the possible inhibitory activity of the dilutant extract. The agar cup diffusion method was used to determine the sensitivities of different fungal strains. 20 mL of SDA was taken into sterile universal bottles. These were then inoculated with 1.0 mL of an overnight culture of the test organism, mixed gently, and poured into sterile Petri dishes. After setting, wells of 7 mm were punctured with the help of a sterilized cork borer having a diameter of 7 mm into the pre-solidified SDA plates containing the test organism.[21] Using the micropipette, 50 µL of each extract was poured into the different wells of the inoculated plates. Standard antifungal was used as a positive control, and fungal plates were incubated at 32 °C for 24 hours. The diameter of the zone of inhibition was measured. Each experiment was done in duplicate, and mean values were taken. Antifungal activity was measured by the diameter (mm) of the clear inhibitory zone formed around the well.

3. STATISTICAL ANALYSIS

Using mathematical and statistical methods, the primary goal of data assessment is to summarise and understand a specific data collection.[22] The data sets are evaluated statistically by measuring variances, correlation coefficients, slopes, means, and standard deviations.

4. RESULTS AND DISCUSSION

4.1. HPLC method

4.1.1. Method Development and Optimization for the HPLC Method

Curcuminoids are water-insoluble but have good solubility in methanol; therefore, considering the solubility aspect, methanol was chosen as the diluent for standards and samples. Also, as the extraction efficiency of the extract obtained by refluxing for twenty minutes is similar to that of Soxhlet extraction, refluxing was decided as the mode for sample preparation considering the ease and shorter time for sample preparation. Several methods are reported for estimating curcuminoids in different curcuma species. However, most methods use the octadecyl (C18) column; only some use the phenyl column, and there are no reports for estimating curcuminoids using the octyl (C8) column. When shorter retention times are desired, C8 is employed. Because lower hydrophobicity causes greater retention for non-polar molecules, nonpolar substances travel down the column faster with C8 than with C18. If a reverse-phase matrix with less hydrophobicity is preferred, C8 is chosen over C18.[23] In organic solvents, curcuminoids strongly absorb between 420 and 430 nm; hence, 420 nm was the wavelength used for detection. The mobile phase composition was optimized to a ratio of 52:48 of 0.1% formic acid and acetonitrile. This mobile phase can be used directly in LCMS to identify any unidentified component or mass in the sample because the formic acid modifier is also compatible with LCMS.[24,25] The flow rate and column temperature were adjusted to 1.2 ml/min and 40 °C for optimal peak shape, symmetry, and resolution between the peaks. In addition, a 10 µl injection volume was chosen for higher method sensitivity and improved peak shape. BDMC was the first compound to elute with a retention time of approximately 7.845 minutes, followed by DMC and curcumin at 8.466 and 9.151 minutes, respectively. The elution sequence was determined by injecting individual standards and comparing the times they were retained. When measured in relation to curcumin, the relative retention durations of BDMC and DMC are approximately 0.85 and 0.92. Run times of Standard and samples were 12 and 20 minutes individually.

