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Standardization and stability studies of neuroprotective lipid soluble fraction obtained from *Curcuma longa*

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Abstract

The lipid soluble fraction of *curcuma longa*, i.e. herbal medicament (HM) was isolated from rhizome of *curcuma longa* by solvent extraction method. The identification of chemical constituent present in HM was done with GC, GC–MS. The standardization of HM was done using HPLC method on the basis of three-marker compound isolated, i.e. *ar*-turmerone, turmerone and curlone. The effect of temperature, pH and light on stability of marker compounds of HM was studied. The composition of marker compound in HM was found to be 50–60%. The content of curcumnoids, another bioactive constituent present in HM was found to be 0.32–0.55%. The HM was found to be stable at different temperature and pH but light sensitive.

Keywords: *Curcuma longa*; Herbal medicament; Turmerone; Stability; GC–MS; HPLC

1. Introduction

Curcuma longa L. (Zingiberaceae) rhizomes, commonly known as turmeric, have traditionally been used as a source of coloring matter for foods, cosmetics and textiles and as well as medicinal agent in the formulations as mentioned in Indian system of medicine for several common ailments [1]. Turmeric powder, extract and oleoresins are some of the widely used commercial products of the plant and India is one of the largest manufacturers of turmeric and its oleoresins [2,3].

The essential oil of turmeric had shown to possess antimicrobial, antifungal, antiviral [4], anti-inflammatory activity, wound healing and insecticidal activity [5]. Curcuma oil has shown potential effect in oral sub mucous fibrosis in human [6] and currently, under clinical trails for treatment of bronchial asthma [7]. Sesquiterpenoids are the major constituents present in oil and known to possess many biological activities [8,9].

Herbal medicament (HM) – the lipid soluble fraction obtained from *curcuma longa* L. has shown potential neuroprotective activity in neurovascular disorder like stroke [10] and is currently under advance stage of drug development at C.D.R.I, Lucknow. The identification and relative amount of components in turmeric oil have been determined by LC–MS [11,12], GC and GC–MS [13,14]. Unfortunately, the results of relative amount could not be used for evaluating the quality of different samples or batches and determining clinical dose of herbs [15].

Recently few GC–MS method has been reported for quantification of sesquisterpenes in different curcuma species [16,17]. However, long run time, high cost and heat sensitive nature of sesqueterpeoinds are main constraints. Degradation of compounds due to heat liable nature has been reported for sesquiterpenes such as germacrene [18,19], germacrone [20] and furanodiene [21,22]. In the present study, our aim was to develop an accurate, specific and reproducible HPLC method for determination of *ar*-turmerone and turmerone (mixture of α and β isomer) and curlone in HM and to perform stability studies using validated analytical HPLC method. Routine finger printing and identification of different component present in HM was done using GC and GC–MS technique.

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2. Materials

Different batches of herbal medicament (HM) were prepared from rhizomes of *Curcuma longa* (turmeric) procured from the market; botanist of Central Drug Research Institute authenticated *Curcuma longa* sample. All solvents used were of analytical grade unless otherwise stated. Hexane, acetonitrile, ethylacetate procured from E-Merck Ltd. (Mumbai, India) was used throughout the study. The reference standard of *ar*-turmerone, turmerone, and curlone (puity∼98) were separated and purified.

3. Methods

3.1. Preparation of herbal medicament

Dried rhizomes of *Curcuma longa* were powdered (sieved through mesh no. 20), 50 g of this was extracted through percolation using light petroleum ether at 60–80 °C (150 mL for each percolate), six percolates after occasional mechanical stirring were collected, solvent removed below 50 °C under vacuum and the resulting oily residue was dried.

3.2. GC and GC–MS for identification of component

The chemical fingerprinting of HM was regularly done using Gas Chromatograph Perkins Elmer, Japan: equipped with Auto system XL system controller, FID detector. The separation was achieved on a SE-30 column. Temperature at start was 60 °C and was gradually increase to 260 °C at 3 ^{O}C/min and kept at 260^{O}C for 20 min. The injector and detector temperature was kept at 281 °C. The carrier gas was nitrogen (12 mL/min). The samples were diluted with chloroform and 1 μL of sample was injected.

