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RP-HPLC Method Development and Validation for Simultaneous Estimation of Thymoquinone and Curcumin in Dosage form

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aim: The aim of work is to develop and validate simple, specific, accurate, precise HPLC method for the estimation of Curcumin (CUR) and Thymoquinone (THQ) simultaneously in bulk and its formulation as per ICH guidelines for analytical method development and validation.
Study Design: Developing RP-HPLC method using C-18 Inertsil column and optimization of variables for estimation of Thymoquinone and Curcumin in bulk and formulation.
Place and Duration of Study: The present work was carried out at Shri D. D. Vispute College of Pharmacy and Research Center, Panvel in year 2021.
Methodology: The RP-HPLC method was developed with an isocratic condition of mobile phase comprising acetonitrile and water in a ratio of (82:18) v/v, at a flow rate of 0.9 mL/minute over Inertsil ODS, 250× 4.6 mm, 5 µm column, at 30°C column oven temperature. Photodiode array at 256 nm was used for detection
Results: Retention time for curcumin and thymoquinone was found to be 3.5 and 4.3 mins respectively. The method showed excellent linear response in concentration range of 4-18 µg/mland 10-45 µg/mlfor thymoquinone and curcuminrespectively with correlation coefficient (R^2)

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values of0.999 for both. System precision and method precision studies were less than the maximum allowable limit percentage of relative standard deviation ≤ 2.0 i.e., 1.61 % and 1.62 % for curcumin and 0.47 % and 0.42 % for thymoquinone respectively. Mean % Recovery for both the drugs were within acceptance limits. The developed and validated HPLC method is simple, accurate, precise and suitable for analysis as all the results were within acceptance criteria.

Conclusion: The developed RP-HPLC method at single wavelength was validated according to ICH guideline with respect to system suitability, specificity, linearity, accuracy, precision and robustness and can be used for routine quality monitoring of drug in pharmaceutical dosage form.

Keywords:Curcumin; thymoquinone; HPLC.

1. INTRODUCTION

Curcumin is a naturally occurring yellow coloured compound produced by plants of *Curcuma longa* species, belongs to ginger family, *Zingiberaceae*. It possesses anti-oxidant, anti-inflammatory, anticarcinogenic, anti-mutagenic, anti-fungal, anti-viral and anti-cancer activities [1]. *Nigella sativa* Linn., belongs to family *Ranunculaceae*, commonly known as black seed or black cumin. *N. sativa* has shown wide therapeutic properties including hypotensive, anti-nociceptive, uricosuric, choleretic, antifertility, antidiabetic, anti-histaminic, anti-oxidant, anti-inflammatory, anti-microbial, anti-tumor and immunomodulatory effects [2].

Gilani S. J., et al. [3] developed and validated RP-HPLC method for estimation of Thymoquinone (TMQ) in API as well as in noisome. The column used for the study was C-18 (150 mm × 3.9 mm × 5 µm). The method showed linearity range between 6.25 - 100 µg/mL.

Efforts were taken by Iqbal M., et al. [4] to perform Solvent based optimization for extraction and stability of thymoquinone from *Nigella sativa* Linn. and its quantification using RP-HPLC in an isocratic system. The developed methods were only for estimation of thymoquinone individually.

Wichitnithad W., et al. [5] developed a simple isocratic HPLC method for the simultaneous determination of curcuminoids in commercial turmeric extracts on an Alltima C18 column with isocratic elution. The method was validated and applied for quantification of individual curcuminoids in commercial turmeric extracts. Jadhav B.K., et al.[6] developed and validated improved RP-HPLC method for simultaneous determination of curcuminoids. Mobile phase consisted of acetonitrile:0.1% trifluro-acetic acid (50:50) and flow rate was 1.5 mL min−1 and elution were monitored at 420 nm with column (Vydac, RP-18, 250 mm × 4.6 mm, 5 µm).

Literature survey [7,3,4,8,9,5,6] reveals that Kazia M., et al. [7] developed method for determination of Curcumin (CUR) and Thymoquinone (THQ) simultaneously using UHPLC-UV-Vis Spectrophotometer with two wavelengths.

From above examples, it is clear that although many methods have been reported on the quantitative and qualitative estimation of thymoquinone and curcumin in bulk and its formulation individually, very little work has been done on its simultaneous estimation and validation by RP-HPLC method using single wavelength with PDA detector.

