Iqbal et al., 2:2
http://dx.doi.org/10.4172/scientificreports.655

Open Access Scientific Reports

Research Article Open Access

High Performance Liquid Chromatographic Method with Fluorescence Detection for the Estimation of Thymoquinone in *Nigella sativa* Extracts and Marketed Formulations

Muzaffar Iqbal[1,2]*, Prawez Alam[3] and Md Tarique Anwer[4]

[1]Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, Riyadh, KSA, 11451
[2]Bioavailability Research Laboratory, College of Pharmacy, King Saud University, Riyadh, KSA, 11451
[3]College of Pharmacy, Salman Bin Abdulaziz University Al Kharj, KSA -11942
[4]College of Pharmacy, Jazan University, Jazan, KSA

Abstract

A simple, economic, robust, reproducible, selective, and precise high-performance liquid chromatography (HPLC) method developed and validated for the estimation of thymoquinone in two different extract and marketed formulations. The mobile phase composed of 20-mM KH_2PO_4 buffer (pH adjusted to 2.7 ± 0.05 using 50% OPA) and acetonitrile at a ratio of 60:40 eluted at a flow rate of 1 mL/min. Thymoquinone was monitored using florescence detector set at 274 excitation and 340 emission wavelength, having column oven temperature of 40°C and sample cooler temperature of 8°C ± 0.2°C. The linear regression analysis data for the calibration curve shows good linear relationship with correlation coefficient of 0.998 in the concentration range of 0.07-12 µg/mL. The limit of detection and limit of quantification were 0.023 and 0.07 µg/mL, respectively. The developed method was validated for accuracy, precision, reproducibility, and robustness as per ICH guidelines. The proposed method with high degree of precision and accuracy is employed for the estimation of thymoquinone in methanolic and petroleum extract of *Nigella sativa* as well as in formulation. Statistical analysis proved that the method is precise, reproducible, selective, and accurate for the estimation of thymoquinone for quality control purpose.

Keywords: Thymoquinone; *Nigella sativa*; HPLC; Chromatography

Introduction

Nigella sativa is an herbaceous plant belongs to a botanical family Ranunculaceae, grows in the Middle East, Central Europe and Western Asia. It is commonly known as black Seed or Black Caraway Seed and traditionally used for the prevention and cure of many ailments for over 2000 years [1-3]. Its seed oil had been used in Arab traditional herbal medicine for the treatment of arthritis, lung diseases and hypercholesterolemia [4]. Recent studies also showed that the *Nigella sativa* oil may be considered as a potential adjuvant therapy in rheumatoid arthritis [5]. Commercial use of these seeds has been extended too many products including shampoos, oils and soaps [6]. In experimental study, *Nigella sativa* has shown anti-neoplastic and antioxidant affects both *in vitro* and *in vivo* animal model. The antitumor activity of *Nigella sativa* seed was tested on the human hepatoma HepG2 cell line by Thabrew et al. [7]. Aqueous and alcohol extracts of *Nigella sativa* were found to be effective *in vitro* in inactivating MCF-7 breast cancer cells [8], where as methanolic, and chloroform extracts of *Nigella sativa* effectively killed HeLa (human epithelial cervical cancer) cells by inducing apoptosis [9]. Khan and Sultana reported the chemo-preventive effect of *Nigella sativa* against ferric nitrilotriacetate (Fe-NTA)-induced renal oxidative stress, hyper-proliferative response and renal carcinogenesis [10]. Active constituents of black seed identified by HPLC, include thymoquinone, dithymoquinone, thymohydroquinone and thymol [11]. But studies had shown that the biological activity of *Nigella sativa* seeds is mainly attributed to its essential oil component which is pre-dominantly (30-48%) thymoquinone (THQ) [12-14].

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 THQ is principal bioactive component of black seed, simple robust method is required for quantification of this active constituent which has been used for quality control and standardization plant and its formulation as a marker compound. In literature, several analytical methods for the determination of THQ have been reported. Methods include HPTLC method in *Nigella sativa* extracts and formulations [15] polographic [16] and HPLC with UV detection [11,17] in black seed oil and UPLC with UV detection in bulk drug and formulations [18]. Here we developed a simple sensitive, highly precise robust HPLC method with florescence detection for the determination of THQ in plant extracts as well as marketed formulations [19].

Experimental

Chemicals and reagent

THQ (purity >99%), was obtained from Sigma Aldrich USA. Its chemical structure is presented in figure 1. Acetonitrile and methanol of HPLC grade were obtained from Winlab laboratory, UK, whereas orthophasphoric acid and potassium dihydrogen phosphate were obtained from BDH and Avonchem Laboratory England. All reagents were of analytical grade unless stated otherwise. All aqueous solutions and buffers were prepared using water that was purified using Milli-QR Gradient A10R (Millipore, Moscheim Cedex, France) having pore size 0.22 µm.