To identify the individual components, GC–MS system (Shimadzu, QP-5000, Kyoto, Japan), equipped with a 30 m × 0.25 mm i.d. DB-5 fused silica capillary column was used. The electron impact technique (70 eV) was used. The carrier gas was helium (1.7 mL/min), and 1 μL of sample was injected. The injector temperature was 240 °C, and that of the detector was 230 °C. The temperature programming was 50 °C for 5 min, inversed to 180 °C at 4 °C/min and to 280 °C at 15 °C/min, and held at 280 °C for 19 min. Identification of the chemical constituents was based on (i) comparison of substance mass spectra with the GC–MS system data bank (NIST 62 library); (ii) comparison of mass spectra with data in the literature [23,24].

3.3. HPLC method for identification and quantification of marker compound

The HPLC workstation used from Shimadzu, Japan: equipped with SCL-10 AVP system controller, LC-10AT VP twin pumps, SPD-10 A VP UV–VIS detector, and Rheodyne injector with 20 μL injection loop. The separation was achieved on a Luna 5 μ C-18 (250 mm × 4.60 mm) reverse phase analytical column (from Phenomenex). The mobile phase consists of acetonitrile (A) and water (B). The flow rate was 1 mL/min with isocratic flow A:B 85:15. Both the solutions were filtered and degassed before use.

3.4. Preparation of standard solutions

Standard stock solutions 20 mg in 25 mL acetonitrile each of *ar*-turmerone, turmerone and curlone were prepared. Working standards of different concentrations were prepared by dilution of the stock solution with acetonitrile to give solution of *ar*-turmerone, turmerone and curlone in concentration range of 0.2–1, 0.2–1 and 0.2–1 μg/mL, respectively.

3.5. Validation of method

3.5.1. Precision

Precision of the method was carried out at three different concentration levels 200, 400 and 600 ng/spot of *ar*-turmerone, turmerone and curlone. The repeatability of sample application and measurement of peak area for the compound were expressed in terms of relative standard deviation (R.S.D.%) and standard error (S.E.). Method repeatability was obtained from R.S.D. value by repeating the assay six times in same day for intraday precision. Intermediate precision was assessed by the assay of two, six sample sets on different days (interday precision).

3.5.2. Limit of detection and limit of quantitation

In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank acetonitrile was injected six times. The signal to noise ratio was determined. LOD and LOQ were experimentally verified by diluting known concentrations of *ar*-turmerone, turmerone and curlone until the average responses were approximately three or ten times the standard deviation of the responses for six replicate determinations.

3.5.3. Selectivity

The selectivity of the methods was assessed by running the calibration curve in the presence of high concentrations of the other components of the HM. The intercept, slope, standard error of the estimate, correlation coefficient and relative standard deviation at two concentration levels for the HPLC determination of *ar*-turmerone, turmerone and curlone.

3.5.4. Recovery studies

The accuracy of the method was determined from recovery studies. A known but varying amount of standards was added to the pre-analyzed sample and analyzed according to the procedure. The experiment was conducted six times. This was done to check the recovery of the *ar*-turmerone turmerone and curlone at different levels in the herbal medicament.

3.6. HPLC method for quantification of curcumnoids

The HPLC workstation used from Shimadzu, Japan: equipped with SCL-10 AVP system controller, LC-10AT VP

twin pumps, SPD-10 A VP UV–VIS detector, and Rheodyne injector with 20 μL injection loop. The separation was achieved on a Luna 5 μ C-18 (250 mm × 4.60 mm) reverse phase analytical column (from Phenomenex). Solvents were filtered and degassed before use. The mobile phase consists of 0.5% acetic acid (A) and acetonitrile (B) containing 2.5% methanol each. The flow rate was 1 mL/min with isocratic flow A:B 45:65. Stock standard solution of curcumnoids 2 mg in 25 mL methanol (curcumin, demethoxy curcumin and bisdemethoxy curcumin) was prepared in 25 mL methanol. Working standard was prepared in the range of 0.1–0.6 μg/mL. Each concentration was injected in triplicate.