There was need to develop method for determination of thymoquinone and curcumin simultaneously using simple RP-HPLC method as thymoquinone and curcumin are gaining therapeutic superiority. Co-administration of thymoquinone and curcumin not only show therapeutic efficiency but also reduce toxicity. The proposed method over other methods isthat the method was developed and validated RP-HPLC using single wavelength with less run time for routine analysis, which can be easily reproducible.The proposed method was validated according to the ICH guidelines.

2. EXPERIMENTAL

2.1 Materials and Methods

2.1.1Chemicals and Reagents

Thymoquinone [10] and Curcumin [11]standard were obtained from Yucca Enterprises, Mumbai, India. HPLC grade solvents were used in chromatographic separation of Thymoquinone and Curcumin, and a 0.45 µ membrane filter was obtained from Millipore. Thymoquinone and Curcumin capsules (label claim 62.5 mg of Curcumin and 25 mg of Thymoquinone) were used for the analysis.

2.1.2 Instrument

The HPLC system used was JASCO-4000 Extrema having to ChromNAV software for processing the data equipped with a PDA detector and autosampler. The analytical column that was selected for ideal separation was the Inertsil ODS- 250 × 4.6 mm, 5 µm column. Several solvents in different proportions were tested to optimize conditions for the separation of drugs.

2.2 Selection of Analytical Wavelength by UV Analysis

Blank/Diluent: Acetonitrile.

2.2.1 Preparation of standard solution for UV analysis

10 mg of thymoquinone and curcumin was weighed and transferred into two different 10 ml volumetric flask. Added 6 ml of diluent and sonicated well to dissolve. Volume was made up to the 10 ml using diluent and mixed well (1000 µg/ml).

Further 1 ml was diluted from above stock to 100 ml volumetric flask using diluent (10 µg/ml).

2.2.2 Selection of working wavelength

Baseline was taken by scanning at 800 to 200 nm range.

Standard solution of Thymoquinone and Curcumin was scanned at 800 to 200 nm wavelength for determination of workingwavelength by recording the spectra. After scanning, working wavelength was selected by overlaying both the UV Spectra.

2.3 HPLC Method Development

2.3.1 Preparation of Standard Stock Solution

Preparation of Standard Stock Solution: Weighed accurately 10 mg of Thymoquinone and 25 mg of Curcumin Standard and transferred it into 100 ml of ambered colored volumetric flask. Added about 70 ml of diluent and sonicated to dissolve standards. Volume was made up to mark with diluent and mixed well.

Standard stock concentration of Thymoquinone: 100 µg/ml
Standard stock concentration of Curcumin: 250 µg/ml

Preparation of Working Standard Solution: 10 ml was pipetted out from standard stock solution and diluted to 100 ml using diluents and mixed well.Final concentration of standard solution: 10 µg/ml of THQ and 25 µg/ml of CUR.

Preparation of Sample Solution: Weighed accurately net content of 20 capsules. 761 mg (weight of one capsule content) was transferred to 100 ml of volumetric flask. Added about 70 ml of acetonitrile as diluent and sonicated it for 20 mins with intermittent shaking after every 5 mins. Allowed to cool at room temperature and then volume was made up to 100 ml with diluent. (625 µg/ml Curcumin and 250 µg/ml Thymoquinone concentration). Centrifuged it at 3000 rpm for 15 mins and collected the supernatant. Pipetted out 2.0 ml to 50 ml volumetric flask and volume was made up using acetonitrile as diluent to give final test solution concentration of 10 µg/ml THQ and 24.64 µg/ml CUR. Sample solution was filtered through 0.45bµ nylon filter. HPLC vial was filled after discarding initial 4-5 ml of filtrate. 10 µl injected into the HPLC system. The Chromatograms were recorded and % Assay was calculated. The amount of analytes present in the formulation were calculated by using the formula as given below,

Formula:

$$\% \text{ Assay} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{AW}{LC} \times 100$$

Where,

AT: Peak area response of test sample
AS: Peak area response of standard sample
WS: Weight of standard sample
DS: Dilution factor of standard sample
DT: Dilution factor of test sample
WT: Weight of test sample
AW: Average Weight
LC: Label Claim
P: Peak purity

2.3.2 Optimization of mobile phase

Various trials were taken for optimization of mobile phase composition which will result in sharp, resolved peaks with defined baseline. Following Mobile phase compositions were tried:

The mobile phase was selected on the basis of good separation, peak purity index, peak symmetry, theoretical plates, etc. So, number of trails was performed to select a mobile phase.