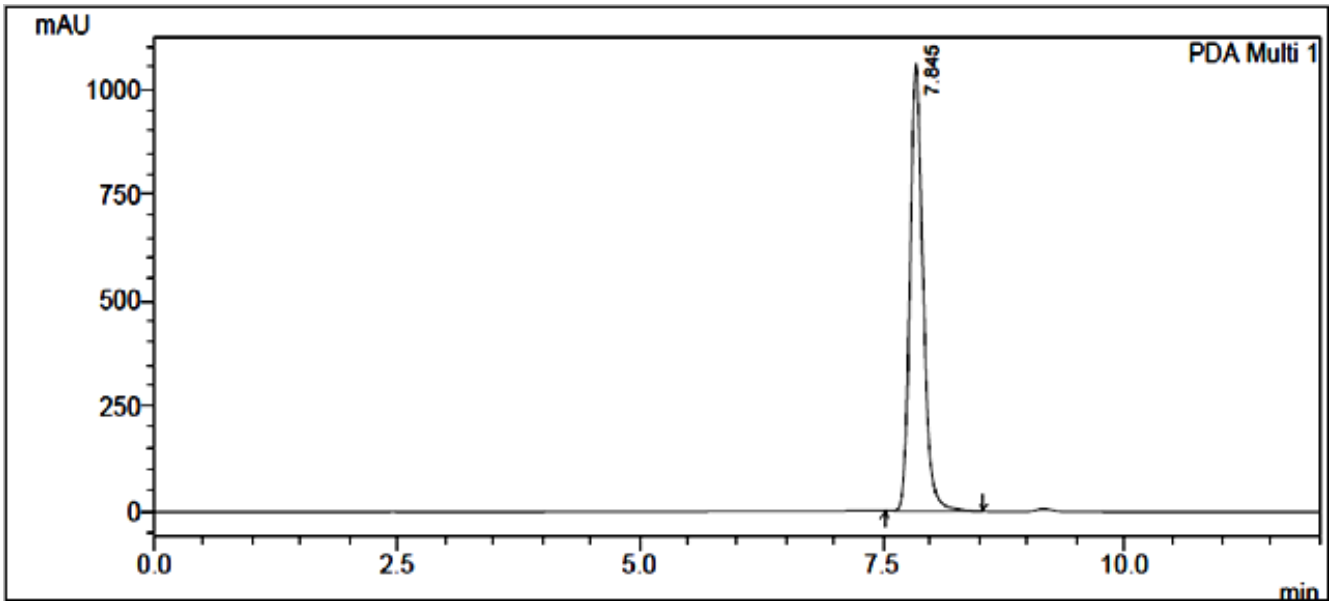


Fig 1: Chromatogram of Bisdemethoxycurcumin standard

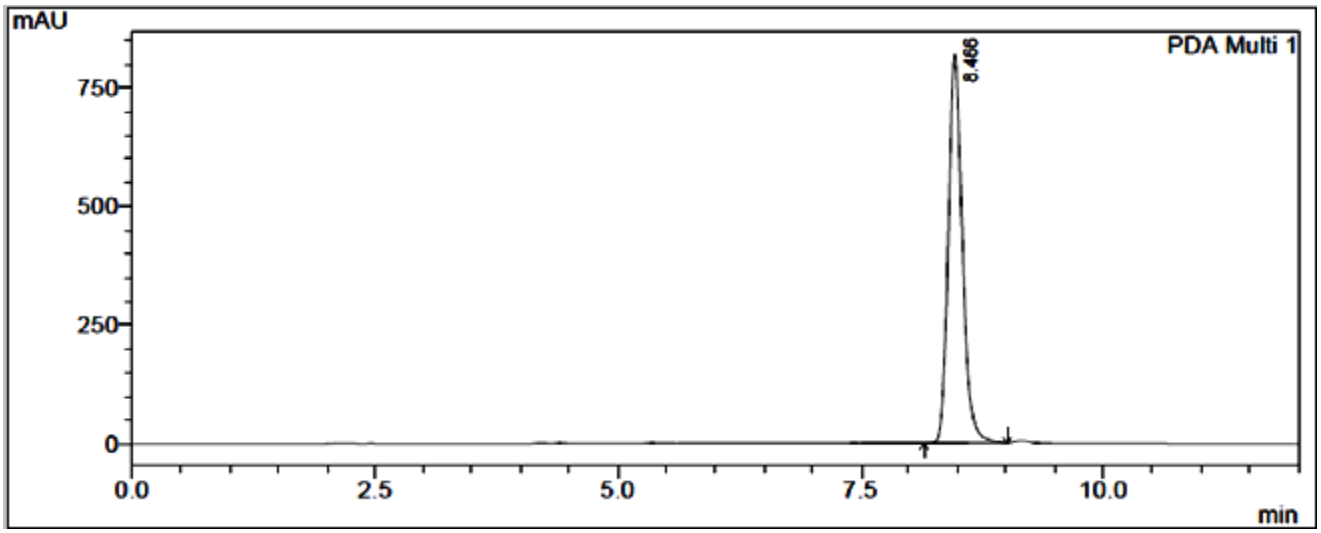


Fig 2: Chromatogram of Demethoxycurcumin standard

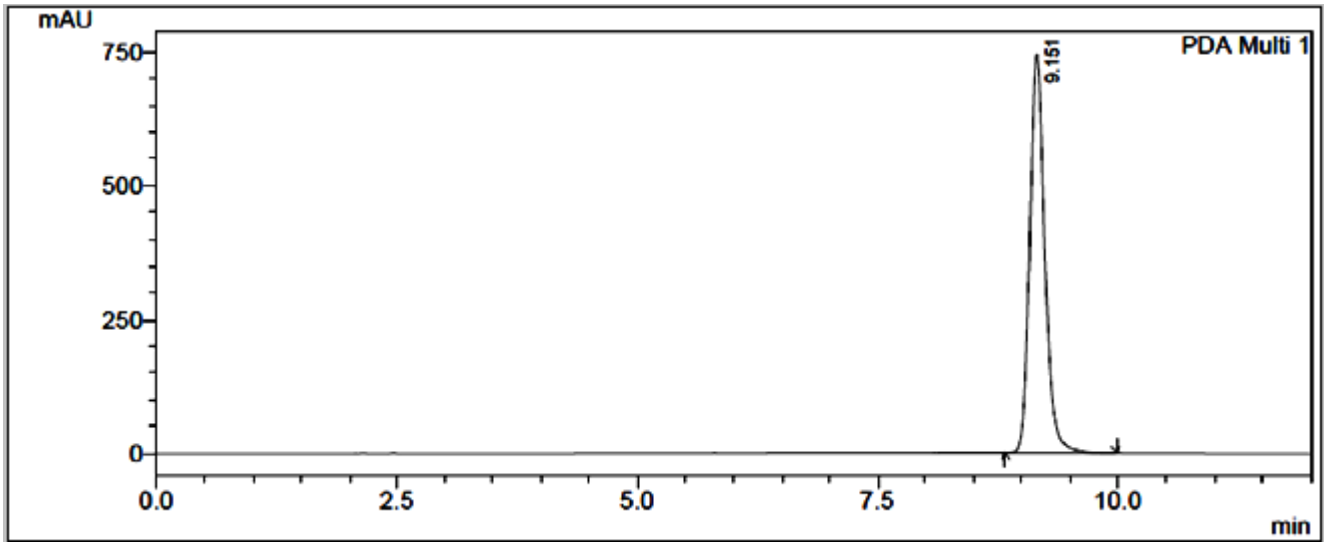


Fig 3: Chromatogram of Curcumin standard

4.1.2. Method Validation

System suitability: The system suitability was evaluated by injecting five replicates of the standard working solution and calculating the mean, standard deviation (S.D.), and relative standard deviation (R.S.D).

Table 3: System suitability results with Peak Area and Retention Time

	BDMC		DMC		Curcumin	
	Retention Time(min)	Peak area	Retention Time(min)	Peak area	Retention Time(min)	Peak area
Mean	7.852	9531886	8.479	8033921	9.166	8047139
S. D	0.002	7240.20	0.002	6782.12	0.002	6871.81
% R.S.D	0.03	0.08	0.03	0.08	0.02	0.09

Table 3 shows that the percentage RSD for retention time and peak area of all the contents are less than 0.1%. Furthermore, the tailing factor of all the curcuminoid peaks, viz., BDMC, DMC, and curcumin, was about 1.2, and the resolution between BDMC and DMC was observed at about 2.38. In contrast, the resolution between DMC and curcumin was about 2.43. Therefore, the data indicated that the system was suitable for analysis, and the optimized method parameters met the system suitability criteria.

