Research Article

Development of HPTLC Fingerprint of Turmeric Samples Collected from Different Geographical Locations and In Some Polyherbal Over The Counter Marketed Formulations

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ABSTRACT

Curcuma longa, commonly known as turmeric is a perennial herb and member of the Zingiberaceae (ginger) family, is cultivated extensively in Asia, India, China, and other countries with a tropical climate. The active constituents of turmeric are the flavonoid curcumin (diferuloylmethane), demethoxycurcumin (DMC), bisdemethoxycurcumin (BDMC) and various volatile oils, including tumerone, atlantone, and zingiberone. Other constituents include sugars, proteins, and resins. The best-researched active constituent is curcumin, which comprises 0.3-5.4 percent of raw turmeric. Turmeric (curcumin) is widely used in traditional Indian system of medicine to treat hepatic disorder, anorexia, cough, diabetic wounds, rheumatoid arthritis, sinusitis and other inflammatory diseases. several analytical techniques have been established for the qualitative and quantitative analysis of Curcuma species including GC-MS, HPLC and HPTLC. Very few studies have been reported to determine curcuminoids in different Curcuma species or in the same species of sample collected from different cultivation regions and this information is critically important for the quality control of related herbal medicines. The present study deals with development of high performance thin layer chromatographic (HPTLC) fingerprint for determination of curcumin, demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) in *C. longa* collected from different geographical region of India and some over the counter polyherbal formulations. Method validation is done as per ICH guidelines and pharmacopoeial specifications.

Keywords: Curcumin, Demethoxycurcumin, Bisdemethoxycurcumin, polyherbal formulations.

INTRODUCTION

Curcuma longa, commonly known as turmeric is a perennial herb and member of the Zingiberaceae (ginger) family, grows to a height of three to five feet and is cultivated extensively in Asia, India, China, and other countries with a tropical climate[1]. Turmeric is used extensively in foods for both its flavor and color, as well as having a long tradition of use in the Chinese and Ayurvedic systems of medicine. The active constituents of turmeric are the flavonoid curcumin (diferuloylmethane), demethoxycurcumin (DMC), bisdemethoxycurcumin (BDMC) and various volatile oils, including tumerone, atlantone, and zingiberone. Other constituents include sugars, proteins, and resins. The best-researched active constituent is curcumin, which comprises 0.3-5.4 percent of raw turmeric[2-3]. Turmeric (curcumin) is widely used in traditional Indian system of medicine to treat hepatic disorder, anorexia, cough, diabetic wounds, rheumatoid arthritis, sinusitis and other inflammatory diseases[4].

In recent years several analytical techniques have been established for the qualitative and quantitative analysis of Curcuma species including GC-MS[5] and HPLC[6-8]. A chromatographic comparison between HPLC and HPTLC method has been reported for quality control of curcumin but very few studies have been reported to determine curcuminoids in different Curcuma species or in the same species of sample collected from different cultivation regions and this information is critically important for the quality control of related herbal medicines[9]. Hence it would be very significant to develop an efficient analytical method that can analyze curcumin of C. longa collected from different geographical regions and some selected over the counter polyherbal formuations. However, to our knowledge. no high performance thin layer chromatographic (HPTLC) method for determination of curcumin and its metabolites in *C. longa* collected from different geographical region of India and some over the counter polyherbal formulations has been ever reported. The present study deals with fulfilling the above objective of development and application of the validated HPTLC methods to analyse marker compounds in

Curcuma longa samples collected from various geographical sources and in selected market formulations. Method validation is done as per ICH guidelines and pharmacopoeial specifications.

MATERIALS AND METHODS

Curcumin, DMC and BDMC standard was procured from Natural Remedies, Bangalore. Silica Gel 60 F254 Aluminium plates (Merck) was used as stationary phase. Chloroform: Ethanol: Glacial acetic acid in the ratio of 9.4:0.5:0.1 was used as mobile phase. Methanol and water were used as solvent. Turmeric plants were procured from five different geographical sources. Gujarat variety was obtained from Biyaran Kendra, Vapi, Gujarat, Tamil Nadu and Andhra Pradesh variety were obtained from Datt Enterprise, Navi-mumbai. Kerala variety was collected from Santosh Ayur Drug, Mumbai and Karnataka variety was obtained from Excelsior Trading Company, Mumbai. The Turmeric samples were authenticated by Archana Khemani, Head, Department of Botany, Zandu Pharmaceuticals Pvt. Ltd. (now Emami, the herbarium of the specimens has been deposited at Botany Department of Zandu Pharmaceuticals Pvt Ltd (now Emami), Vapi, Gujarat.

Marketed formulations Jwarnashak kwath, Chandraprabhavati, Punarnava mandur, Utriplex capsules, Hypondid tablets, Dashang Lepa Churna and Mahasudarshan churna were obtained from local pharmacy stores.

A Camag HPTLC system (Switzerland) comprising of Camag Linomat IV applicator, Camag TLC Scanner 3, Camag winCATS software, version 1.3.3, Hamilton syringe (100µl), Camag, Shimadzu weighing balance, Camag UV cabinet were used for the study.

Preparation of standard and sample solution

Standard

10 mg of Curcumin, DMC and 12 mg BDMC were diluted with 10ml of methanol to give a concentration of 1mg/ml and 1.2mg/ml respectively.

Turmeric Sample

100g of coarsely powdered turmeric samples obtained from different geographical places were subjected to defatting by refluxing for 4 hours with petroleum ether. The material was dried and extracted by refluxing with 100 ml methanol and water respectively for 8 hours. The extract was concentrated over water bath, labeled and stored. 100mg of methanolic and aqueous extracts of all Turmeric samples were dissolved in 10ml of methanol and filtered through Whatmann No 1 filter paper. For quantification 20µl of all sample solutions were spotted along with 1-5µl of the standard solution.