Table 1. Mobile phase composition trails

Sr. No.	Mobile phase composition
1.	Methanol: Water (75:25 %v/v)
2.	Methanol: Water (90:10 %v/v)
3.	ACN: Water (50:50 %v/v)
4.	ACN: Water (70:30 %v/v)
5.	ACN: Methanol: Water (80:10:10 %v/v/v)
6.	ACN: Water (90:10 %v/v)
7.	ACN: Water (82:18 %v/v)

2.4 Validation of Developed HPLC Method [12,13,14,15,16,17,18]

The developed analytical method was validated to confirm that the present method was suitable for its intended purpose as described in ICH guidelines Q2 (R1).

System Suitability: A system suitability test was performed to evaluate the chromatographic parameters (capacity factor, separation factor, number of theoretical plates, asymmetry of the peak and resolution between two peaks). Six injections of the standard solution (10 µg/ml of THQ and 25 µg/ml of CUR) were analysed.

Specificity: Blank, standard solution and sample solution were injected and checked for any interference at main peaks.

Linearity: Linearity was performed by preparing a standard solution at seven different concentration levels of the drugs (4- 18 µg/ml and 10- 45 µg/ml Thymoquinone and Curcumin respectively). The correlation coefficient value was calculated using linear equation obtained from plotting graph against concentration vs peak response.

Accuracy: Recovery study was performed for determining the accuracy of the method. Sample solution was analysed in triplicate for each concentration level (80%, 100% and 120%).

Precision: System Precision was performed by injecting six replicating injections of standard solution as well as method precision was performed by injecting six different sample solutions. % RSD was calculated.

Robustness: Robustness of the method is its ability to remain unaffected by small changes in a variety of parameters. Prepared three sample solutions and analysed under the condition by changing wavelength (±2 nm), oven temperature (±5°C), and flow rate (±0.1ml/min). Calculated the % RSD of variable situations and effect was analysed.

Limit of Detection and Limit of Quantitation: Limit of Detection is the lowest amount of analyte in a sample which can be detected but not quantitated, and Limit of Quantitation is the lowest amount of analyte in the sample that can be quantitatively determined and were calculated by using the formula;

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

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

Where,

σ = Standard deviation of the response
S = Slope of the calibration curve.

3. RESULTS AND DISCUSSION

3.1 Optimized Chromatographic Conditions

The mobile phase selected was a mixture of acetonitrile and water in the proportion of 82:18% v/v at a flow rate of 0.9 mL/min as it resolves the height with retention times of 3.56 min and 4.37 min for curcumin and thymoquinone respectively. Standard drug solutions were scanned over a range from 800 to 200 nm, and detection was carried out at 256 nm as both the drugs showed reasonably good response with characteristic UV spectrum as exhibited in Fig 1 and optimized chromatographic conditions were tabulated in Table 3.

Table2. Robustness parameters

Sr No.	Robustness parameters	Optimized	(+ change)	(- change)
1	Wavelength (nm)	256	258	254
2	Flow rate (ml/min)	0.9	0.8	1.0
3	Column oven temperature (°C)	30	25	35