4. Stability studies

4.1. Effect of temperature

Stability studies of HM were conducted at different temperatures 37, 50, 60 °C in stability chamber. The samples kept at different temperatures were withdrawn at regular time intervals and content of major marker compound *ar*-turmerone in HM was analyzed by HPLC. This study was done for 15 weeks and after that the net decomposition of the *ar*-turmerone and change in ratio of other component present in HM at each temperature was studied. Reaction kinetics was studied with the help of LINREG programme.

4.2. Effect of pH

Acid–base degradation studies were carried out to determine the pH at which stability of HM was maximum. This information is required for the preparation of the final dosage forms. Effect of pH on this compound was studied using following method. About 1 mL each of stock solution of HM was taken in 10 mL volumetric flasks and the volumes were made up with buffers of pH 3–9. Samples were withdrawn at different time intervals and 20 μL was injected on to the HPLC column to analyze as described above. Effect of different pH on the stability of HM was studied based on change in concentration of marker compounds.

4.3. Photochemical stability

The *ar*-turmerone (20 mg) and HM (60 mg) containing approx 20 mg of *ar*-turmerone were exposed to direct sunlight and UV chamber at 254 nm for 24 h. After 24 h exposure the HM and *ar*-turmerone were diluted with acetonitrile and injected into HPLC and then chromatograms were recorded as described in Section 2.2. In all degradation studies, the average peak area of *ar*-turmerone after application 1 μg/mL of HM in three replicates and change in ratio of other component with respect to marker compound was determined.

5. Results and discussion

5.1. Extraction of HM

Normally, curcuma oil is obtained by steam distillation process. Since we are interested in the biological activity, we adopted solvent extraction process using nonpolar organic solvent like hexane. The lipid soluble fraction (HM) obtained by this process gave better yield 4.9% as compared to oil obtained from steam distillation process having yield of 2.1% approx. The chemical constituents and their composition obtained by these extraction processes were different than those obtained by steam distillation.

5.2. GC–MS/GC chemical profiling of HM

Standardization/chemical finger printing of HM was performed to check the purity, quality and batch-to-batch variation. Chemical profiling was done using GC (Fig. 1) and GC–MS (Fig. 2). Table 1 shows the major substances found in the HM, their mass fragmentation pattern and relative percentage in HM. The chemical profiles for the HM in different batches were almost similar. Therefore, batch-to-batch variation in chemical composition of HM is negligible. Major characteristic constituents found in HM where *ar*-turmerone, α-turmerone, β-turmerone and curlone, four major curcumenes: curcumene, zingiberene, bisabolene and sesquiphellandrene and other minor constituents like zederone, germacrone, curdione, curcumerone,

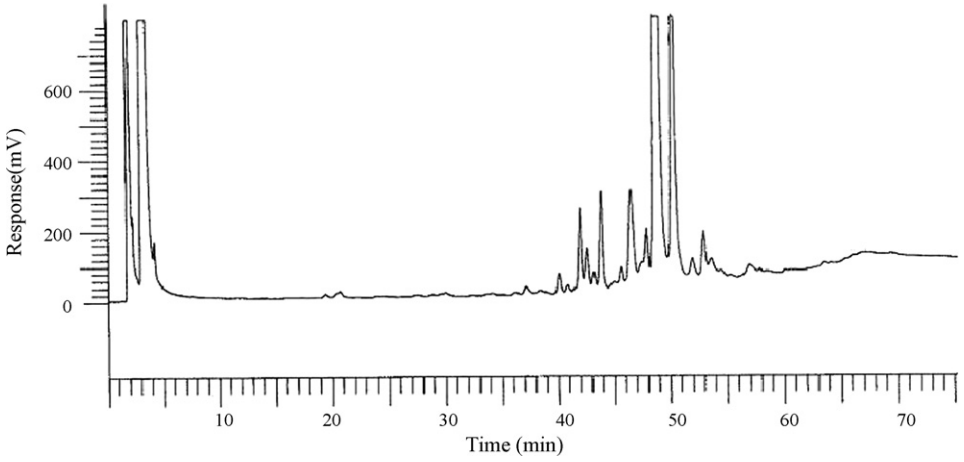

Fig. 1. GC chromatograph of HM.