Marketed formulations

Four different dosage forms were chosen for estimation of Curcumin, DMC and BDMC. Of these three are tablets (Chandraprabhavati, Punarnava mandur and Hypondid tablets), one capsules (Utriplex capsules) two churna (Dashang Lepa Churna and Mahasudarshan churna) and one kwath (Jwarnashak kwath). The average weight of the tablets was determined by weighing 20 tablets. In the case of capsule the average weight determined by weighing 20 capsules. 10 coated tablets were weighed, soaked in water to remove coating; dried in oven at 105 °C, weighed again, powdered and macerated with 30ml methanol for 24 hrs. Similar procedure was carried out for uncoated tablets without soaking in water. Hard gelatin shells were removed, 10 capsules were emptied and the powder within was weighed. This was dissolved in 30ml methanol for 24 hrs, 2 g of the churna and kwath samples each were macerated with 30ml methanol for 24 hrs. All were then filtered through Whatmann No.1 filter paper

Chromatographic conditions

The chromatographic estimation was performed using the following conditions, stationary phase was precoated silica gel 60 F254 aluminium sheets (20 x 10 cm & 10 x 10 cm) and the mobile phase used was Chloroform: Ethanol: Glacial acetic acid in the ratio of 9.4:0.5:0.1 v/v/v. The chamber saturation time employed was 20 minutes and the developing distance was 8.5cm. Scanning wavelength of 425 nm with a slit dimension of 8.0 x 0.40 mm and scanning speed of 20 mm/s and data resolution of 100 µm/step were employed.

Method validation

The method was validated in compliance with ICH guidelines with respect to Prevalidation, Limit of Detection, Limit of Quantification, Linearity, Precision and Accuracy.

1. Sample solution stability

Chromatography of the sample solutions standing for different period of time (48 hrs, 24hrs, 4hrs, lhr) before development was carried out. The sample solution was prepared and stored at room temperature. The solution was spotted after different time intervals. The chromatogram was developed and scanned at 425nm. The peak areas obtained were

compared to study the variation of the standard solution.

b) Stability of sample on plate

Sample solution was applied to different plates and development was done after different time intervals (48 hrs, 24hrs, 4hrs, lhr) Respective chromatogram was developed and scanned at 425nm The peak areas obtained were compared to study the variation of the standard solution on the plate.

3.5.1 Linearity

The linearity of responses for Curcumin, and BDMC were assessed in the range of 1.0 -5.0 µg/spot and for DMC 1.2-6.0 µg/spot. Five different concentrations of the standards solutions were applied five times to study the linearity.

3.5.2 Accuracy and recovery studies

Both accuracy and recovery were studied. Accuracy of the method was tested by taking three concentrations and three determinations of each analytical concentration. The recovery study was carried out by addition of known amounts of standards to the product. Standards added were 80%, 100% and 120% of the 1 µg. Three determinations were done to study the recovery. The % recovery of curcumin was compared with the actual amounts.

3.5.3 Precision

3.5.3.1 System precision

The system precision study basically focuses on the exactness of the instrument.

Repeatability of sample application and measurement of the peak area were studied. Six determinations at a concentration of 2.0 µg/spot, for Curcumin, and BDMC and 2.4 µg/spot for DMC were applied. The repeatability of sample application and the repeatability of measurements of the peak area were evaluated by comparing their coefficient of variations which are obtained from the peak area measurements.

3.5.3.2 Method precision

To study the precision of the method, both intra - day and inter - day precision were applied. Intra- day precision was studied by taking three different concentrations 1.0, 2.0 and 3.0µg/spot of Curcumin, and BDMC and 1.2, 2.4 and 3.6 µg/spot for DMC were applied three times to see variation in their peak areas within a day. For inter-day precision the same concentrations were applied but their peak area variation was studied for three different days.

3.5.4 Limit of detection and quantification

Detection and quantification limits were calculated from the calibration equations obtained from the experiment. The determinations of the detection and quantitation limits were based on the standard deviation of the response and the slope. The slope was estimated from the calibration curve of the analyte and the estimate of the standard deviation was carried out from the standard deviation of the y intercept.

3.5.5 Robustness

In order to study the robustness of the method, slight but deliberate changes were made in some parameters. Parameters such as; the mobile phase composition, total mobile phase amount, time from application to development and time from development to scanning were used to study the robustness. Concentrations of 2.0 µg/spot of Curcumin, and BDMC and 2.4 µg/spot for DMC were applied for the analysis.

Estimation of Curcumin, DMC and BDMC

For quantification 20µl of all sample solutions were spotted along with standard solution. The chromatograms were developed and scanned at 425nm. The amount of Curcumin present in each extract was calculated by comparing the peak area of standard and respective samples.

The following formula was used to quantify the active constituent,

$$\% \text{ Curcumin, DMC \& BDMC} = \frac{\text{Area of sample}}{\text{Area of standard}} \times \frac{\text{conc of standard}}{\text{conc. of sample}} \times 100$$

Results of quantification of methanolic and aqueous extracts of Turmeric samples and its marketed formulations are reported in Table 4. The image of the HPTLC pattern recorded at 366nm for calibration, turmeric samples and marketed formulations and 3D view of curcumin 95%linearity at 425 nm is shown in figures 1-6.

RESULTS AND DISCUSSIONS

The mobile phase containing Chloroform: Ethanol: Glacial acetic acid in the ratio of 9.4:0.5:0.1 gave sharp and symmetric peaks and better spot characteristics for Curcumin, BDMC and DMC. The spots at 0.69 ± 0.04, 0.57 ± 0.014 and 0.38 ± 0.03 respectively

were identified as Curcumin, DMC and BDMC with the help of chromatograms of their individual standards. %RSD values less than 2 indicates that Curcumin, DMC and BDMC are stable for 48 hours in solution as well as on plate. However, it can be suggested that, the increasing value of %RSD with time is an indication that the solution will lose it stability after some time.