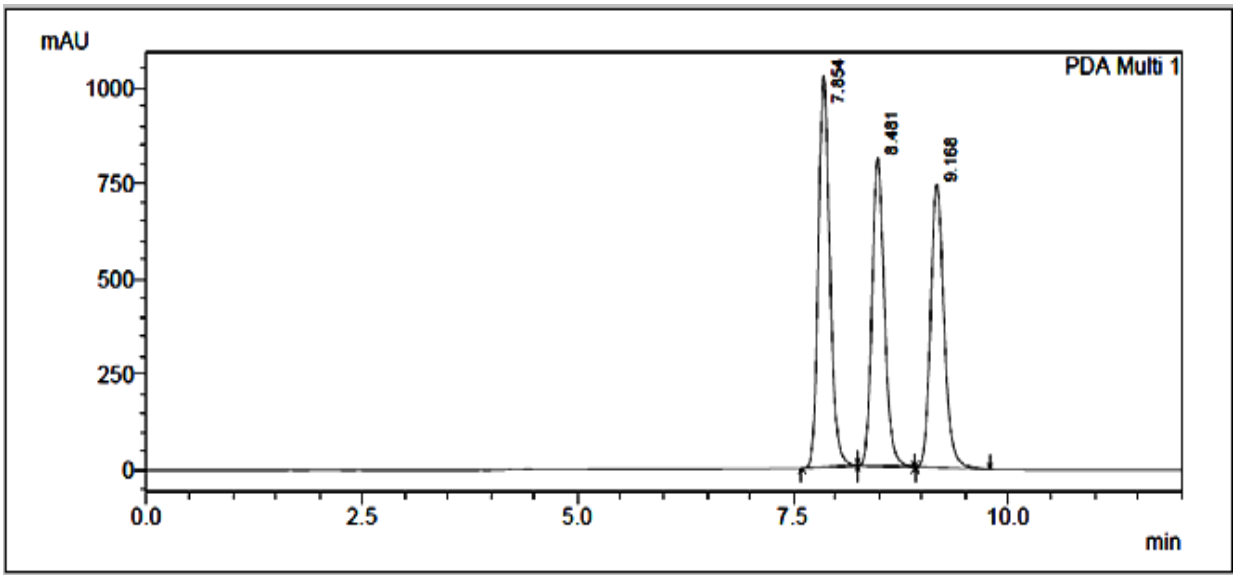


Fig 4: Chromatogram of Standard Working Solution

Specificity: Blank (diluent methanol) was injected to evaluate specificity, and no peak was observed in the blank injection at the retention times of the BDMC, DMC, and curcumin peaks.

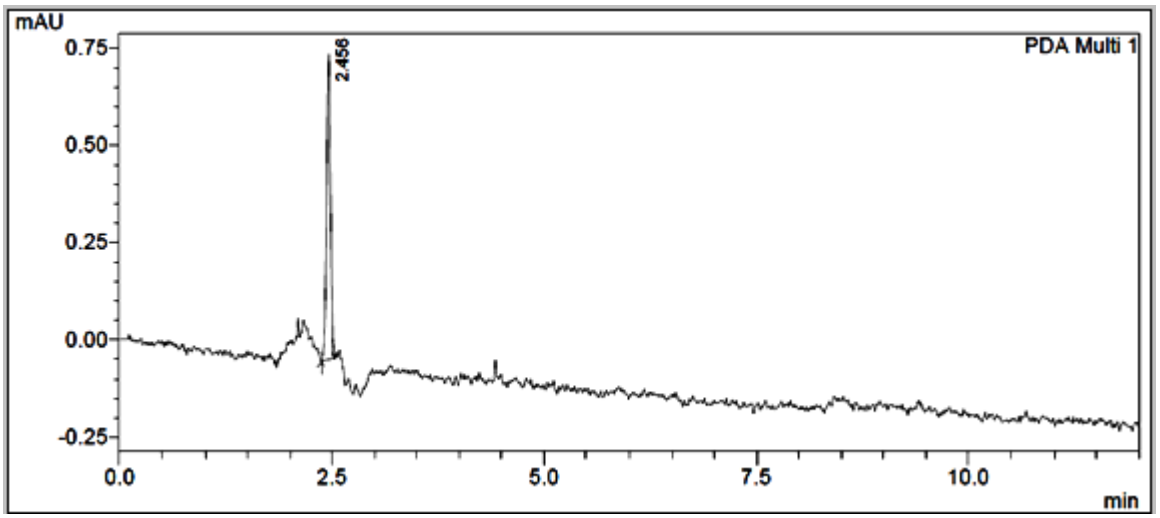


Fig 5: Blank (Diluent) specificity chromatogram

Precision: Repeatability as a part of precision was performed by injecting six sample preparations, and the mean, SD, and RSD of the peak area and peak area percentages were assessed.

Table 4: Precision data of the sample

Sample Preparation	Peak Area			Peak Area %		
	BDMC	DMC	Curcumin	BDMC	DMC	Curcumin
Mean	26950	560944	114603	3.724	77.416	15.798
S. D	3238.44	74387.82	16403.18	0.06	0.36	0.26
% R. S. D	12.02	13.26	14.31	1.59	0.46	1.65

Table 4 displays the mean, SD, and RSD of the peak area and peak area percentages of BDMC, DMC, and curcumin peaks.

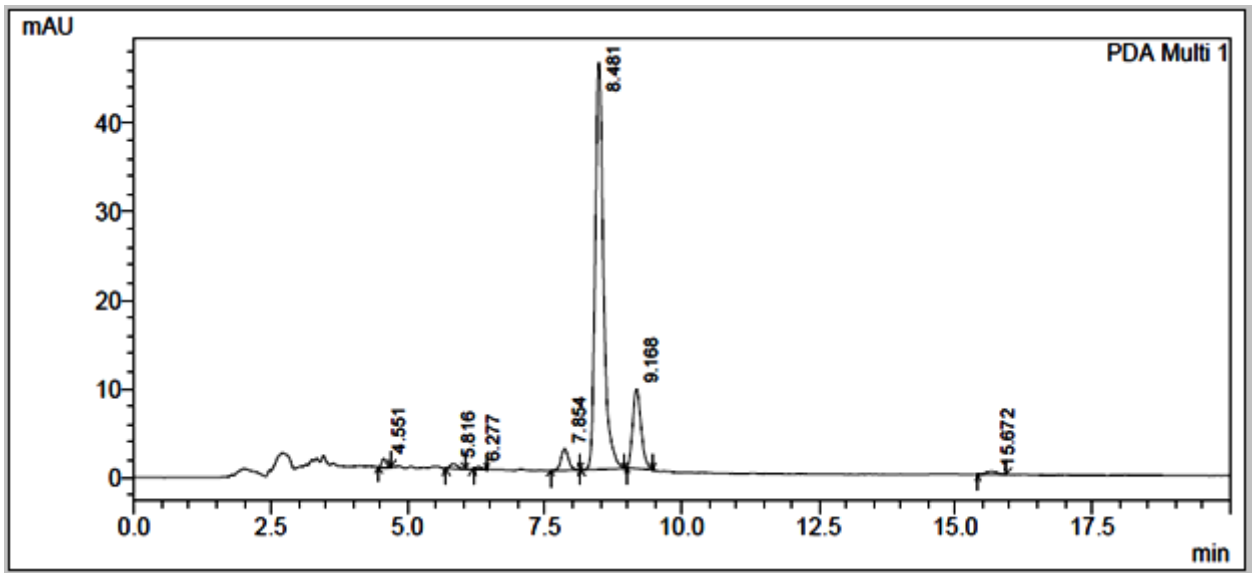
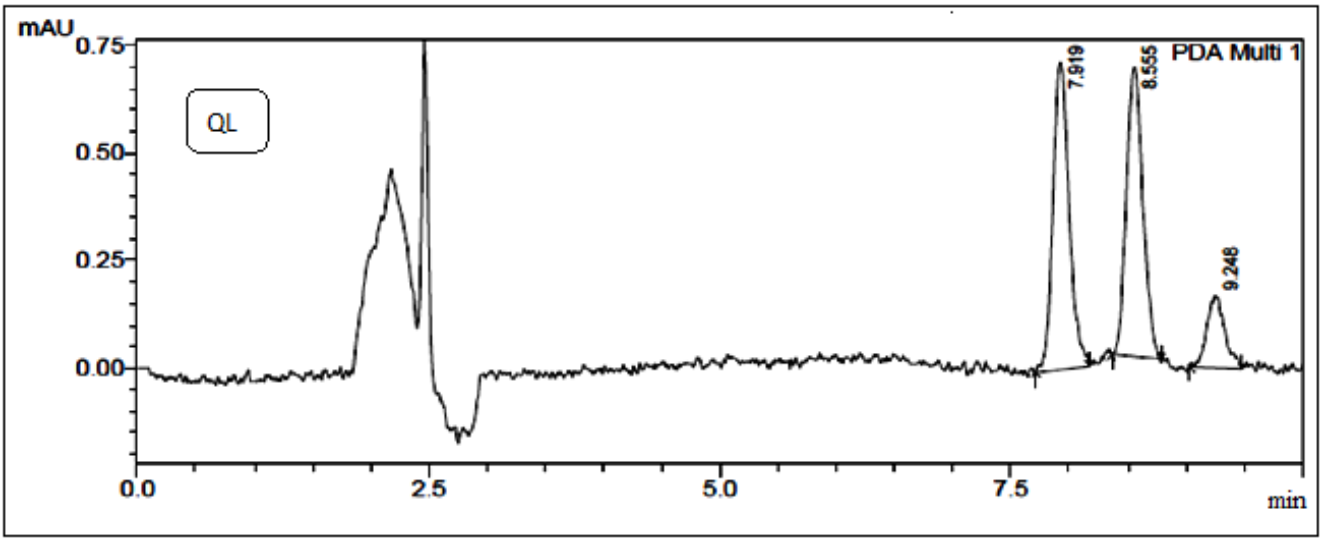