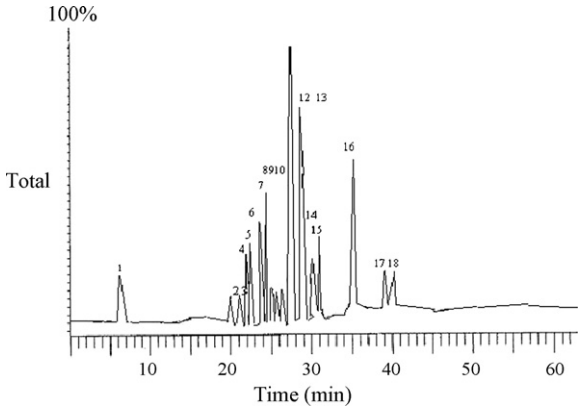

Fig. 2. GC–MS chromatograph of HM.

zederondiol and isozederendiol and three curcuminoids: monodemethoxycurcumin, didemethoxycurcumin, curcumin. The structure of different component present in HM is given in (Fig. 3).

Some chemical constituent present in HM in significant quantity in HM like *ar*-turmerone, turmerone and curlone were isolated by CC followed by preparative HPLC. These isolated pure constituents were used as marker constituents and later quantified using validated HPLC method. Initially, an attempt was made to develop a HPLC method for determining all the three active marker compounds and other minor constituents in single run. The HPLC conditions providing clear separation of different marker constituents and other components was established (Fig. 4).

5.3. Optimization of the chromatographic conditions

The experimental variable optimized to accomplish adequate separation in eluting the analytes were the composition of the mobile phase and the type of column. The influence of the percentage of acetonitrile in binary aqueous mixtures used as mobile phase to separate analytes in a Lichrosorb Diol column was studied in the range 20–100% (v/v) acetonitrile. The retention time of turmerone was long when low percentage of acetonitrile was used, but an increase of acetonitrile content in the mobile phase resulted in good separation of the turmerone chromatographic peaks with shorter retention times. However, overlapping of *ar*-turmerone and turmerone peaks were obtained when acetonitrile content was increased above 85%. Therefore, high acetonitirle content (85% v/v) was used, and this gave rise to clear base line separation of three isolated marker compounds peaks. Isomers α-turmerone and β-turmerone were quantified as a single component because base line separation of this isomer mixture was not possible. The retention time for *ar*-turmerone, turmerone and curlone was found to be 9.0, 11.5 and 14.0, respectively.

5.4. Validation of the methods

The proposed chromatographic methods were validated according to the ICH guideline by considering the following criteria: non-interference of peaks; sensitivity; linearity of the response and precision. The linearity of the response was studied by running the standard curve of *ar*-turmerone, turmerone and curlone solutions of the analyte without the other components of the HM. Standard solutions were injected in triplicate. The linearity was in range from 0.1 to 1 μg/mL for marker compounds. The slope and intercept of the linear segment standard error of the mean (S.E.M.) and correlation coefficient (*r*) are listed in Table 2. The lower detection limits were 25, 50 and 25 ng/mL for *ar*-turmerone, turmerone and curlone, respectively.

The selectivity of the methods was assessed by running the calibration curve of *ar*-turmerone, turmerone and curlone in the presence of high concentrations of the other components of the HM (Table 2). The accuracy of the method was determined from recovery studies. The average recovery percentage values were found to be 97.73%, 98.51% and 97.77% for *ar*-turmerone and turmerone and curlone, respectively. The results were shown in Table 3.