The method gave a good linearity curve in the range of 1.0 – 5.0 µg for Curcumin and its one of the two metabolites DMC and between 1.2–6 µg for BDMC with correlation coefficient of 0.998 ± 0.0002 for curcumin , 0.996 ± 0.0002 for BDMC and 0.999 ± 1.451E-05 for DMC. The average recovery values of Curcumin, BDMC and DMC were found in the range 98.62 to 100.92%, 98.84 to 100.35 %and 98.80 to 100.80% which are in accordance with ICH limits of 80% - 120%. The LOD of Curcumin, DMC and BDMC were found to be 0.056 µg/spot, 0.054 µg/spot and 0.018 µg/spot respectively. These were the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The LOQ of Curcumin, DMC and BDMC were 0.171 µg/spot, 0.164 µg/spot and 0.056 µg/spot respectively. These were the lowest concentration of drugs, accurately detected and integrated by the instrument. The low values of S.D. and % RSD along with unchanged R_f values of Curcumin DMC and BDMC obtained after introducing small deliberate changes in the method indicated the robustness of the developed HPTLC method. Different validation parameters of the proposed HPTLC method are summarized in table no. 1-3

The amount of Curcumin in turmeric samples collected from various geographical sources were spotted, developed and calculated by comparing peak area of standard and sample solutions. % of Curcumin was found to be in the range of 8.43 – 10.55% in methanolic extracts. Curcumin was not detected in aqueous extracts. Turmeric sample obtained from Andhra Pradesh was found to be best with highest content of Curcumin of 10.55% in methanolic extract. % of DMC was found to be in the range of 4.64 – 6.59% and 0.019 – 0.031% in methanolic and aqueous extracts. Turmeric sample obtained from Andhra Pradesh was found to be best with highest content of DMC of 6.59% in methanolic extract, whereas aqueous extract of Gujarat variety showed maximum DMC content of 0.031%. BDMC was found to be in the range of 1.98 – 2.56% in methanolic extracts. BDMC was not detected in aqueous extracts. Turmeric sample obtained from Andhra Pradesh was found to be best with highest content of BDMC of 2.56 % in methanolic extract. The amounts of Curcumin , BDMC and DMC obtained in different turmeric samples is presented in figure7.

The proposed method was applied for the determination in commercial polyherbal formulations containing turmeric. Three replicates of determinations were made and satisfactory results were obtained with good separation. Hence the method was suitable for routine analysis of Curcumin, BDMC and DMC in turmeric containing formulations. The amounts of Curcumin , BDMC and DMC obtained in different dosage forms are reported in the table no. 4. and in figure8.

CONCLUSION

Variation in content has been observed. The reason may be like climatic conditions, water level, soil and its texture, fertility etc. The present investigation shows that Curcumin, DMC and BDMC content vary with their respective root source. Turmeric can be grown in diverse tropical conditions from sea level to 1500 m above sea level, at a temperature range of 20-35°C with an annual rainfall of 1500 mm or more, under rainfed or irrigated conditions. Though it can be grown on different types of soils, it thrives best in well-drained sandy or clay loam soils with a pH range of 4.5-7.5 with good organic status.

A simple, rapid, accurate and convenient method was developed for estimation of Curcumin by HPTLC. This method was used to standardize different Ayurvedic polyherbal formulations containing Turmeric. This developed and validated HPTLC method can be used to determine batch to batch variations and routine analysis by herbal manufacturers of Turmeric formulations. Thus, these analytical standardization techniques facilitate manufacturers to market their plant based medicines with defined content of respective bioactives and to ensure its quality.

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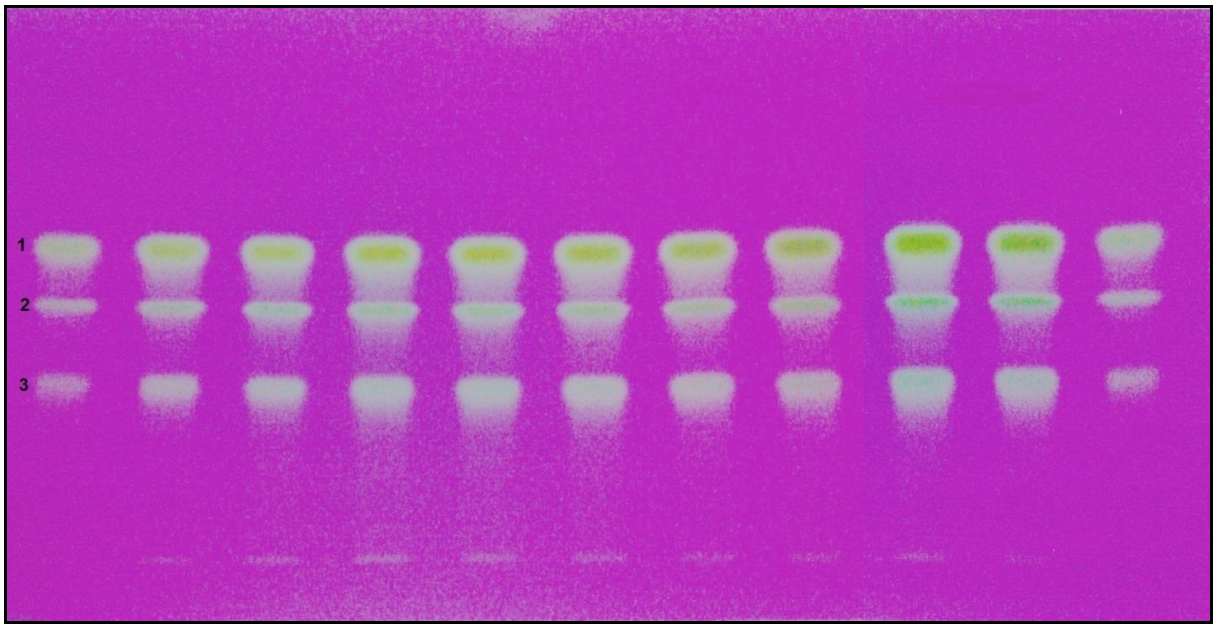