Fig 6: Precision chromatogram of Sample

Quantitation Limit (QL) and Detection Limit (DL) (sensitivity): Since this is an assay method, the QL was set around 0.05 µg/mL. The QLs obtained for BDMC, DMC, and curcumin were 0.050 µg/mL, 0.049 µg/mL, and 0.046 µg/mL, respectively. The DLs obtained for BDMC, DMC, and curcumin were 0.0151 µg/mL, 0.0147 µg/mL, and 0.0139 µg/mL, respectively. The concentrations are presented in Table 5.

Table 5: QL and DL details

	BDMC		DMC		Curcumin	
	µg/mL	% w.r.t standard concentration	µg/mL	% w.r.t standard concentration	µg/mL	% w.r.t standard concentration
QL	0.050	0.048	0.049	0.048	0.046	0.044
DL	0.0150	0.0143	0.0147	0.0144	0.0138	0.0131



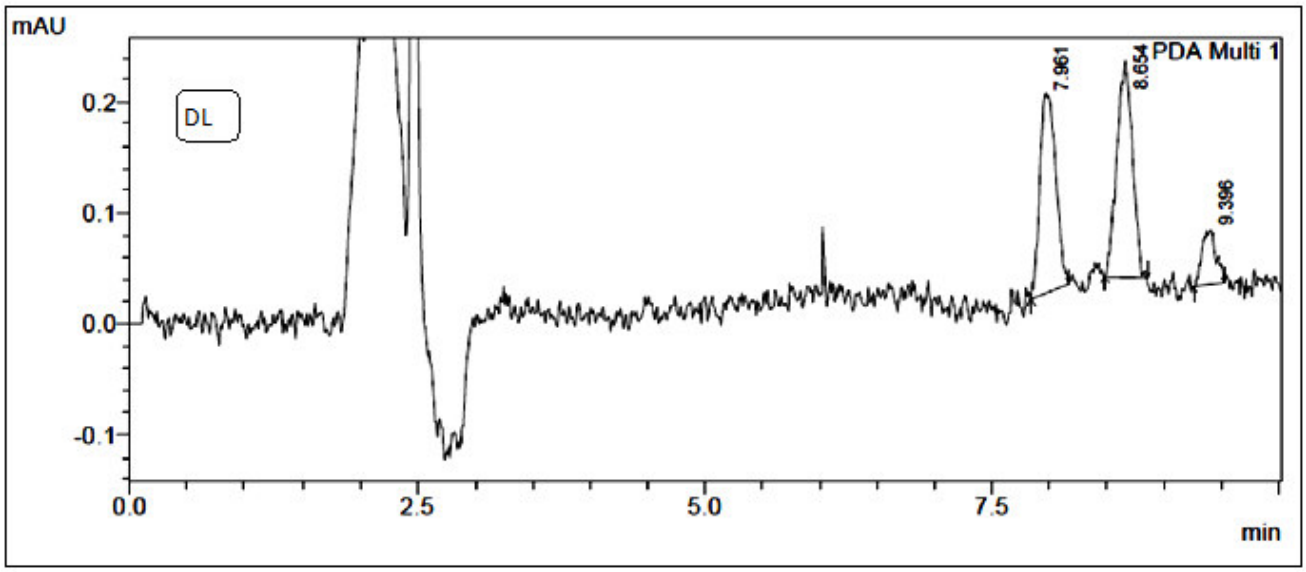


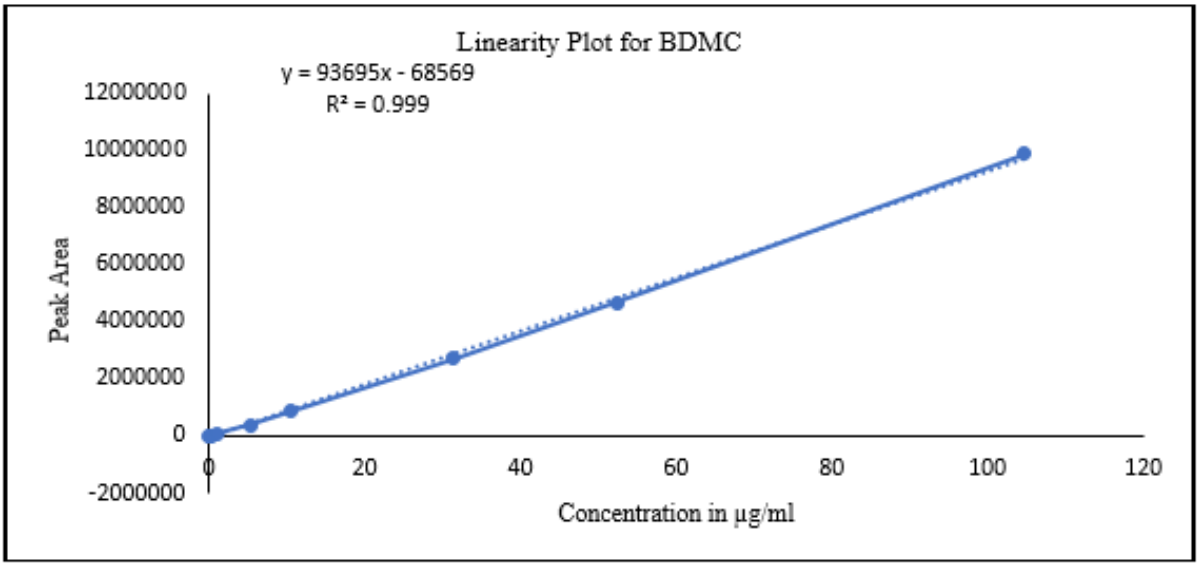
Fig 7: QL and DL chromatogram

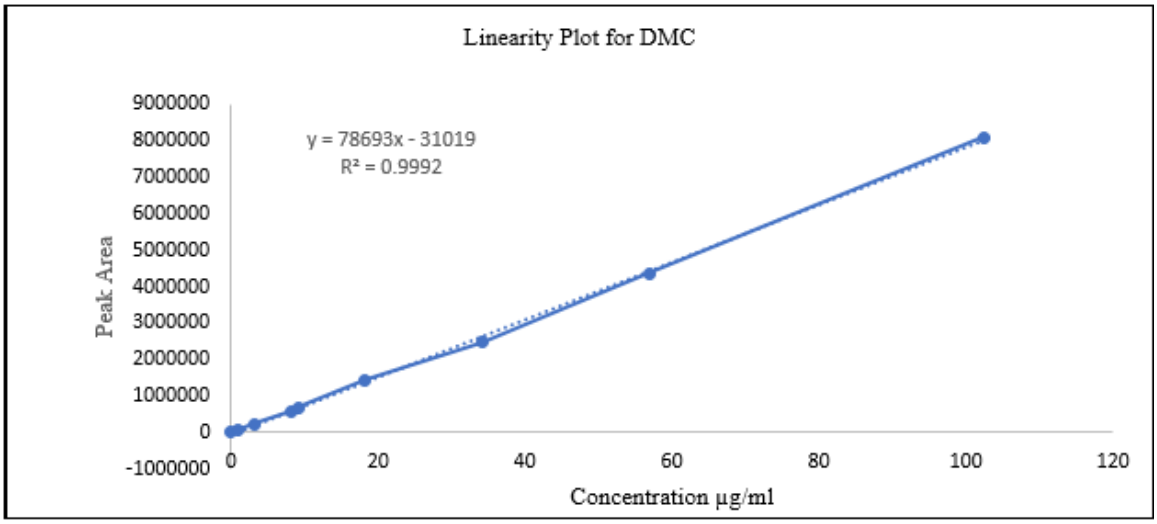
Linearity: The correlation coefficient was used to assess the created calibration curve.

Table 6: Regression data for linearity of BDMC, DMC, and curcumin

