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# HPTLC densitometric method for analysis of thymoquinone in *Nigella sativa* extracts and marketed formulations

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## PEER REVIEW

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**Comments**

This is a valuable research work in which author have quantified the thymoquinone in *N. sativa* extract and marketed formulation. Statically data from the accuracy, precision and limit of detection and quantification indicates that the proposed densitometric HPTLC can be used in analysis of thymoquinone in extract and marketed formulations.

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## ABSTRACT

**Objective:** To develop a sensitive and accurate high-performance thin layer chromatography method and to determine the quantity of thymoquinone in in two different *N. sativa* extracts and marketed formulations.

**Methods:** Thymoquinone was separated on aluminum-backed silica gel 60 F254 plates with *n*-hexane-ethyl acetate 8:2 (% v/v) as mobile phase.

**Results:** A compact band was obtained for thymoquinone at  $R_f$  value of  $0.48 \pm 0.04$ . The calibration plot was linear in the range of 50–700 ng/spot of thymoquinone and the correlation coefficient of 0.9989 was indicative of good linear dependence of peak area on concentration. Limit of detection (8.67 ng/spot), limit of quantification (17.43 ng/spot) accuracy (less than 2) and recovery (ranging from 98.39–99.17) were found satisfactory.

**Conclusions:** The developed high-performance thin layer chromatography densitometric method was found cheap, selective, precise and accurate and can be used for routine analysis of *N. sativa* extracts and marketed formulations.

## KEYWORDS

Thymoquinone, HPTLC densitometry, ICH guidelines, Qualitative, Quantitative

## 1. Introduction

*Nigella sativa* Linn. (Ranunculaceae) (*N. sativa*), commonly known as black seed or black cumin, is an herbaceous plant, mainly grows in the Middle East, Central Europe and Western Asia. It is widely used in indigenous system of medicine for treatment of numerous disorders for over 2000 years<sup>[1–3]</sup>. Its seed oil had been widely used in Arab traditional of medicine for the treatment of arthritis, lung diseases and hypercholesterolemia<sup>[4]</sup>. Some of the reported pharmacological properties of *N. sativa* include hypotensive, anti-nociceptive, uricosuric, choleric, antifertility, anti-diabetic, anti-histaminic, anti-oxidant, anti-inflammatory, anti-microbial, anti-tumor and immunomodulatory effects<sup>[5]</sup>.

Most pharmacological properties of the whole seeds or their extracts of *N. sativa* are mainly attributed to the its volatile oil, of which thymoquinone, about 27%–57%, is the most abundant component<sup>[6–8]</sup>. 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 thymoquinone is principal bioactive component of *N. sativa* seed, simple and robust method is required for quantification of this active constituent which has been used for quality control and standardization of crude drug and its formulation as a marker compound. A thorough literature survey revealed that, several analytical techniques for the determination of thymoquinone have been reported. These methods include high-performance

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thin layer chromatography (HPTLC) method in *N. sativa* extracts and formulations[9], holographic[10] and high performance liquid chromatography with UV detection in black seed oil[11,12], and ultra performance liquid chromatography with UV detection in bulk drug and formulations[13].

## 2. Materials and methods

### 2.1. Plant material

*N. sativa* seeds was purchased from local market of Al Kharj, Kingdom of Saudi Arabia. The plant was collected and identified by Dr. Mohammad Atiqur Rahman, taxonomist of Medicinal, Aromatic and Poisonous Plants Research Center (MAPPRC), College of Pharmacy, King Saud University, Saudi Arabia. A voucher specimen No. 13987 was deposited in the Herbarium of the College of Pharmacy, King Saud University, Saudi Arabia.

### 2.2. Chemicals and reagents

Standard thymoquinone was purchased from Sigma Aldrich, USA. All the solvents were of HPLC grade and other chemicals used were of analytical reagent grade. Baraka capsule, black seed rub and *N. sativa* oil were also purchased from a local market of Riyadh, Kingdom of Saudi Arabia.

Accurately weighed 1 mg of standard thymoquinone (purity 99%) was dissolved in MeOH in a 10 mL volumetric flask to gives concentration of 100 µg/mL. This solution was used as a reference solution (stock solution) for thymoquinone.