T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11

Fig. 1: HPTLC PATTERN OF CURCUMIN-95% LINEARITY AT 366 NM

1- Curcumin , 2- DMC, 3- Bisdesmethoxycurcumin

T1,T2- Curcumin 95% 1.0 µg; T3,T4- Curcumin 95% 2.0 µg; T5,T6- Curcumin 95% 3.0 µg; T7,T8- Curcumin 95% 4.0 µg; T9,T10- Curcumin 95%5.0 µg; T11- Curcumin 95% 1.0 µg

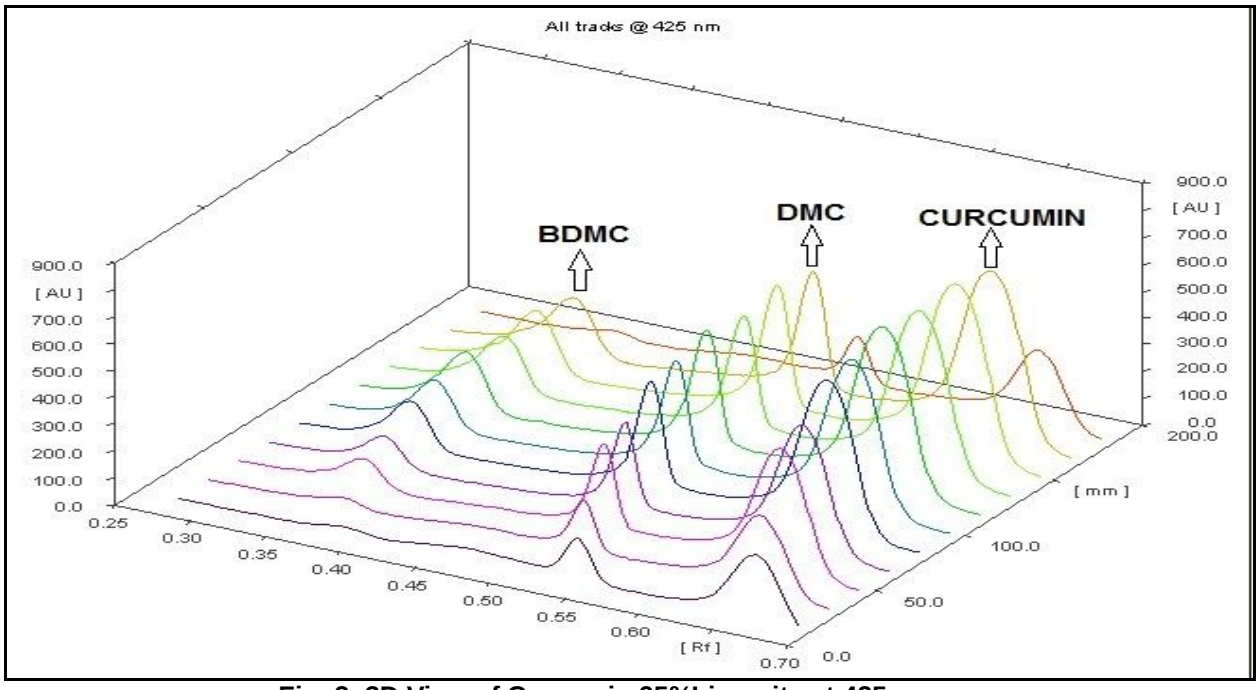

Fig. 2: 3D View of Curcumin 95%Linearity at 425 nm

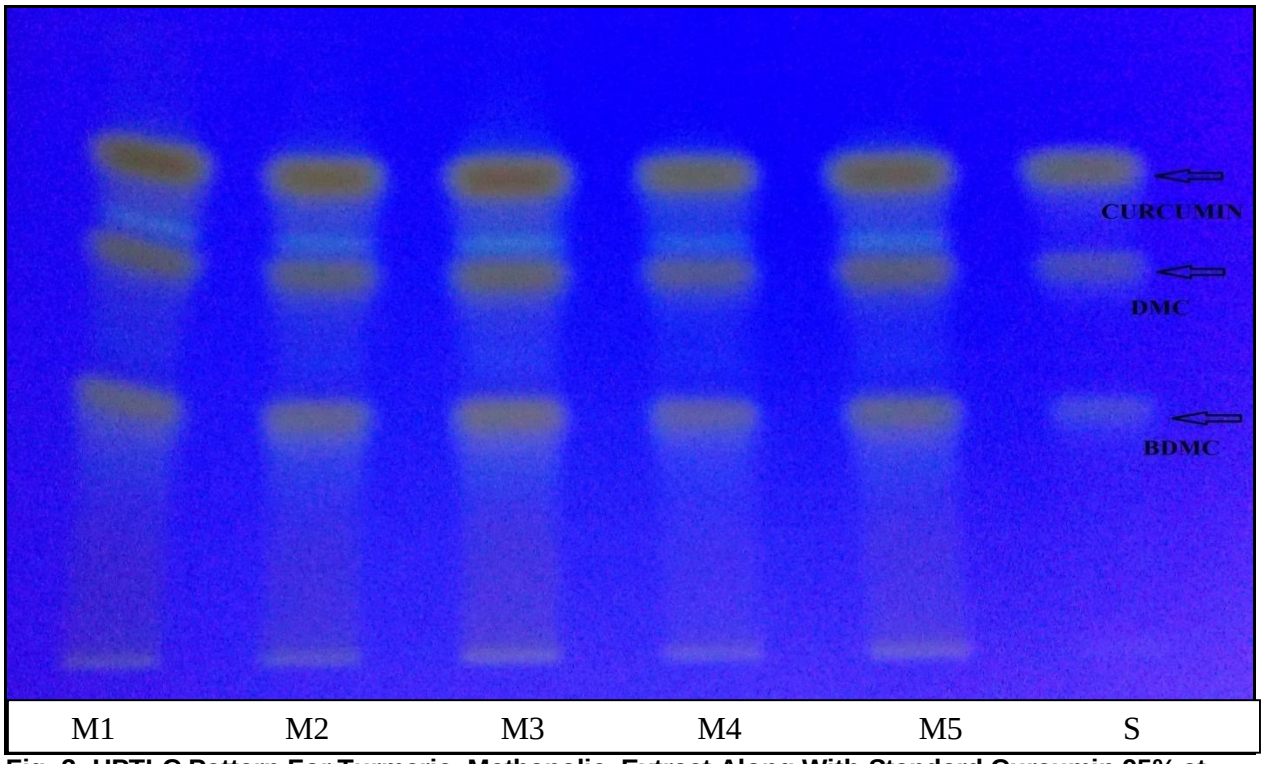

Fig. 3: HPTLC Pattern For Turmeric Methanolic Extract Along With Standard Curcumin 95% at 366 Nm