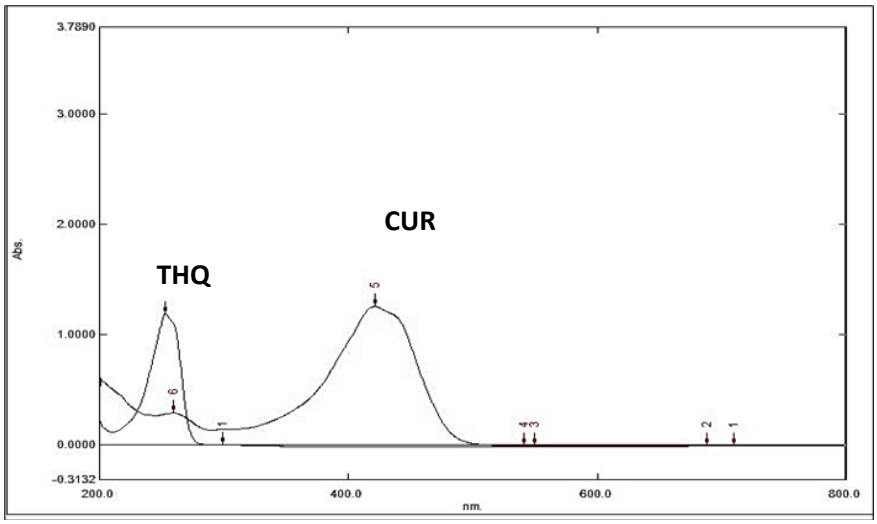

Fig. 1. Overlay of UV spectrum of thymoquinone and curcumin

Table 3. Optimized chromatographic conditions

Column	Inertsil ODS- 4.6 × 250 mm, 5 µm
Column oven temperature	30°C
Sampler temperature	25°C
Run time	6 min
Flow rate	0.9 ml/min
Wavelength	256 nm
Injection volume	10 µl
Diluent	Acetonitrile
Mobile phase	Acetonitrile: Water (82:18 %v/v)
Retention Time	3.56 mins (CUR) & 4.37 mins (THQ)

3.2 Assay Method Development

The amount of Curcumin and Thymoquinone present in analysed dosage form was found to 99.4% and 100.3% respectively. Amount of thymoquinone and curcumin present in dosage form was successfully estimated using developed assay method using RP-HPLC. The chromatogram of the standard solution and sample solution were exhibited in Figs. 2 and 3 respectively.

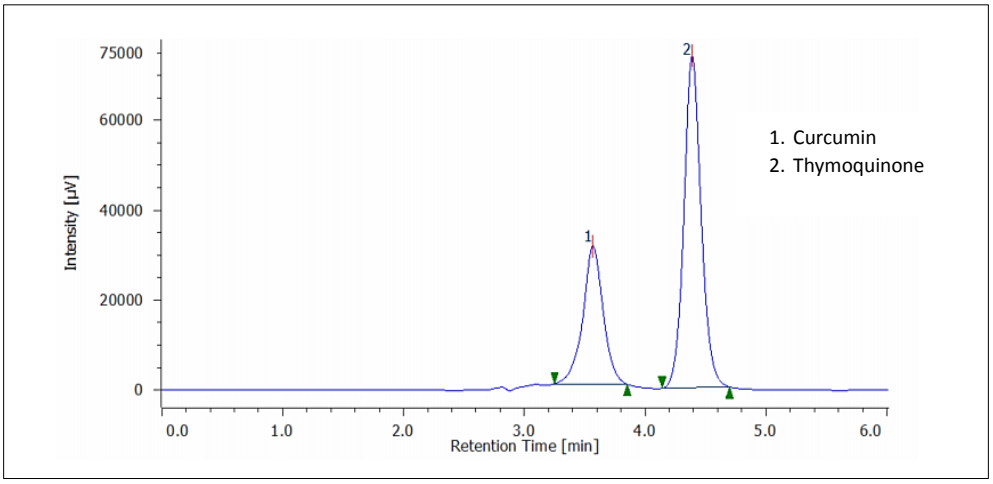

Fig. 2.Chromatogram of curcumin and thymoquinone obtained from the standard solution