	BDMC	DMC	Curcumin
Level	0.050 to 104.664 µg/mL	0.049 to 102.343 µg/mL	0.046 to 105.661 µg/mL
Slope	93695	78693	76760
Intercept	-68569	-31019	-9515.8
Correlation coefficient (R^2)	0.9990	0.9992	1.000

From the calibration curves, slope, intercept, and correlation coefficient (R^2) were calculated. The values obtained for regression are tabulated in Table 6. The peak areas were linear in the range of 0.05 to 104.664 µg/mL for BDMC, 0.049 to 102.343 µg/mL for DMC, and 0.046 to 105.661 µg/mL for curcumin, with the correlation coefficient (R^2) of the calibration curves consistently higher than 0.999.





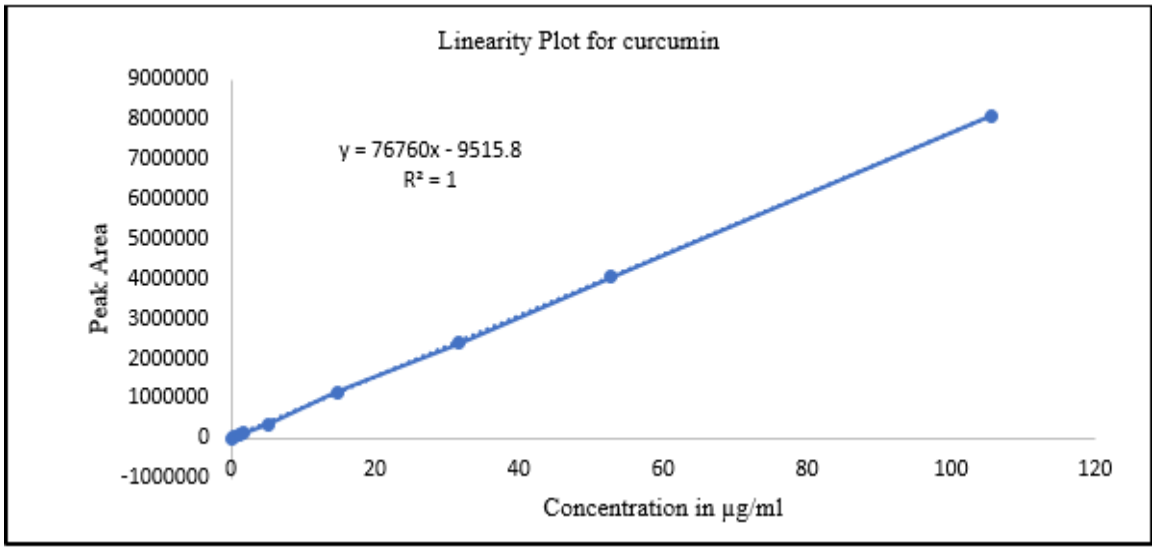


Fig 5: Linearity plots of BDMC, DMC and curcumin

Accuracy: Accuracy was performed on two levels, 50% and 150%, in triplicate, and the percentage of the contents present in the sample in mg/g was calculated against the contents found in the precision experiment at 100%, and the recoveries were calculated. The mean recoveries are tabulated below.