M1 – Methanolic extract of Gujarat variety; M2 – Methanolic extract of Tamil Nadu variety
M3 – Methanolic extract of Andhra Pradesh variety; M4 – Methanolic extract of Kerala variety
M5 – Methanolic extract of Karnataka variety; S – Curcumin 95% standard

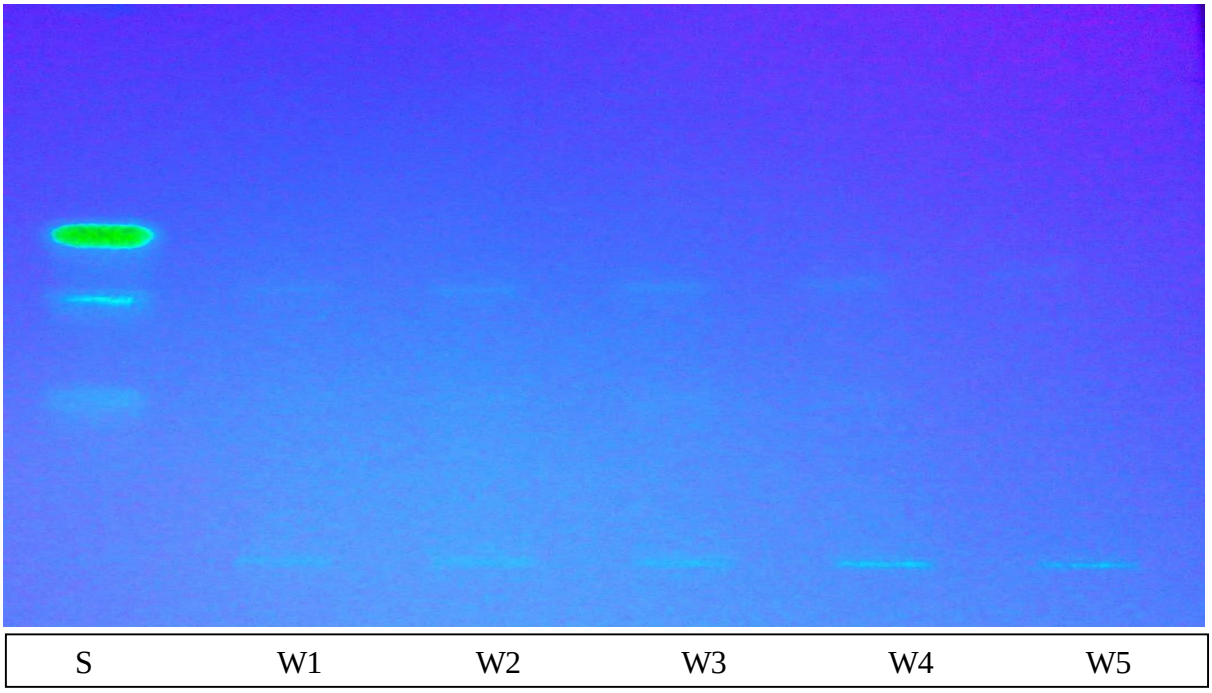

Fig. 4: HPTLC pattern for turmeric aqueous extract along with standard curcumin 95% at 366 nm

S – Curcumin 95% standard; W1 – Aqueous extract of Gujarat variety
W2 – Aqueous extract of Tamil Nadu variety; W3 – Aqueous extract of Andhra Pradesh variety
W4 – Aqueous extract of Kerala variety; W5 - Aqueous extract of Karnataka variety