Table 1
Chemical constituents of HM by GC–MS

Peak no.	Retention time (min)	Total area (%)	Mass *m*/*z*	Compound
1	21.60	0.9	161	NE: 161(M+, 7), 41(100), 53(12), 67(24), 79(28), 93(30), 105 (15), 133(12)
2	22.13	0.6	202	NE: 202(M+, 11), 41(95), 69(100), 91(30), 145(3)
3	22.96	3.3	202	Curcumene
4	23.40	4.1	204	Zingiberene
5	23.76	1.2	204	B-bisabolene
6	24.23	5.4	204	Sesquiphellandrene
7	24.86	0.7	187	NE: 187(M+, 18), 41(100), 67(75), 91(75), 105(40), 119(26), 131(20), 145(18)
8	26.06	1.8	216	Curzerene
9	27	0.9	120	NE: 120(M+, 100), 41(18), 55(30), 91(42), 105(25)
10	27.86	31.7	216	*ar*-Turmerone
11	27.96	11.5	218	α-Turmerone
12	28.73	14.3	218	β-Turmerone
13	29.63	2.9	218	Curlone
14	30.33	2.1	218	Germecrone
15	34.73	1.1	236	Curdione
16	38.46	1.44	234	Curcumenol

NE: Not identified (with mass fragmentation).