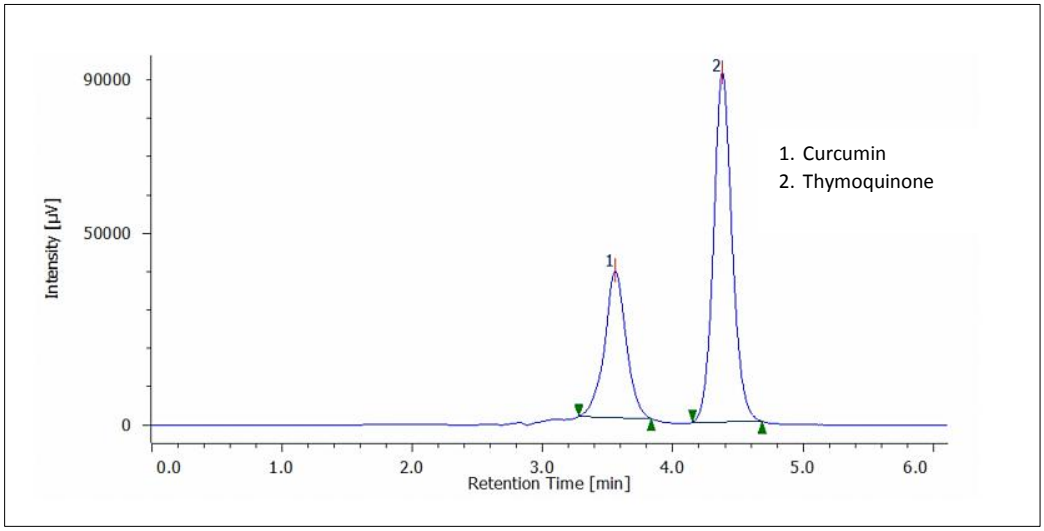

Fig. 3.Chromatogram of curcumin and thymoquinone obtained from the sample solution

3.3 Validation of Developed Method

System Suitability Test: Tailing factor found to be less than 2.0. Theoretical plates were more than 1500. Resolution between two peaks was 3.156, which is more than 2. % RSD for drug peak areas of six standard replicates found to be less than 2. Parameters for SST were listed in Table 4.

Specificity: No interference of mobile phase, solvents, diluents and impurities were observed. Hence, the method was found to be Specific.

Linearity: Thymoquinone and Curcumin was found to be linear in a concentration range of 4-18 µg/ml and 10- 45 µg/ml respectively. The correlation coefficient value was found to be 0.999 for both. Linearity data and calibration curve are reported in the Table 5 and Fig. 4.

Accuracy: Mean % Recovery for Curcumin was found to be 99.52% (80%), 99.01% (100%), 100.85% (120%) whereas for Thymoquinone it was 98.88% (80%), 100.49% (100%), 101.54% (120%). The results from recovery study for accuracy determination are given in the Table 6. The percentage recovery was found to be within the limit 98 to 102 %.

Table 4. Data for system suitability study

Drug	Mean Retention time	NTP	Tailing Factor	% RSD	Resolution
Curcumin	3.563	2597	0.991	0.45	3.156
Thymoquinone	4.367	4934	1.112	0.25	

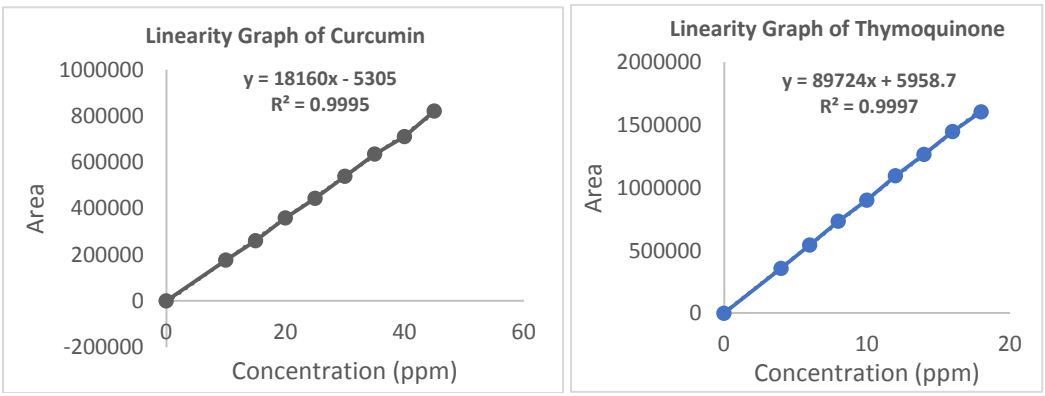

Fig. 4. Linearity graph of curcumin and thymoquinone

Table 5. Linearity Data of curcumin and thymoquinone

Sr. No.	Curcumin		Thymoquinone	
	Concentration (µg/ml)	Peak area response	Concentration (µg/ml)	Peak area response
1	10	176431	4	357496
2	15	260399	6	543326
3	20	358940	8	733336
4	25	443942	10	900731
5	30	539176	12	1095226
6	35	635369	14	1265102
7	40	710984	16	1447231
8	45	821464	18	1603955
Correlation coefficient	**0.9995**		**0.9997**	
Y-intercept	**y = 18160x - 5305**		**y = 89724x + 5958.7**	