Table 7: Accuracy data

	Mean % recovery
BDMC	108.1
DMC	97.6
Curcumin	98.8
Total curcuminoids	98.5

The mean percentage recovery values for BDMC, DMC, curcumin, and total curcuminoids from 50% to 150% levels were discovered to be between 90% and 110%, according to data in Table 7. It proves the accuracy of the HPLC approach for quantifying curcuminoids.

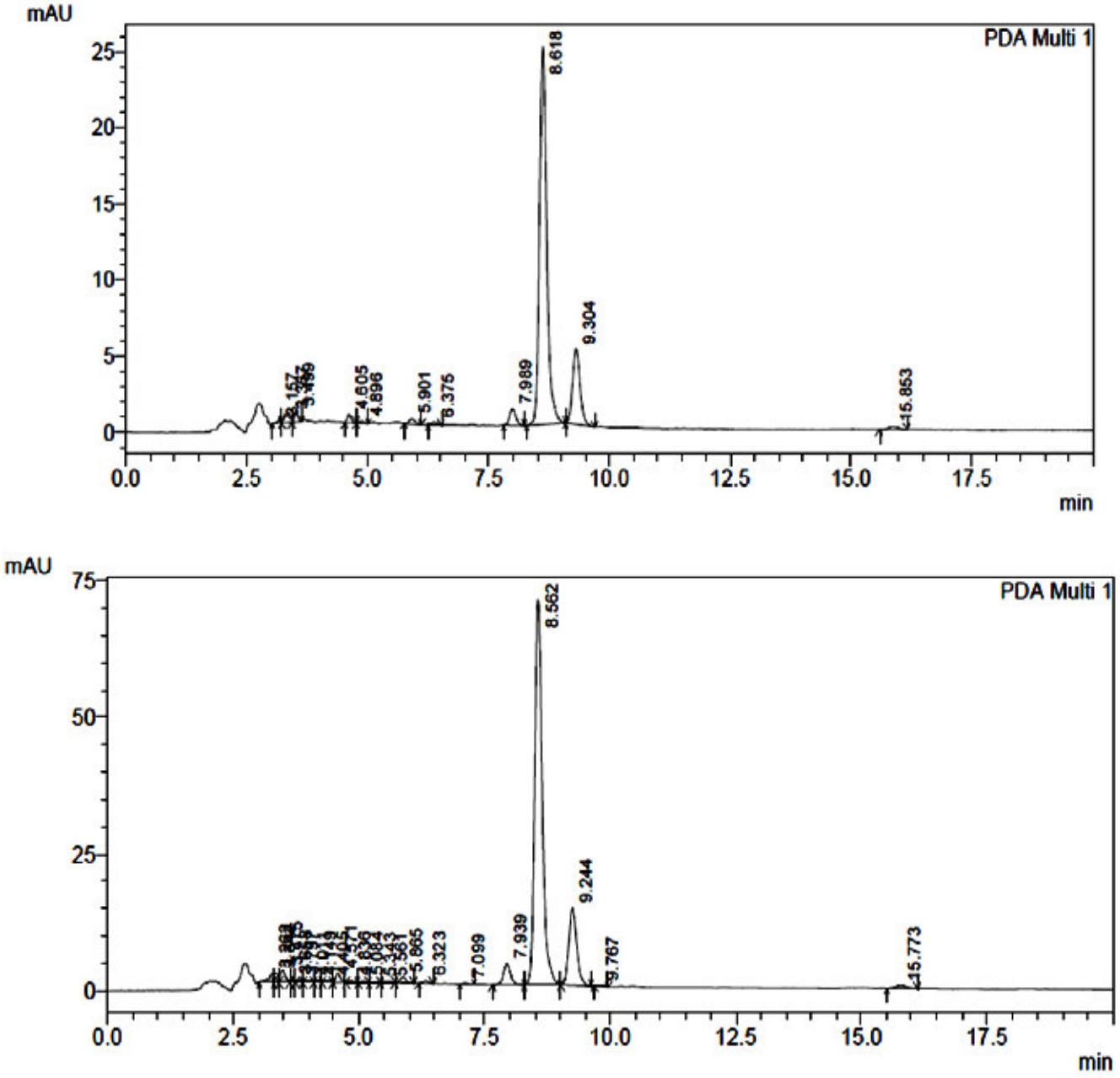


Fig 6: Accuracy chromatogram of 50% and 150%

Robustness: As a part of the robustness study, flow rate changes, column temperature changes, and mobile phase compositional changes were studied by injecting replicate injections of standards, and for evaluation, the retention time (RT), relative retention times (RRT), and tailing factors of peaks were compared with the precision data of standards. The results are tabulated below in Table 8.

Table 8: Robustness data

Temperature (°C)	BDMC			DMC				Curcumin			
	RT	RRT	Tailing Factor	RT	RRT	Tailing Factor	USP Resolution (Between BDMC and DMC)	RT	RRT	Tailing Factor	USP Resolution (Between DMC and Curcumin)
40	7.854	0.857	1.2	8.481	0.925	1.2	2.4	9.168	1.000	1.2	2.4
35	8.250	0.862	1.3	8.887	0.928	1.2	2.2	9.576	1.000	1.2	2.3
45	7.400	0.845	1.2	8.042	0.919	1.2	2.5	8.753	1.000	1.2	2.6
Flow (ml/min)											
1.2	7.933	0.856	1.2	8.568	0.925	1.2	2.4	9.263	1.000	1.2	2.4
1.1	9.300	0.866	1.3	9.986	0.930	1.3	2.5	10.736	1.000	1.2	2.6

1.3	7.374	0.856	1.2	7.966	0.925	1.2	2.4	8.616	1.000	1.2	2.4
Mobile phase ratio (0.1% formic acid: acetonitrile)											
52:48	7.933	0.856	1.2	8.568	0.925	1.2	2.4	9.263	1.000	1.2	2.4
55:45	8.224	0.815	1.2	9.115	0.903	1.2	2.4	10.094	1.000	1.2	2.5
49:51	6.984	0.878	1.3	7.448	0.936	1.3	2.2	7.956	1.000	1.3	2.2

The robustness data from Table 8 unveiled that even though the retention times of curcuminoid peaks varied due to changes in temperature, flow, and mobile phase composition, their relative retention times remained mostly unchanged. The resolution between peaks was somewhat better at 45 °C temperature and 1.1mL/min flow, whereas the mobile phase with reduced aqueous content (49%) had less resolution. All conditions were determined to have the same tailing factor. The data from the method validation demonstrated that the method was precise, specific, sensitive, linear, accurate, and robust and can be used to estimate curcuminoids in extracts of *Curcuma angustifolia* Roxb. The method validation study presented is preliminary for reaffirming the method's intended use. Furthermore, additional studies on the method can be performed as desired for specific plant-related requirements.