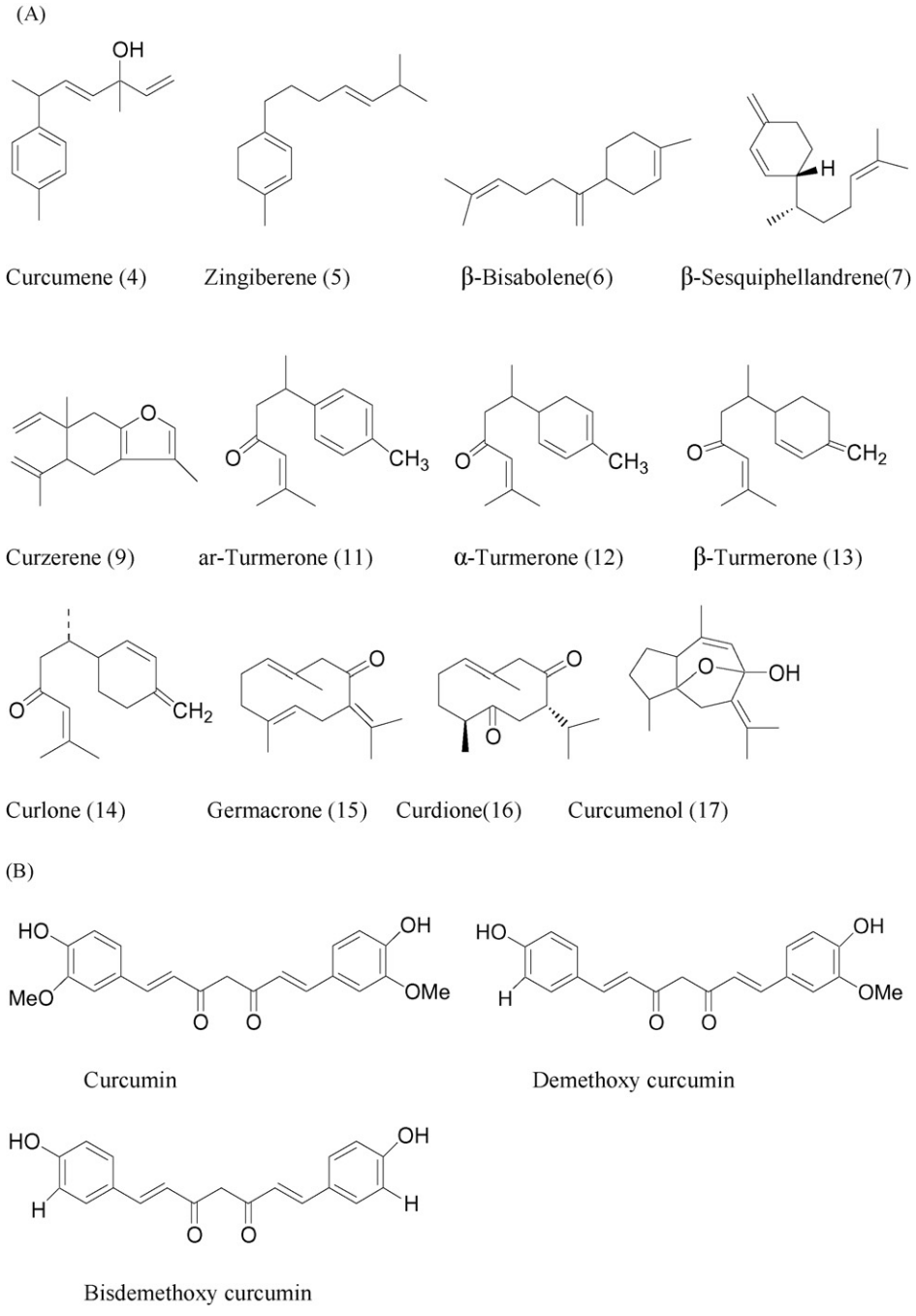

Fig. 3. (A) Structure of compounds in HM characterized by GC–MS. (B) Structure of curcumnoids present in HM.

The repeatability, calculated as the R.S.D. of nine successive injections on the same day (interday), was 1.98%, 2.45% and 1.78% for *ar*-turmerone, turmerone and curlone, respectively. The reproducibility, calculated, as the R.S.D. of successive injections of six solutions carried out on three different days (intraday), was 2.0%, 2.78% and 2.34% for *ar*-turmerone, turmerone and curlone, respectively. The analysis of variance (ANOVA) for these replicates is summarized in Table 4.

Table 2
Linear regression data for calibration curve o f marker compounds in absence and presence of other components of HM

Marker compound	Slope ± S.D.	Intercept ± S.D.	S.E. of estimate	r^2	Slope ± S.D.	Intercept ± S.D.	S.E. of estimate	r^2
ar-Turmerone	4.03 ± 0.22	10.06 ± 0.56	25.24	0.997	4.76 ± 0.67	11.56 ± 0.33	27.56	0.997
Turmerone	4.90 ± 0.39	13.01 ± 0.15	21.29	0.995	5.27 ± 0.34	13.80 ± 0.78	22.22	0.995
Curlone	3.65 ± 0.71	12.99 ± 0.54	18.44	0.993	4.23 ± 0.50	12.14 ± 0.96	19.50	0.993