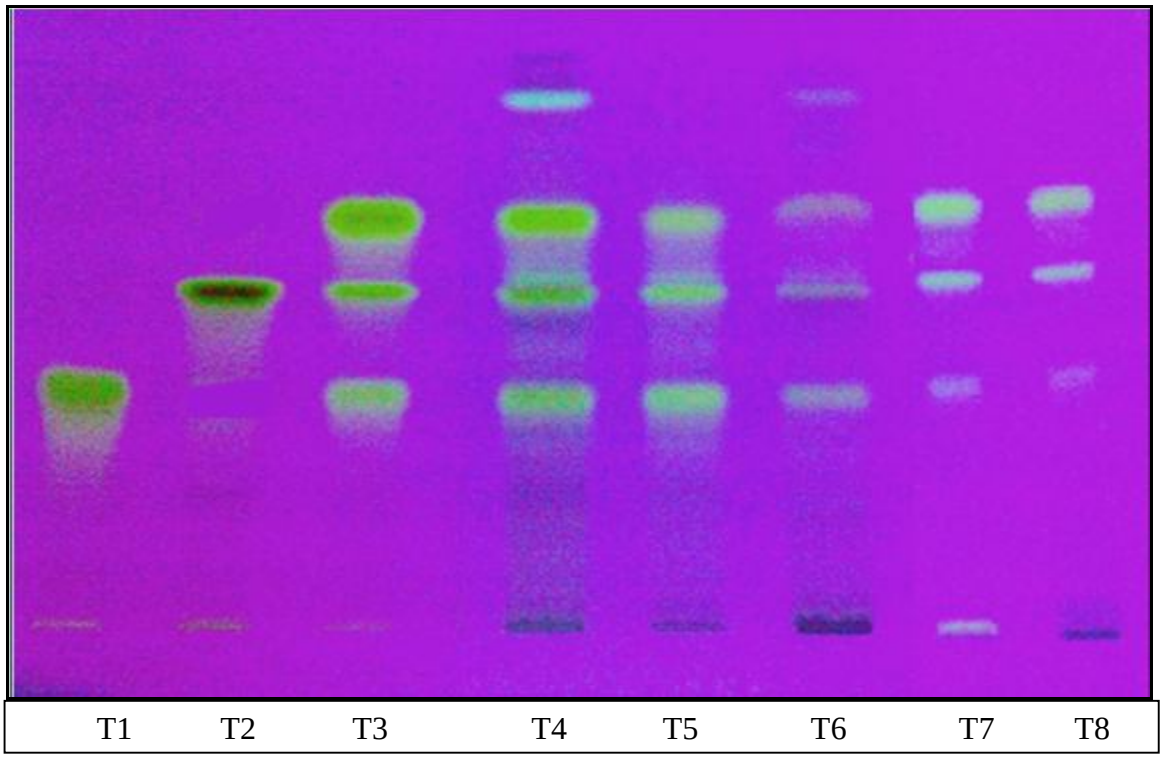

Fig. 5: HPTLC pattern for turmeric marketed formulations along with standard curcumin 95% at 366 nm

T-1= BDMC, T-2 = DMC, T-3 = CURCUMIN 95%, T-4= JWARNASHAK KWATH, T-5=TURMERIC, T-6 =CHANDRAPRABHAVATI, T-7 =UTRIPLEX CAPSULE, T-8 = HYPONIDD TABLET

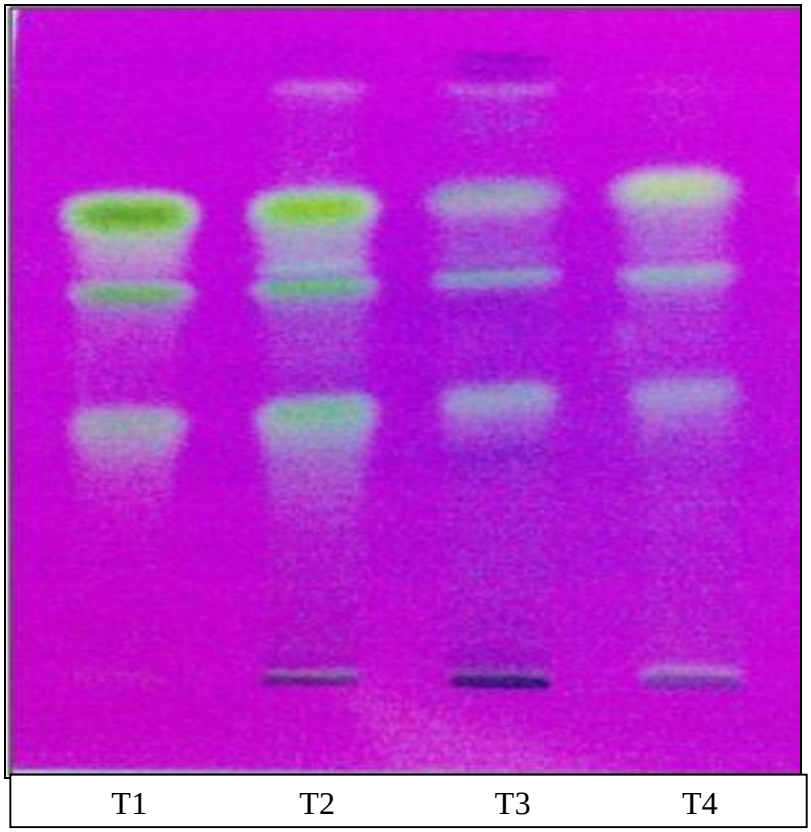

Fig. 6: HPTLC pattern for turmeric marketed formulations along with standard curcumin 95% at 366 nm