4.1.3. Application of the method to market finished product (MFP)

The validated method was applied to a marketed formulation with *Curcuma angustifolia* Roxb. rhizome powder as one of its ingredients. The label claim stated on the pack consisted of many ingredients, and the rhizome powder of *C. angustifolia* was mentioned among them with a label claim: "Every 10 grams of granules contains 0.189 g of rhizome powder of *C. angustifolia*". Since the exact concentration of curcuminoids was not mentioned on the label, the experiment detected the curcuminoids present in the MFP, which are attributed to the *C. angustifolia* rhizomes added to it as an ingredient. The chromatograms of the formulation sample (as such) and samples spiked with curcuminoids standard are shown below.

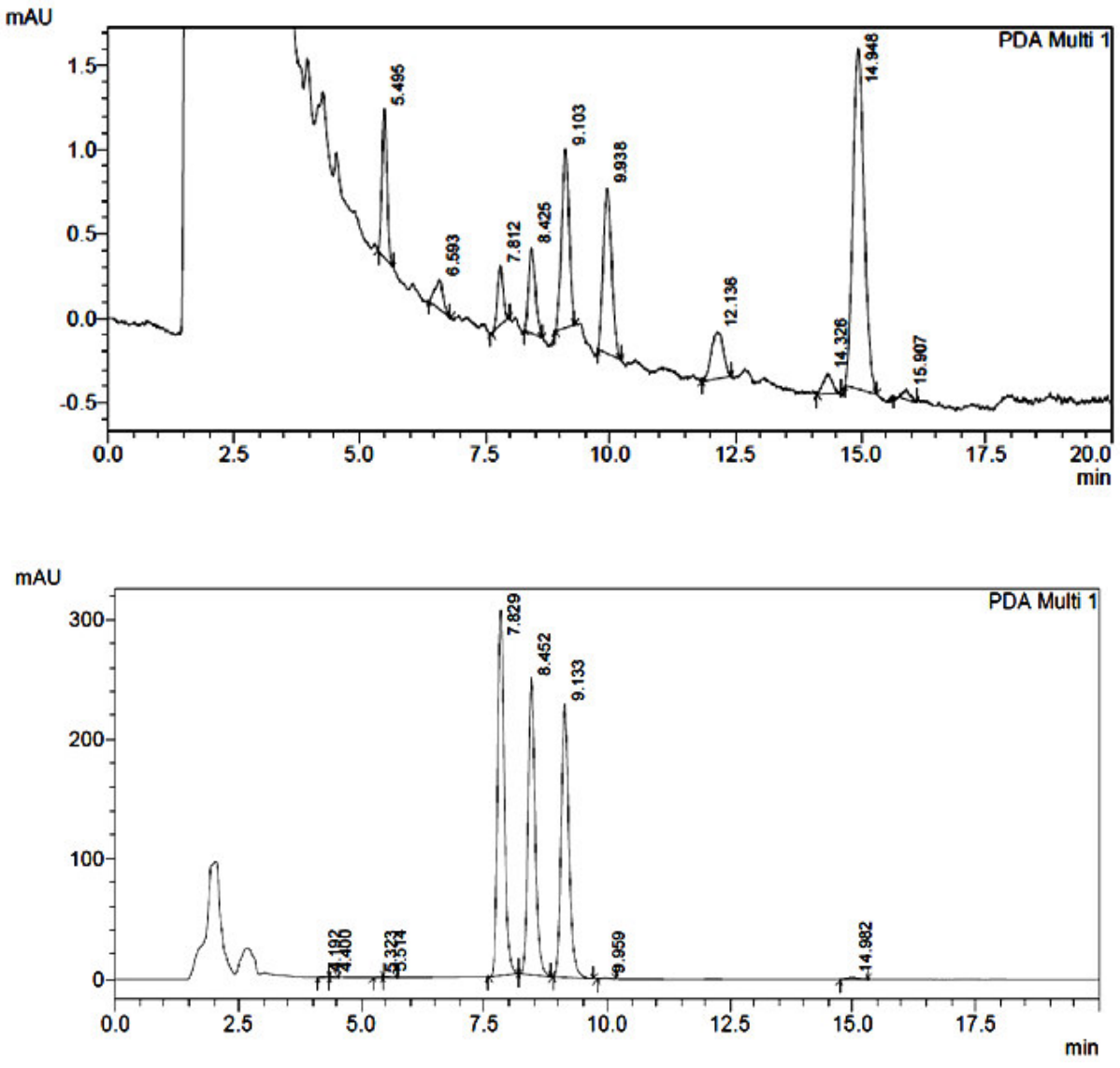


Fig 7: MFP as such sample chromatogram and spiked chromatogram

Several pieces of literature have been published detailing the developed method's applicability to marketed formulations.[26–29] This study is a preliminary work for the applicability of the HPLC method on a formulation containing *Curcuma angustifolia* Roxb. rhizome powder. As a result, with high efficiency, this method can be used to quantify curcuminoids in *Curcuma angustifolia* Roxb. rhizome extracts.

4.2. *In vitro* Antifungal activity

4.2.1. Minimum Inhibitory Concentration (MIC) of the extracts

The Minimum Inhibitory Concentration (MIC) for methanol extracts of *Curcuma angustifolia* Roxb. against *Candida albicans* was found to be 625 µg/mL, and against *Candida glabrata*, it was 2500 µg/mL. (Table 9)

Table 9: MIC in µg/mL for *C. angustifolia*

Strain	Minimum Inhibitory Concentration (MIC)
Candida albicans	625 µg/mL
Candida glabrata	2500 µg/mL

The data exhibited in Table 9 showed variable MIC values for *C. angustifolia* extracts against both *Candida albicans* and *Candida glabrata*. The extract's inhibitory activities against fungal strains may be influenced by the nature of its constituents, resulting in varying values for various strains.