### 2.3. Sample preparation for analysis of thymoquinone in extract of *N. sativa* and marketed formulations

Five grams of powdered seeds of *N. sativa* were extracted by percolation at room temperature with methanol till exhaustion. Another 5 g were separately extracted similarly with petroleum ether. The solvent was evaporated to dryness under reduced pressure by use of a rotary vacuum evaporator and the residue was separately dissolved in methanol in 50 mL volumetric flask. Accurately 1 mL of the *N. sativa* oil was separately dissolved in 10 mL methanol in volumetric flasks. Accurately weighed 5 g of black–seed rub was also dissolved in 50 mL methanol, filtered and used for analysis. Accurately weighed 1.622 g of the Baraka capsules was separately dissolved in 25 mL methanol in volumetric flasks.

### 2.4. Chromatographic conditions

HPTLC densitometric analysis was performed on 10 cm×20

cm aluminium–backed plates coated with 0.2 mm layers of silica gel 60 F254 (E–Merck, Germany). Samples were applied to the TLC plates as 6 mm bands using a Camag Automatic TLC Sampler 4 sample applicator (Switzerland) fitted with a Camag microlitre syringe. A constant application rate of 150 nL/s was used. Linear ascending development of the plates to a distance of 80 mm was performed with hexane: ethyl acetate 8:2 (% , v/v) as mobile phase in a Camag Automatic Developing Chamber 2 previously saturated with mobile phase vapour for 30 min at 22 °C. After development, the plates were scanned at 259 nm using a Camag TLC scanner in absorbance mode, using the deuterium lamp. The slit dimensions were 4.00 mm×0.45 mm and the scanning speed was 20 mm/s.

### 2.5. Method validation

The proposed HPTLC method was validated according to the guidelines of International Conference on Harmonization (ICH)[14]. The linearity of the method for thymoquinone was checked between 50 and 700 ng/spot and concentration was plotted against peak area.

Accuracy, as recovery, was determined by the standard addition method. Pre–analyzed samples of thymoquinone (200 ng/spot) were spiked with extra thymoquinone standard (0, 50, 100, and 150%) and the mixtures were reanalyzed. Percentage recovery and relative standard deviation (RSD, %) were 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 thymoquinone at three different amounts (300, 400, and 500 ng/spot) in six replicate.

Robustness of the proposed TLC densitometric method was determined to evaluate the influence of small deliberate changes in the chromatographic conditions during determination of thymoquinone. Robustness was determined by changing the polarity of the mobile phase.

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:

$$\text{LOD}=3.3\times\text{SD}/\text{S}$$

$$\text{LOQ}=10\times\text{SD}/\text{S}$$

### 2.6. Quantification of thymoquinone in extracts of *N. sativa* and marketed formulations

The test samples were applied and chromatograms were obtained under the same conditions as for analysis of standard thymoquinone. The area of the peak corresponding

to the  $R_f$  value of thymoquinone standard was recorded and the amount present was calculated from the regression equation obtained from the calibration plot.

### 3. Results

#### 3.1. Method development

The mobile phase composition was optimized to establish a suitable and accurate densitometric HPTLC method for analysis of thymoquinone. The mobile phase *n*-hexane: ethyl acetate 8:2 (% v/v) resulted in a sharp, symmetrical, and well resolved peak at  $R_f$  value of (0.48±0.04) (Figure 1). UV spectra measured for the bands showed maximum absorbance at approximately 259 nm.

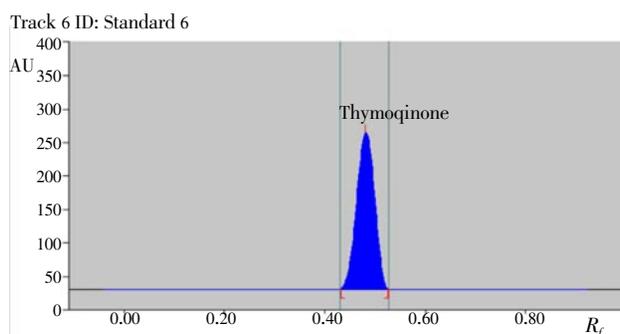


Figure 1. HPTLC chromatogram of standard thymoquinone.

#### 3.2. Calibration curve

The calibration plot of peak area against amount of thymoquinone was linear in the range 50–700 ng/spot. Linear regression data for the plot confirmed the good linear relationship (Table 1). The correlation coefficient ( $R^2$ ) was 0.9989 which was highly significant ( $P < 0.05$ ). The linear regression equation was  $Y = 13.092x + 1212.1$ , where  $Y$  is response and  $x$  is amount of thymoquinone (Figure 2).

