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Standardization and Validation of *Nigella sativa* Seed Extract Using High Performance Thin Layer Chromatography

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ABSTRACT

Objective: To develop a sensitive, reliable, accurate and simplified quantitative high-performance thin layer chromatography (HPTLC) method and to determine the quantity of thymoquinone (TQ) in *Nigella sativa* seed extract (NSE). **Methods:** TQ (TQ) was separated on aluminum-backed silica gel 60 F254 plates (Merck Germany) with Toluene: Petroleum ether 8:2 (%, v/v) as mobile phase. **Results:** At R_f value of 0.71±0.04 a compact band was observed. The linearity was in range of 10 to 40µg/spot of TQ and the correlation coefficient of 0.9987 indicated good linear dependence of peak area on concentration. Limit of detection (0.2 µg/spot), limit of quantification (0.4µg/spot) accuracy (less than 2) were found satisfactory. **Conclusions:** The developed HPTLC densitometric method was found to be cheap, selective, precise and accurate and can be used for routine analysis of NSE.

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1. INTRODUCTION

Nigella sativa Linn. (Ranunculaceae), mostly known as black seed or black cumin, It is indigenous to the Mediterranean region. It has wide use in indigenous system of medicine for over 3000 years. Black seed oil is been widely used in for the treatment of arthritis, lung diseases and hypercholesterolemia. There are many reported pharmacological properties of *N. sativa*, for instance, hypotensive, anti-nociceptive, uricosuric, choleretic, antifertility, antidiabetic, anti-histaminic, anti-oxidant, anti-inflammatory, anti-microbial, anti-tumor and immunomodulatory effects. Most pharmacological properties of the black seed extract are mainly attributed to its volatile oil, out of which TQ (TQ), about 20%-50%, is the most active component. Standardization of herbal formulations in terms of quality of raw materials, manufacturing practices and composition is important to ensure quality and optimum levels of active principles for their bio-potency. Since TQ is principle bioactive component of *N. Sativa* seed, simple and robust method is required for quantification of TQ which has been used for quality control and standardization of crude drug. A thorough literature survey revealed that, several analytical techniques for the determination of TQ.

MATERIALS AND METHODS

2.1. Plant material:

Nigella sativa Linn. (Black seeds) seeds were purchased from the local market Vashi, Navi-Mumbai, Maharashtra in the month of August. Authentication of the collected material was carried out at GuruNanak Khalsa College, Mumbai by Dr. Harshad Pandit and its identity was confirmed to be *Nigella sativa* Linn. (Black seeds), family Ranunculaceae with herbarium accession no. #182603.

2.2. Chemicals and reagents:

Standard TQ was purchased from Sigma Aldrich, USA. All the solvents were HPLC grade and other chemicals used were of analytical reagent grade. Accurately weighed 1 mg of standard TQ (purity 99%) was dissolved in methanol in a 10mL volumetric flask to give concentration of 100 µg/mL. This solution was used as a reference solution (stock solution) for TQ.

2.3. Sample preparation:

For analysis of TQ in extract of *N. sativa* 7 grams of powdered seeds of *N. sativa* were extracted by ultrasonication at room temperature with n-hexane:water (9:1). The solvent was evaporated to dryness under reduced pressure by use of a rotary vacuum evaporator and the residue was dissolved in methanol in 25 mL volumetric flask separately. Accurately 1 mL of the *N. sativa* oil was separately dissolved in 10 mL methanol in volumetric flasks, filtered and used for analysis.

2.4. Chromatographic conditions:

HPTLC densitometric analysis was performed on 10cm x 10cm aluminium-backed plates coated with 0.2 mm layers of silica gel 60 F254 (Merck, Germany). Samples were applied to the TLC plates as 10 mm bands using a Camag Linomat V Applicator, a sample applicator (Switzerland) fitted with a Camag 100 microlitre syringe. A constant application rate was used. Linear ascending development of the plates to a distance of 65 mm was performed with Toluene: Petroleum ether 8:2 (%, v/v) as mobile phase in a CAMAG twin trough chamber previously saturated with mobile phase vapor for 20 minutes at 25°C. After development, the plates were scanned at 254 nm using a Densitometer (TLC scanner) in absorbance mode, using a deuterium lamp.