4.2.2. Antifungal assay

The values obtained for the zone of inhibition for fluconazole (positive control) and methanolic extracts of *Curcuma angustifolia* Roxb. against *Candida albicans* and *Candida glabrata* strains are tabulated below in Tables 10 and 11

Table 10: Zone of inhibition values against *Candida albicans*

Candida albicans strain	Zone of inhibition		
Samples	**I (mm)**	**II (mm)**	**Mean (mm)**
Fluconazole	15.0	14.9	14.95
methanol extract	16.0	16.0	16.0

Table 11: Zone of inhibition values against *Candida glabrata*

Candida glabrata strain	Zone of inhibition		
Samples	**I (mm)**	**II (mm)**	**Mean (mm)**
Fluconazole	26.0	26.0	26.0
methanol extract	19.0	19.0	19.0

Table 10 shows that the methanolic extract and fluconazole have 16.0 and 14.95 mm inhibition zones against *Candida albicans*, respectively. Table 11 showed that methanolic extract and fluconazole had inhibition zones of 19.0 and 26.0 mm, respectively, against *Candida glabrata*.

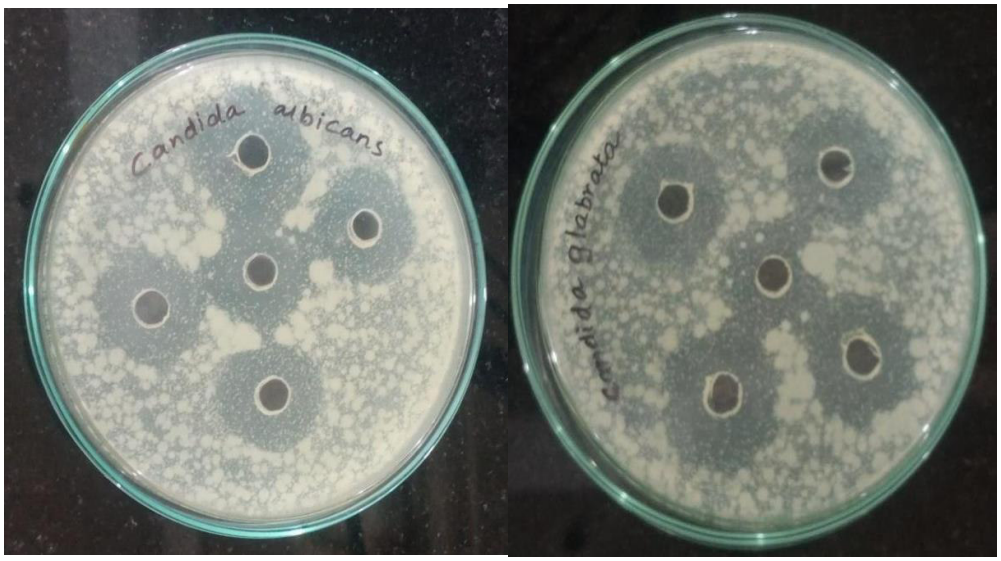


Fig 8: Images of plates of the antifungal assay for *candida albicans* and *candida glabrata*

The antifungal activity was carried out at the determined minimum inhibitory concentration. For the *Candida albicans* antifungal assay activity study, the methanolic extract of *C. angustifolia* exhibits a higher zone of inhibition distance than the control. Consequently, it may possess better antifungal capability than the control. In studies involving *Candida glabrata*, the extract demonstrates a smaller zone of inhibition than the positive control. The encouraging results obtained against *Candida albicans* for the methanolic extract establish that this plant species can also alleviate fungal infections caused by this strain. Additionally, *C. angustifolia* extracts exhibit antifungal efficacy against the *Candida glabrata* strain. The methanolic extract investigated in this study is a new source of treatment for disorders caused by candida strains. The positive control employed for the study can demonstrate some adverse effects; however, naturally occurring chemicals might not show the same level of toxicity. Several Curcuma species have demonstrated anti-fungal properties.[30–35] Research on the antifungal properties of *Curcuma angustifolia* Ro. leaf extracts,[36,37] stem extracts (aquatic and ethyl acetate),[38] rhizome extracts (ethanol, acetone, and petroleum ether),[37] and essential oils[39,40] has been published. This is one of the first studies for a methanolic extract of *Curcuma angustifolia* Roxb. rhizome extract using *Candida glabrata* strain. As a part of the assessment of structure-related activity, curcuminoids can be structurally modified at the following four positions: (a) the active methylene group; (b) double bonds; (c) the diketo group; and (d) hydroxyl groups on aryl side chains.[41] According to published research, a molecule with a half-analogous structure to curcumin, containing an aromatic ring with methoxyl and phenolic hydroxyl groups and an unsaturated carbonyl moiety, plays a significant role in the antifungal activity.[42] The literature suggests that the beta-keto-enol site often has the advantage of being active against drug-resistant variations and all virus genotypes.[43,44] The presence of curcuminoids, namely BDMC, DMC, and curcumin, in the methanolic extract may be attributed to the extract's antifungal activity. The present antifungal assay investigation has established that the methanolic extract of *Curcuma angustifolia* Roxb. rhizome exhibited notable antifungal activity against *Candida albicans* and *Candida glabrata* strains. It is possible to plan additional research on these plant species to isolate the active ingredients and put them through anti-candidal, pharmacological, and clinical tests, which would further strengthen the findings of this study.

5. CONCLUSION

The reversed-phase HPLC method was developed for quantifying curcuminoids, specifically bis-demethoxycurcumin (BDMC), demethoxycurcumin (DMC), and curcumin, in *Curcuma angustifolia* Roxb. rhizome extract. The established HPLC assay method demonstrated good compound separation and was precise, sensitive, linear, accurate, robust, and specific. The HPLC method could estimate markers in *Curcuma angustifolia* Roxb. rhizomes. The *in vitro* antifungal study demonstrated that *Curcuma angustifolia* rhizome extracts are antifungal. The extracts contain phytoconstituents that treat *Candida albicans* and *Candida glabrata* diseases. This study reaffirms using plant extracts to treat antifungal infections, offering a new way to treat diseases using natural products. *Curcuma angustifolia* Roxb. could be transformed into antifungal herbal drugs to treat human fungal illnesses.

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7. AUTHORS CONTRIBUTION STATEMENT

The experimental work was planned under the guidance of Dr. Madhavi R Badole. Pravith P Warrier performed the study execution and report compilation.

8. CONFLICT OF INTEREST

Conflict of interest declared none.

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