#### 3.3. Method validation

##### 3.3.1. Precision

The accuracy of the method, as recovery, was 98.39%–99.17%, with RSD values in the range 0.62–1.10. These results indicated the accuracy of the method (Table 2). Results from determination of repeatability and intermediate precision, expressed as SD (%) are shown in Table 3. RSD is in the range 0.39–1.06 for repeatability and 0.52–1.17 for intermediate precision. These low values indicated that the method was precise[15].

Table 1

Linear regression data for the calibration curve of thymoquinone ( $n=6$ ).

Linearity range (ng/spot)	50–700
Regression equation	$Y = 13.092x + 1212.1$
Correlation coefficient	0.9989
Slope±SD	13.0920±0.3948
Intercept±SD	1212.10±270.39
Standard error of slope	0.228
Standard error of intercept	156.12
95% confidence interval of slope	8.303–9.319
95% confidence interval of intercept	805.83–1501.50

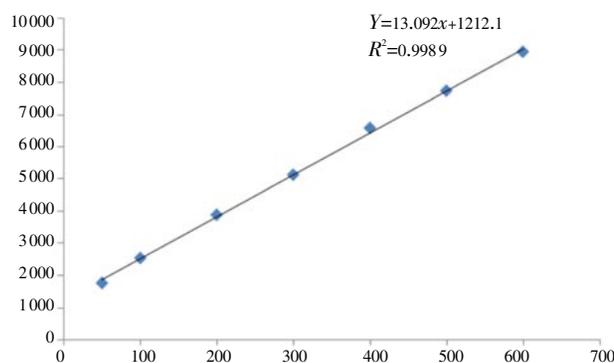


Figure 2. Regression curve of standard thymoquinone.

Table 2

Accuracy of the proposed method ( $n=6$ ).

Excess drug added to analyte (%)	Theoretical content (ng)	Conc. found (ng) ±SD	% Recovery	%RSD
0	200	198.33±1.21	99.17	0.61
50	300	295.17±3.25	98.39	1.10
100	400	395.00±3.16	98.75	0.80
150	500	495.33±3.93	99.07	0.79

Conc.: Concentrations.

Table 3

Precision of the proposed method.

Conc. (ng/spot)	Repeatability (Intraday precision)			Intermediate precision (Interday)		
	Area±SD ( $n=6$ )	Standard error	% RSD	Area±SD ( $n=6$ )	Standard error	% RSD
300	5131.83±54.25	22.15	1.06	5126.17±59.97	24.49	1.17
400	6588.33±34.69	14.16	0.53	6588.67±34.06	13.91	0.52
500	7743.67±29.86	12.19	0.39	7627.83±48.43	18.77	0.63

##### 3.3.2. Robustness of the method

Results of robustness are shown in Table 4. Low values of RSD (0.46%–1.29%) were obtained after introducing small deliberate change into the densitometric TLC procedure, proving the robustness of the proposed HPTLC method[16].

Table 4

Robustness of the proposed HPTLC method.

Conc. (ng/spot)	Mobile phase composition (Hexane: ethyl acetate)			Results		
	Original	Used	Δ	Area±SD ( $n=6$ )	% RSD	$R_f$
300	8:2	8:2.1	−0.1	5166±24	0.46	0.49
		8:2.0	0.0	5127±66	1.29	0.48
		8:1.9	+0.1	5149±26	0.51	0.47

### 3.3.3. Limit of detection and quantification

LOD and LOQ of the proposed method was found to be 8.67 and 17.43 ng/spot, for thymoquinone, which indicated that the proposed method can be used in wide range for detection and quantification of thymoquinone effectively<sup>[17]</sup>.

### 3.4. Quantification

Thymoquinone peaks from methanolic extract, petroleum ether extract and marketed formulation were identified by comparing their single spot at  $R_f=0.40\pm 0.04$  (Figures 3 and 4) values with those obtained by chromatography of the standard under the same conditions. The thymoquinone content in methanolic extract, petroleum ether extracts of *N. sativa* marketed formulations were quantified by use of the linear regression equation and concentration, which are presented in Table 5.