2.5. Method validation:

The proposed HPTLC method was validated according to the guidelines of International Conference on Harmonization (ICH). The linearity of the method for TQ was checked between 10µg/spot to 40µg/spot and concentration was plotted against peak area. Accuracy, as recovery, was determined by the standard addition method. Relative standard deviation (RSD, %) was calculated for each concentration level. Precision was assessed by determination of repeatability and intermediate precision. Repeatability of sample was determined as intra-day variation whereas intermediate precision was determined by assessment of inter-day variation for analysis of TQ at three different concentrations viz; 20µg/spot, 30µg/spot and 40µg/spot in six replicate. Limit of detection (LOD) and limit of quantification (LOQ) were determined by standard deviation (SD) method. They were determined from the slope of the calibration (S) curve and SD of the blank sample using following equations:

LOD=3.3xSD/S

LOQ=10 xSD/S

2.6. Quantification of TQ in extracts of *N. sativa*

The test samples were applied and chromatograms were obtained under the same conditions as for analysis of standard TQ. The area of the peak corresponding to the Rf value of TQ standard was recorded and the amount present was calculated from the regression equation obtained from the calibration plot.

3. RESULT AND DISCUSSION

3.1. Method development:

The mobile phase composition was optimized to establish a suitable and accurate densitometric HPTLC method for analysis of TQ. The mobile phase toluene: petroleum ether 8:2 (%, v/v) resulted in a sharp, symmetrical, and well resolved peak at Rf value of (0.71±0.04) (Figure 1). UV spectra measured for the bands showed maximum absorbance at approximately 254 nm.

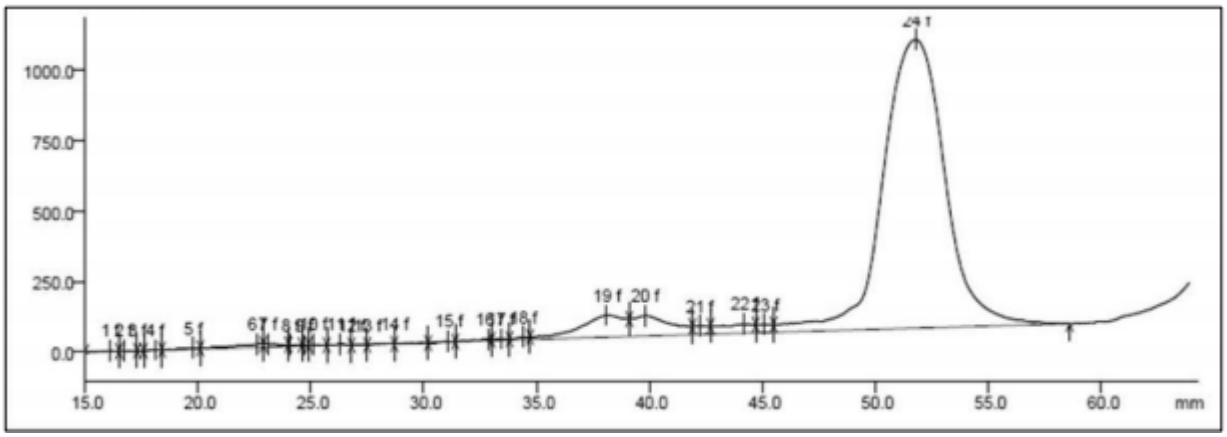


Figure 1: HPTLC chromatogram of standard TQ.

3.2. Calibration curve:

The calibration plot of peak area against amount of TQ was linear in the range 10-40 µg/spot. Linear regression data for the plot confirmed the good linear relationship (Table 1). The correlation coefficient (R^2) was 0.9987 which was highly significant ($P<0.05$).