T-1 = CURCUMIN 95%, T-2 = DASHANG LEPA CHURNA, T-3 = MAHASUDARSHAN CHURNA, T-4 = PUNARNAVADI MANDUR TABLET

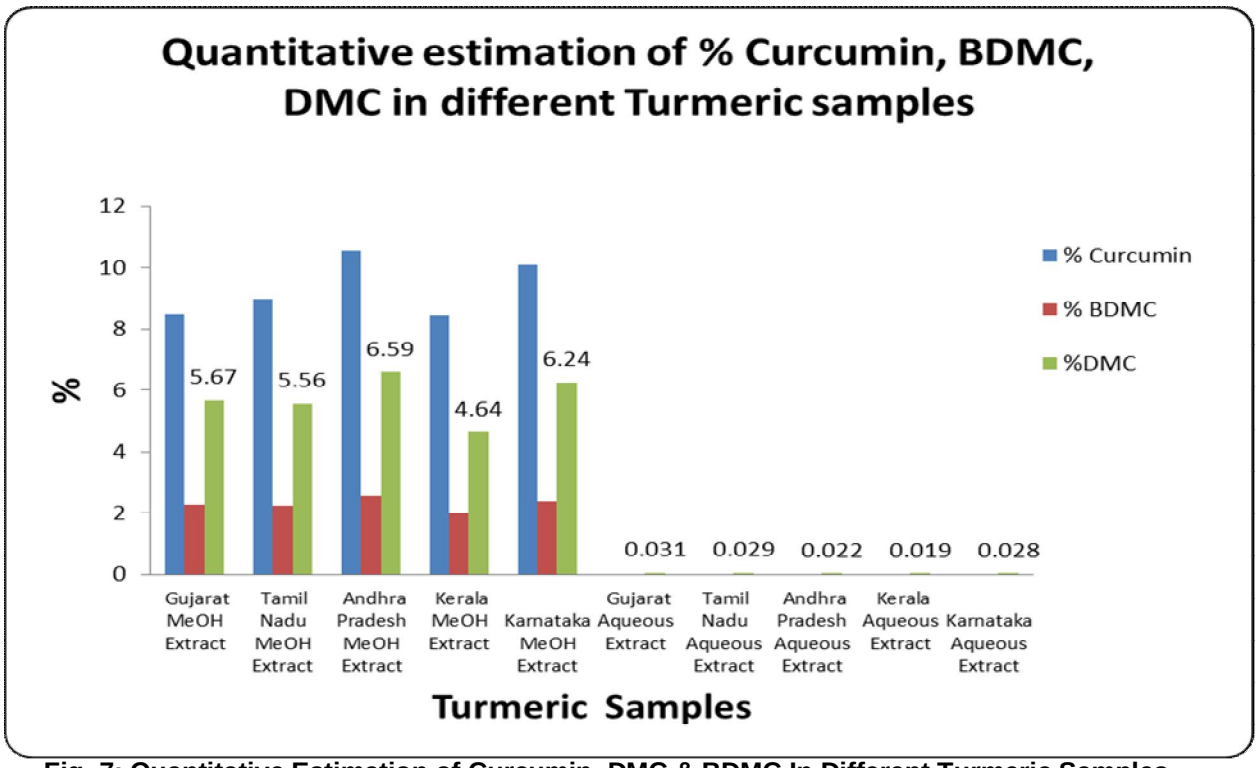

Fig. 7: Quantitative Estimation of Curcumin, DMC & BDMC In Different Turmeric Samples

%DMC values is mentioned in the above chart as in aqueous extracts, curcumin and BDMC were not observed

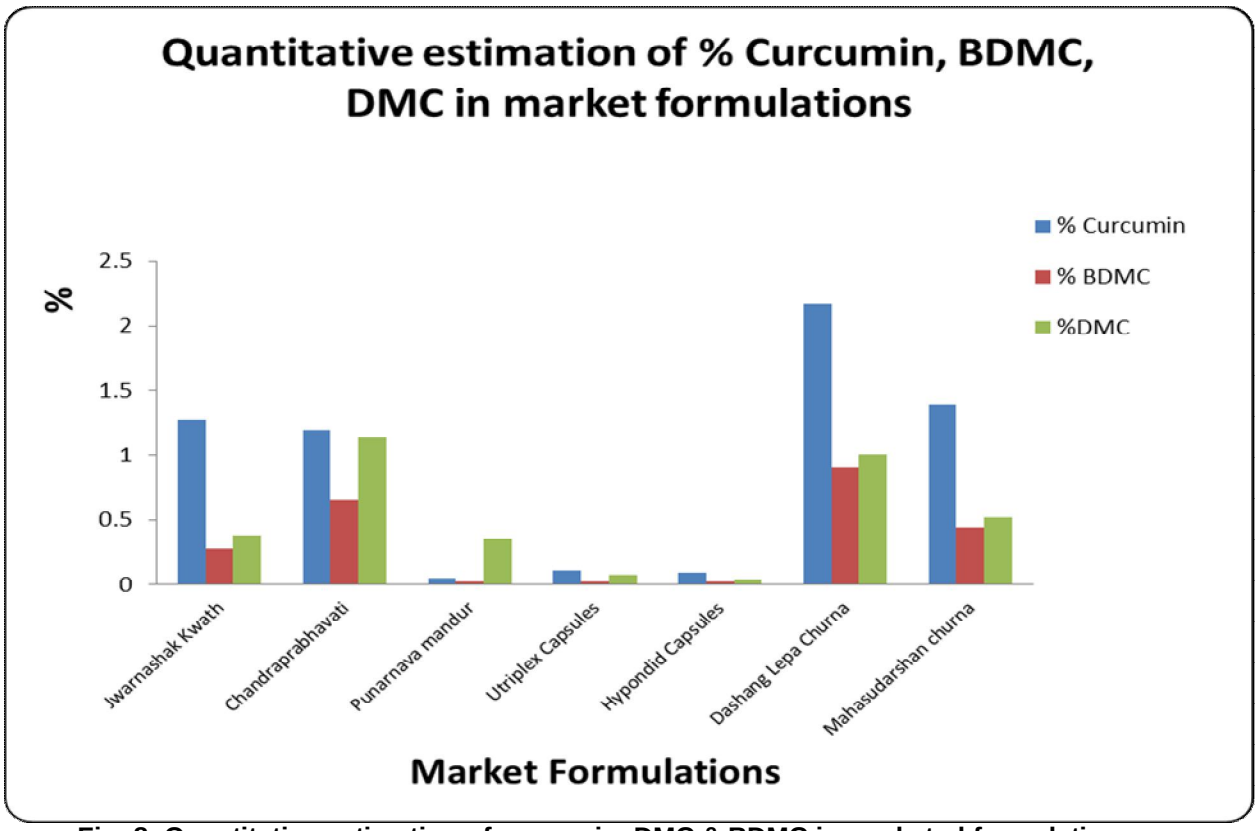

Fig. 8: Quantitative estimation of curcumin, DMC & BDMC in marketed formulations

Table 1: Summary of validation parameters for estimation of curcumin by HPTLC method