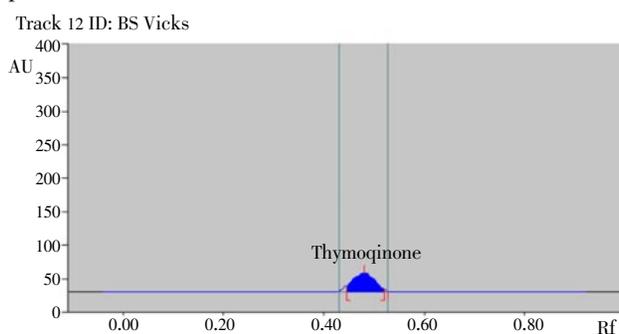


Figure 3. HPTLC chromatogram of formulation.

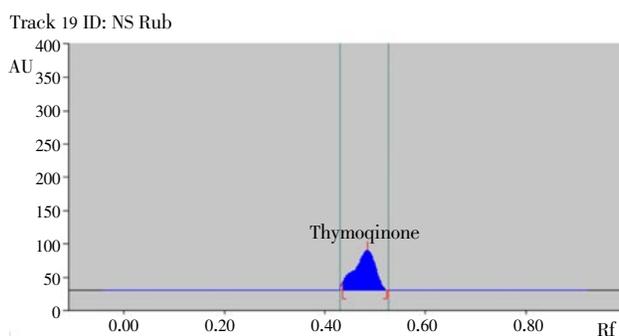


Figure 4. HPTLC chromatogram of formulation.

Table 5

Contents of thymoquinone in methanolic, petroleum ether extracts of *N. sativa* and marketed formulations.

Samples	Contents mean±SD (% w/w)	% RSD
Methanolic extract	2.23±0.18	11.13
Petroleum ether extract	4.67±2.12	4.56
<i>N. sativa</i> oil	3.23±3.45	5.51
Baraka capsule	3.41±1.16	5.43
Black seed rub	2.48±0.34	3.78

## 4. Discussion

A validated HPTLC method has been developed to determine the quantity of thymoquinone in in two different *N. sativa*

extracts and marketed formulations. The mobile phase *n*-hexane: ethyl acetate 8:2 (% v/v) resulted in a sharp, symmetrical, and well resolved peak at  $R_f$  value of 0.48. Linear regression data for the plot confirmed the good linear relationship and the resulting equation was operational in the concentration range of 50–700 ng/spot. The method was accurate (98.39%–99.17%), with RSD values in the range 0.79–1.10 after spiking the thymoquinone with different concentrations of standard. The HPTLC method developed for quantitation of thymoquinone was found to be simple, accurate, reproducible and sensitive and is applicable to the analysis of a wide variety of black seed-containing herbal formulations. Also it will find wide applications in standardization and quality control of herbal raw materials as well as formulations. Statistical data proves that the method is reproducible and selective for the analysis of thymoquinone with added advantages of short time, minimal sample preparation, in addition to the low cost.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Acknowledgements

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### Comments

#### Background

Standardisation 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. HPTLC has recently emerged as a preferred analytical tool for fingerprints and quantification of marker compounds in herbal drugs because of its simplicity, sensitivity, accuracy, suitability for high-throughput screening, reliability in quantification of analytes at micro and even nanogram levels and cost-effectiveness.

#### Research frontiers

The present work developed and validated an HPTLC

method for determination of thymoquinone in *N. sativa* extracts and marketed formulations.

### Related reports

No similar reports were found in the literature regarding the quantification of thymoquinone in *N. sativa* extracts and marketed formulations.

### Innovations & breakthroughs

The present work developed and validated an HPTLC method for determination of thymoquinone in *N. sativa* extracts and commercially available marketed herbal formulations

### Applications

The method is reproducible and selective for the analysis of thymoquinone with added advantages of low cost of reagents, speed and minimal sample preparation, satisfactory precision and accuracy. This method may be recommended for quality control to establish the authenticity of *N. sativa* containing formulations and can also be used in the quality standardisation of *N. sativa* extracts for pharmaceutical production.

### Peer review

This is a valuable research work in which author have quantified the thymoquinone in *N. sativa* extract and marketed formulation. Statically data from the accuracy, precision and limit of detection and quantification indicates that the proposed densitometric HPTLC can be used in analysis of thymoquinone in extract and marketed formulations.

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