Table 6. Accuracy data of curcumin and thymoquinone by HPLC method

Level (%)	Curcumin				Thymoquinone			
	Amount Added (µg/ml)	Amount recovered (µg/ml)	Average % Recovery	Mean%	Amount Added (µg/ml)	Amount recovered (µg/ml)	Average % Recovery	Mean %
80	36	35.31	98.09	99.52	14.4	14.24	98.88	98.92
80	36	36.62	101.72		14.4	14.31	99.36	
80	36	35.55	99.52		14.4	14.19	98.53	
100	40	39.66	99.16	99.01	16	16.26	101.60	100.49
100	40	39.38	98.45		16	15.68	98.00	
100	40	39.77	99.43		16	16.30	98.92	
120	44	44.88	102.00	100.85	17.6	17.81	101.20	101.54
120	44	44.49	101.12		17.6	17.91	101.75	
120	44	43.76	99.45		17.6	17.90	101.68	

Precision: The % RSD for system and method precision was found to be 1.61% and 1.62% for curcumin and 0.47% and 0.42% for thymoquinone respectively which is within acceptance criteria i.e., less than 2%. Hence, developed method was found to be precise.

Robustness: The developed method was found to be robust as% RSD were within limit (less than 2%) at different wavelengths, flow rates and column oven temperature for Robustness study conducted.

LOD & LOQ: LOD and LOQ was calculated and tabulated in Table 10.

Table 7. System precision data

n	Curcumin (Area)	Thymoquinone (Area)
1	350734	738393
2	350461	732805
3	350676	734596
4	364429	731998
5	355126	739486
6	359077	731098
Average	355083.8	734729.3
Std Dev	5709.64	3476.088
%RSD	**1.61**	**0.47**

Table8. Method precision data

n	Curcumin (Assay %)	Thymoquinone (Assay %)
1	99.76	99.67
2	102.59	100.64
3	101.47	99.76
4	98.77	100.44
5	98.82	99.63
6	98.75	99.99
Average	100.27	100.03
Std Dev	1.62	0.42
% RSD	**1.62**	**0.42**

Table 9. Data for robustness study

Parameter		% RSD
254 nm	AREA CUR	**0.07**
	AREA THQ	**0.01**
	Rt CUR	**0.03**
	Rt THQ	**0.06**
	NTP CUR	**0.84**
	NTP THQ	**0.28**
258 nm	AREA CUR	**0.26**
	AREA THQ	**0.13**
	Rt CUR	**0.02**
	Rt THQ	**0.05**
	NTP CUR	**0.44**
	NTP THQ	**0.77**
0.8 ml/min	AREA CUR	**0.16**
	AREA THQ	**0.12**
	Rt CUR	**0.14**
	Rt THQ	**0.04**
	NTP CUR	**0.23**
	NTP THQ	**0.08**
1 ml/min	AREA CUR	**0.21**
	AREA THQ	**0.32**
	Rt CUR	**0.04**
	Rt THQ	**0.06**
	NTP CUR	**1.00**
	NTP THQ	**0.11**
25°C	AREA CUR	**0.07**
	AREA THQ	**0.02**
	Rt CUR	**0.63**
	Rt THQ	**0.35**
	NTP CUR	**1.19**
	NTP THQ	**0.68**
35°C	AREA CUR	**1.18**
	AREA THQ	**0.02**
	Rt CUR	**0.11**
	Rt THQ	**0.02**
	NTP CUR	**0.04**
	NTP THQ	**0.20**

Table 10. LOD & LOQ data

	Curcumin (µg/ml)	Thymoquinone (µg/ml)
LOD	0.67	0.25
LOQ	2.04	0.77

4. CONCLUSION

A simple and novel RP-HPLC method for the simultaneous determination of thymoquinone and curcuminin pharmaceutical dosage form. The developed method was validated as per ICH Q2 (R1) guidelines. This method can be employed for routine quality control analysis of curcumin and thymoquinonesamples simultaneously The proposed method can further be studied for its application in LC-MS/MS method development and validation.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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