Table 1: Linearity of TQ

CONC	10µg	20 µg	30 µg	40 µg
AREA	530.771	1828.008	2657.883	3250.056
	532.234	1834.445	2634.756	3275.376
	529.912	1876.865	2674.562	3257.543
AVG	530.9723	1846.439	2655.734	3260.992
SD	1.17402	26.54524	19.98985	13.00752
%RSD	0.221108	1.437645	0.752705	0.398882

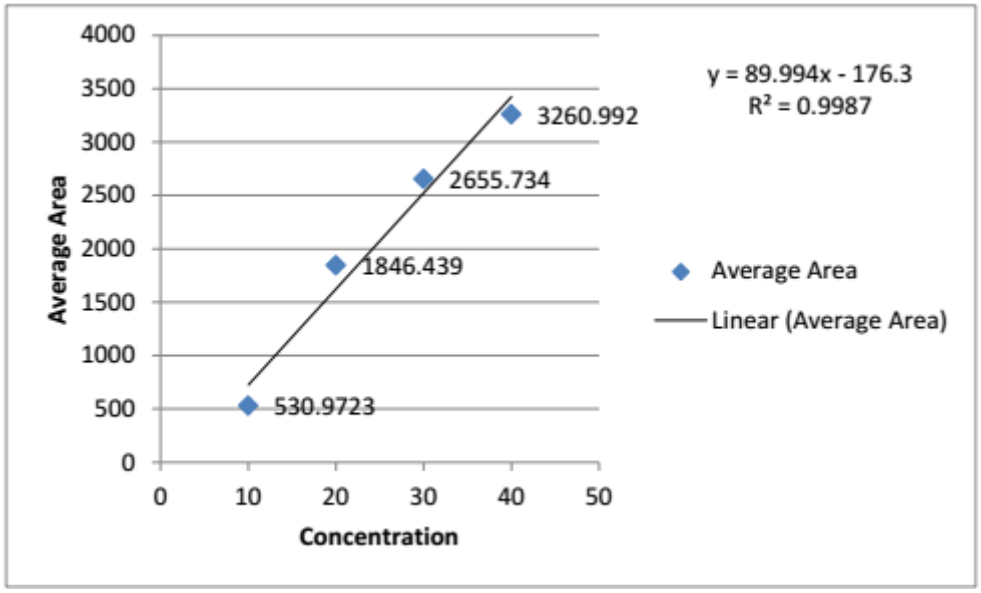


Figure 2: Linearity curve of TQ

3.3. Method validation:

3.3.1. Precision:

Precision is reported in terms of relative standard deviation (%RSD) over the range of quantitation for a single experiment in which standards are assayed in replicate (intraday) and for a series of experiments in which standards are assayed in over several experiments (interday). Accuracy of the method was proved by finding ratio of mean of back-calculated

concentration against actual concentration. The results of Intraday and Interday accuracy/precision are shown in table 2 and 3 respectively.

Table 2: Intraday Precision and Accuracy

S.No	Conc. Per spot		
	20 µg	30 µg	40 µg
1	19.56	29.34	39.74
2	19.42	30.12	40.11
3	19.89	29.59	40.04
MEAN	19.6233333	29.6833333	39.96333
SD	0.24131584	0.398288	0.196554
%RSD	1.22973928	1.34179002	0.491835
%ACCURACY	98.1166667	98.9444444	99.90833

Table 3: Interday Precision and Accuracy

Sr.No.	CONCENTRATION		
	20µg	30 µg	40 µg
1	19.56	29.34	39.74
2	19.42	30.12	40.11
3	19.89	29.59	40.04
4	20.11	30.84	39.89
5	21.11	30.66	40.92
6	19.59	29.83	40.27
MEAN	19.94666667	30.06333333	40.16166667
SD	0.622468205	0.593992144	0.41373502
%RSD	3.120662794	1.975802673	1.03017393
% ACCURACY	99.73333333	100.2111111	100.4041667

3.3.2. Specificity:

The specificity of the method was ascertained by analyzing standard and the extract. There were no interfering spots of the plant constituents at the Rf Value of the markers. The absorption spectra of standard marker (Rf=0.71±0.04) and the corresponding spot present in seed extract matched exactly, indicating no interference by the other plant constituents.