Parameter	Curcumin
Λ max (nm)	425
Linearity range (µg/spot)	1.0-5.0
Correlation Coefficient	0.998 ± 0.0002151
Regression equation	y = 3029.36 x + 5468.11
Limit of detection (µg/spot)	0.056
Limit of Quantification (µg/spot)	0.171
Recovery (Mean ±S.D.)	99.64 ± 0.293
Precision (% RSD)	
Repeatability of application (n=6)	0.283
Repeatability of measurement (n=6)	0.267
Intra-day*	0.248
Inter-day**	0.231
Robustness	Robust

Table 2: Summary of validation parameters for estimation of DMC by HPTLC method

Parameter	DMC
λ max (nm)	425
Linearity range (µg/spot)	1.2 - 6.0
Correlation Coefficient	0.999 ± 1.451E-05
Regression equation	y = 1715.38 x + 13970.64
Limit of detection (µg/spot)	0.054
Limit of Quantification (µg/spot)	0.164
Recovery (Mean ±S.D.)	99.53 ± 0.230
Precision (% RSD)	
Repeatability of application (n=6)	0.149
Repeatability of measurement (n=6)	0.153
Intra-day*	0.089
Inter-day**	0.096
Robustness	Robust

Table 3: Summary of validation parameters for estimation of BDMC by HPTLC method

Parameter	BDMC
Λ max (nm)	425
Linearity range (µg/spot)	1.0-5.0
Correlation Coefficient	0.996 ± 0.0002
Regression equation	y = 4677.61x + 5013.66
Limit of detection (µg/spot)	0.018
Limit of Quantification (µg/spot)	0.056

Recovery (Mean ±S.D.)	99.60 ± 0.233
Precision (% RSD)	
Repeatability of application (n=6)	0.168
Repeatability of measurement (n=6)	0.171
Intra-day*	0.171
Inter-day**	0.159
Robustness	Robust

* Mean of three concentrations in triplicates in the same day.
** Mean of three concentrations in triplicates in three different days.

Table 4: Percent content of curcumin, dmc & bdmc in marketed formulations

S. No.	Sample Name	% Curcumin ± S.D	% DMC ± S.D	% BDMC ± S.D
1	Jwarnashak Kwath	1.27 ± 0.021	0.38 ± 0.031	0.27 ± 0.008
2	Chandraprabhavati	1.19 ± 0.040	1.14 ± 0.040	0.65 ± 0.062
3	Punarnava mandur	0.05 ± 0.012	0.35 ± 0.010	0.02 ± 0.002
4	Utriplex Capsules	0.11 ± 0.015	0.07 ± 0.004	0.02 ± 0.003
5	Hypondid Capsules	0.09 ± 0.015	0.04 ± 0.003	0.02 ± 0.002
6	Dashang Lepa Churna	2.17 ± 0.067	1.01 ± 0.078	0.90 ± 0.021
7	Mahasudarshan churna	1.39 ± 0.031	0.52 ± 0.068	0.44 ± 0.033

REFERENCES

1. Dobelis IN, ed. Magic and Medicine of Plants. Pleasantville, NY: Reader's Digest Association, Inc. 1986.
2. Leung A. Encyclopedia of Common Natural Ingredients Used in Food, Drugs, and Cosmetics. New York, NY: John Wiley; 1980:313-314.
3. Merina Benny Antony. Indigenous Medicinal Plants: their extracts and isolates as a value added export product. Journal Agro bios 2003; 1:39-41.
4. Asha J, Rongqiqn W, Mian Z, Ping Wang, Mechanism of anti inflammatory effect of curcumin: PPAR-r Acitivation, Hindawi Publishing Corporation PPAR Research, 2007: 1: Article ID-89369.
5. Yang FQ, Li SP, Zhao J, Lao SC. Wang YT. Optimization of GC-MS conditions based on resolution and stability of analytes for simultaneous determination of nine sesquiterpenoids in three species of Curcuma rhizomes. J Pharm Biomed Anal 2007; 43: 73-82.
6. Jadhav BK. Mahadik KR. Paradkar AR. Development and validation of improved reversed phase- HPLC method for simultaneous determination of curcumin, DMC, and bis-demethoxvcurcumin. Chromatographia 2007; 65: 483-488.
7. Javaprakasha GK. Rao LJM, Sakariah KK. Improved HPLC method for the determination of curcumin. (DMC and BDMC. J Agric Food Chem 2002; 50: 3668—3672.
8. Wisut W. Nutthapon J. Sunibhondn P'. Pornchai RA. simple isocratic HPLC method for the simultaneous determination of curcuminoids in commercial turmeric extracts. Phytochem Anal 2009; 20: 314—319.
9. Rui L, Cheng X, Min Y, Hui-Fang L. Xing Z and De-An G. Qualitative and quantitative analysis of curcuminoids in herb al medicines derived from Curcuma species. Food Chem. 2011; 126:1890—95