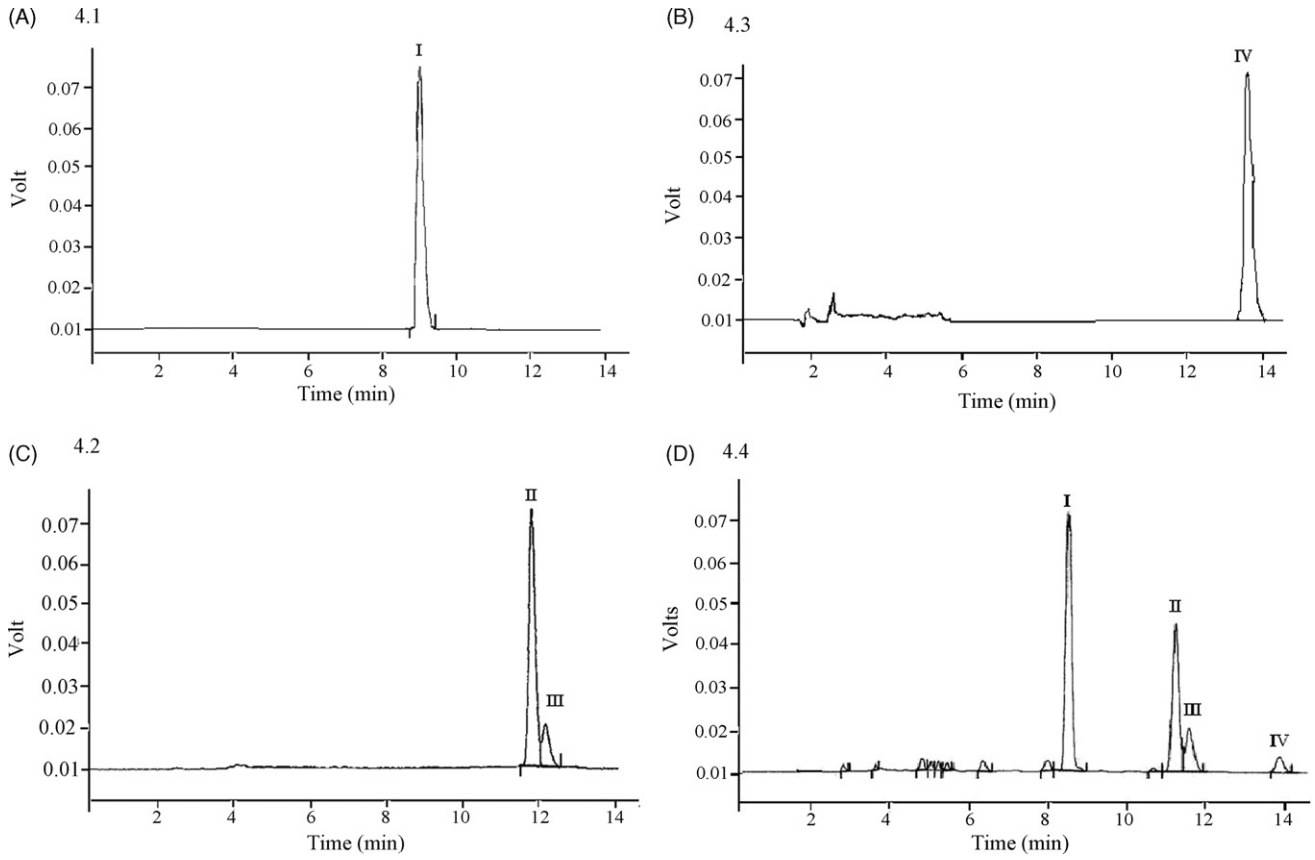

Fig. 4. HPLC chromatograph of (A) *ar*-turmerone I; (B) α-turmerone II, β-turmerone III; (C) curlone IV; (D) HM.

5.5. Application of the method

The performance of the method was tested by applying it to the determination of content of marker compound in different batches of HM for regular quality control prior to their detailed biological evaluation. There was good correlation between concentration found for *ar*-turmerone, turmerone and curlone by HPLC and GC, respectively. Using this validated HPLC method, the relative contents of *ar*-turmerone, (α and β)-turmerone and curlone were estimated in HM. The results of these analyses for different batches are summarized in Table 5. Different chemical component were observed in chromatogram of the HM; these components appear in the chromatogram at significantly different *RT* values. Marker compound *ar*-turmerone, turmerone and curlone gives well distinguish peak at distinct retention time. There was no interference in the analysis of marker compound from other components.

Table 3
Recovery

Marker	Spiked (μg)	Recovered (μg)	Recovery (%)
ar-Turmerone	250	242	96.80
	500	490	98.00
	750	738	98.40
Turmerone	250	251	100.40
	500	485	97.00
	750	736	98.13
Curlone	250	244	97.60
	500	492	98.40
	750	730	97.33

Table 4
Analysis of variance ANOVA for the proposed method

	ar-Turmerone	Turmerone	Curlone
Interday variance	0.0045	0.0067	0.0056
Intraday varinace	0.0076	0.0089	0.0059
LOD	25	50	25
LOQ	50	100	50
F-ratio	1.06	1.23	1.12
Mean value	0.65	1.45	1.01
Precision Interday R.S.D. (%)	1.98	2.45	1.78
Precision intraday R.S.D. (%)	2.0	2.78	2.34

Table 5
Marker compounds in different batches of HM by validated HPLC method

Batch	*ar*-Turmerone (%)	Turmerone (%)	Curlone (%)	Curcumnoids (%)
HM-1	27.75	30.70	4.70	0.55
HM-2	26.09	36.65	3.95	0.53
HM-3	27.71	31.89	5.10	0.40
HM-4	27.09	33.50	3.66	0.48
HM-5	30.01	34.50	3.85	0.32