3.3.3. Stability:

Stability in sample solution: Solution of 50µg/ml of TQ was prepared from the stock solution and stored at cold temperature for 1 week. This solution was compared with fresh solution of 50µg/ml and then applied on the same plate in triplicate. After development, the chromatogram was evaluated for additional spot. (Table 4)

Table 4: Stability of TQ

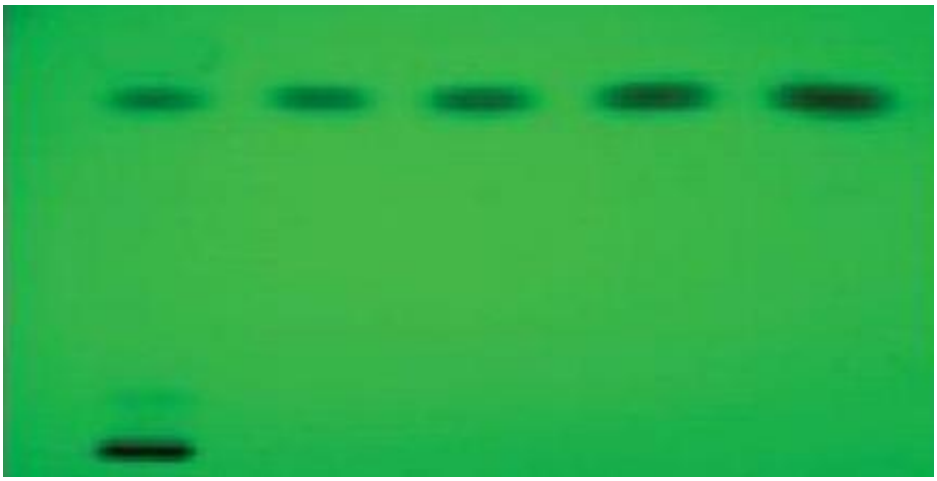
Sr. no	TQ Concentration (µg) per spot	
	Fresh (10µg)	Stability (10µg)
1	10.13	9.87
2	10.17	10.12
3	10.19	10.07
MEAN	10.16	10.02
±S.D	0.03	0.13
% RSD	0.3	1.32
% STABILITY	101.63	100.2

3.4. Quantification:

TQ peaks from extract were identified by comparing single spot at Rf =0.71±0.04 values with those obtained by chromatography of the standard under the same conditions (Table 5). The TQ content in NSE was quantified by use of the linear regression equation and concentration. (Figure 3).

Table 5: Rf values of TQ

Lane no.	Type of Solution	Conc. (µg)	Rf values
1	TQ Std	10	0.7
2	TQ Std	20	0.71
3	TQ Std	30	0.7
4	TQ Std	40	0.7
5	Sample solution	1250	0.73



Extract 10 µg 20 µg 30 µg 40 µg

Figure 3: Estimation Of TQ in *Nigella sativa* seed extract

3.3.4. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Lowest amount of analyte that could be detected is to be determined on the basis of signal to noise ratio. It was determined by spotting on plate concentrations of TQ. The lowest amount of analyte that could be detected was found to be 0.2 µg per spot.

The lowest amount of TQ that could be quantitatively determined with definite precision and accuracy was calculated on the basis of signal to noise ratio. LOQ was found to be 0.4 µg per spot.

4. CONCLUSION

The quantity of TQ in NSE was determined by a validated HPTLC method. The mobile phase Toluene: Petroleum ether 8:2 (%, v/v) resulted in a well resolved peak at Rf value of 0.71. A good linear relationship was confirmed using linear regression data. The statistical data confers that the method is reproducible and selective for the analysis of TQ with added advantages of, minimal/easy sample preparation, short time and most importantly it is cost effective. The method was accurate, with RSD values less than 2 after spiking the TQ with different concentrations of standard. The HPTLC method developed for quantitation of TQ was found to be simple, accurate, reproducible and sensitive.

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