Table 6
Effect of temperature on *ar*-turmerone (HM) content

Temperature (°C)	Reaction rate constant	Shelf-life (weeks)	Half-life (weeks)
37	5.268×10^{-3}	19.99	131.50
50	1.318×10^{-2}	7.99	52.59
60	1.38×10^{-2}	7.64	50.24

Table 7
Photostability of HM

Parameter	Time (h)	*ar*-Turemerone (remaining (%))	Ratio of *ar*-turmerone/turmerone (area)	Ratio of *ar*-turmerone/curlone (area)
	0	100	1.9	9.65
Sunlight	24	95.93	7.9	Undetected
UV exposure	24	97.66	7.14	66.91

5.6. Quantification of curcumnoids

Curcumnoids especially curcumin is reported to exhibit many pharmacological activities. Therefore, amount of curcumnoids present in HM can be critical. The percentage of curcumnoids in HM was estimated by reported HPLC method [5]. The curcumniods bisdemethoxy curcumin, demethoxy curcumin and curcumin give well distinguished peaks at distinct retention time of 9.2, 9.6 and 10.7, respectively, as depicted in Fig. 5. The amount of curcumnoids present in HM was found to be in range of 0.32–0.55% (Table 5).

5.7. Effect of temperature

The samples were kept at 37, 50 and 60 °C for different duration of time. The content of major marker *ar*-turmerone was determined in the samples by HPLC. The reaction rate constant was calculated by LINREG program. The *ar*-turmerone was found to follow first order kinetics. Stability profile of HM at different temperature with respect to *ar*-turmerone content is depicted in Table 6. Energy of activation, half-life and Shelf-life was found to be 37.188 kJ/mol, 212 weeks (about 4.25 years) and 32.25 weeks at 25 °C.

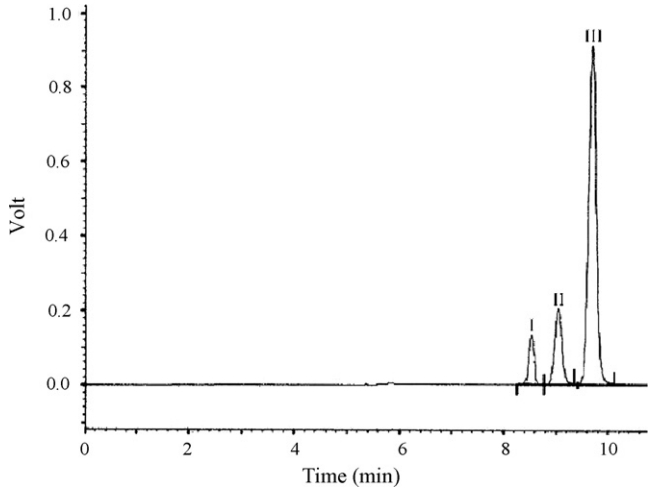

Fig. 5. HPLC chromatograph of Curcumnoids: (I) bis-demethoxycurcumin; (II) demethoxy curcumin; (III) curcumin.

5.8. Effect of pH

The results of stability studies at different pH (at 30 ± 2 °C) indicated that the HM is stable at different pH range. The HM was incubated at acidic as well as basic pH and changes in different component was assess at different time duration by HPLC. No significant changes in chromatograph of HM can be visualized. The change in concentration of marker constituents with time was minimal (data not shown). Therefore, any liquid formulation of HM should be stable at wide pH range. Therefore, pH-based degradation of marker components seems to be minimum and designing of other drug delivery systems for this HM at varied pH may be possible.

5.9. Effect of light

Herbal medicament is light sensitive. Although percentage change in concentration of *ar*-turmerone (marker compound) is not significant when exposed to sunlight or UV light for 24 h. The change in content of turmerone and curlone is significant as indicated in Table 7. No peak for curlone was detected after 24 h exposure to sunlight. Work is in progress in our laboratory to identify any photo-degradation product of turmerone and curlone in HM. There is change in color intensity of HM at 50 and 60 °C, this effect may be mainly due to degradation of curcumnoids present in HM. The quality and marker concentration in HM are well protected when kept in dark or away from direct exposure of sun or UV light.

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