***Corresponding author:** Muzaffar Iqbal, Department of Pharmaceutical Chemistry College of Pharmacy, King Saud University, Riyadh, KSA, Fax: +966-1-467-6220; E-mail: muziqbal@gmail.com; muziqbal@ksu.edu.sa

Received Janary 09, 2013; **Published** February 20, 2013

Citation: Iqbal M, Alam P, Anwer MT (2013) High Performance Liquid Chromatographic Method with Fluorescence Detection for the Estimation of Thymoquinone in *Nigella sativa* Extracts and Marketed Formulations. 2: 655 doi:10.4172/scientificreports.655

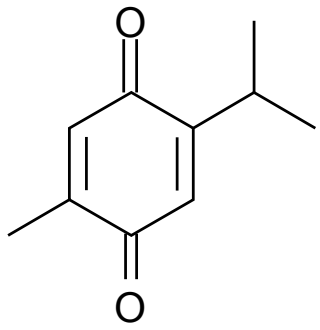

Figure 1: Chemical structure of THQ.

Plant material and marketed formulations

Crude black seed was purchased from a local market and authenticated by a Taxonomist at the College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. Baraka capsule, black seed rub and *Nigella sativa* oil were also purchased from a local market. The powdered seeds of Nigelia sativa (5 g) were extracted by percolation at room temperature with methanol till exhaustion. Another 5 gm 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 *Nigella sativa* oil was separately dissolved in 10 mL methanol in volumetric flasks. Accurately weighed 5 gram of black-seed rub also dissolved in 50 ml methanol and filtered it and used for analysis. Accurately weighed 1.622 g of the Baraka capsules was separately dissolved in 25 mL methanol in volumetric flasks.

Instrumentation and chromatographic conditions

The analysis was performed on Waters Alliance HPLC systems 2695 separation module connected to Waters 2475 Multi Lambda florescence detector. The LC system consisted of a water binary pump, model 1525 (Milford, MA, USA), with an autosampler model 717. Waters quaternary solvent delivery system was used to operate the isocratic flow. Data acquisition was carried out using Empower software. The chromatographic separations were performed using a symmetry LC-18 stainless steel column (150 mm × 3.9 mm, 5 µm). An isocratic mobile phase consisted of 20-mM KH_2PO_4 buffer (pH adjusted to 2.7 ± 0.05 using 50% orthophosphoric acid) and acetonitrile at a ratio of 60:40 eluted at a flow rate of 1 mL/min. Prepared buffer (20-mM KH_2PO_4) was filtered through a Millipore membrane filter (0.2 µm) from Nihon, Millipore (Yonezawa, Japan) and degassed in an ultrasonic bath for 5 min. The injection volume was 25 µL and effluent was monitored using florescence detection set at 274 excitation and 340 emission wavelength, having column oven temperature of 40°C and sample cooler temperature of 8°C ± 0.2°C. Calibration curves were constructed by calculating the area *vs*. concentration of standard analyte.

Preparation of stock, calibration and quality control sample

10 mg of THQ was dissolved in 10 ml of methanol to produce the stock solution of 1 mg/ml respectively. Serial dilution of THQ was made with the help of MS Exel 2007 in water: methanol (50:50) solution to obtain the calibration range of 0.07–12 µg/ml. Serial dilution of THQ stock solution also made to obtained four different concentration levels of 0.07, 0.24, 3, and 10 µg/mL which was used for quality control sample.

Linearity and standard curve

Calibration curves of THQ were constructed by using the analyte peak area versus nominal concentrations of the analytes. The linearity of the method was determined by analysis of standard plots associated with a six-point standard calibration curve of 0.07-12 µg/ml. The data were subjected to statistical analysis using a linear-regression model. Least squares linear regression analysis of the data gave slope, intercept and correlation coefficient data. Curves were best fitted using a least square linear regression model $y = mx + b$, weighted by $1/x^2$, in which y is the peak area ratio, m is slope of the calibration curve, b is the y-axis intercept of the calibration curve, and x is the analyte concentration. Back calculations were made from these curves to determine the concentration of THQ in each calibration standard, and the resulting calculated parameters were used to determine concentrations of analyte in quality control or unknown samples in plant extracts and formulations. The correlation coefficient $R^2>0.98$ was desirable for all the calibration curves.

Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantitation (LOQ) were determined by standard deviation (S y/x) method. For the determination of LOD and LOQ, blank sample was injected in triplicate to the chromatograph, and then peak area of this blank was recorded. The LOD and LOQ were determined using the slope of the calibration curve and $S_{y/x}$ of the blank sample by following formulae: $LOD = 3.3\times S_{y/x}/S$ and $LOQ = 10\times S_{y/x}/S$; where $S_{y/x}$ is the standard deviation of the blank response and S is the slope of the calibration curve.

Accuracy as recovery

Recovery was determined by standard addition method. The pre analyzed samples of THQ (10 µg/mL) were spiked with the extra 0, 50, 100, and 150% of the standard THQ, and the mixtures were reanalyzed by the proposed method. The percent (%) recovery of samples, percentage relative standard deviation (% RSD), and standard error were calculated at each concentration level.

Precision and accuracy

The within-run and between-run accuracy and precision of the assays were determined by assaying calculated four quality control samples in triplicate over a period of three days. The concentrations represented the entire range of the calibration curves. The lowest level was the expected limit of quantitation (LOQ) for each analyte. The second level was within 3 times of LOQ. Calibration curves were prepared and analyzed daily and linear models were used to determine concentrations in the quality control samples. The nine measured concentrations per concentration level (triplicates from three runs) were subjected to estimate the between-run precision. Percent accuracy was determined (using the data from the precision assessment) as the closeness of spiked samples to the nominal value of in-house standards.

Reproducibility

Reproducibility of the method was checked by obtaining precision on a different instrument, which was analyzed by another person in a different laboratory. Both intra-day and inter-day precision was calculated at four different concentration levels 0.07, 0.24, 3, and 10 µg/mL in triplicates.

Robustness

Robustness was carried out to evaluate the influence of small

but deliberate variation in the chromatographic conditions for the determination of THQ. Robustness of the method was determined by changing the flow rate (1.1 and 0.9 mL/min) and ratio (58:42 and 62:38) of mobile phase.

Quantification of THQ in extracts and formulations

Different concentrations (25, 50, 100 and 200 µl) of methanolic (5 gm/50 ml), petroleum extracts (5 gm/50 ml) and Baraka capsule (1.62 gm/25 ml) were diluted with methanol to make up the volume 1 ml. Similarly *Nigella sativa* oil (1 mg/ml) was first diluted with methanol to get concentration of 100 µg/ml. Then 10, 20, 40 and 80 µl of it was further diluted with methanol to make up the volume 1 ml. Vortex all above samples vigorously and 25 µl of it injected and chromatograms were obtained in the same conditions as that of standard THQ. The peak area corresponding to the Rt of standard THQ was recorded, and content of the same was calculated from the regression equation obtained from calibration curve.

Results

Optimization of chromatographic condition

The LC method carried out in this study aimed at developing a reverse phase sensitive chromatographic system capable of eluting and resolving THQ and from a standard solution *Nigella sativa* extracts and marketed formulations. The preliminary investigations were directed toward the effect of several variables on the system suitability of the method. The parameters assessed included the type and percentage of organic modifiers, the flow rate, the type and the concentration of the buffer and the pH of the mobile phase.

Initially experiment was started on HPLC connected with photo diode array detector at different detection wavelength. But sensitivity of analyte was very low as no detection happened at ng/ml level. Then analyte was scanned on spectroflourimeter, and the compound was identified as florescence sensitive at excitation 274 and emission 340 wavelengths. The robustness test showed that to create a chromatogram with a minimal variation in separation, one should strictly control the fraction of organic modifier and the pH of the mobile phase. Buffers (disodium monohydrogen phosphate, Potassium dihydrogen orthophosphate) at different pH with varying concentrations of organic phases (methanol, acetonitrile) were tried. Optimum resolution and appropriate retention times of analyte was obtained with potassium dihydrogen orthophosphate buffer and acetonitrile as an organic modifier. So the experiment started by utilizing isocratic flow condition by 20 mM potassium dihydrogen orthophosphate and acetonitrile at different concentration ratio and different pH range between (2.5 to 6.5) by orthophosphoric acid. Adjusting the pH to 2.9 of 20-mM KH_2PO_4 buffer and its flow with acetonitrile at a ratio of 60:40 associated with better resolution of analyte.

Calibration curve and linearity

The calibration curve area versus concentration (µg/mL) was found linear in the range of 0.07-10 µg/mL. Statistical calculations were done at 5% level of significance. One-way analysis of variance (ANOVA) test was performed to compare the results. The linear regression data for the calibration curve showed a good linear relationship over the concentration ranges of 0.07-12 µg/mL with respect to peak area as shown in table 1. The correlation coefficient value (R2) was found to be 0.998, which was highly significant (Table 1) ($p<0.05$). The linear regression equation was $y = 788235x + 34591$. No significant differences were observed in the slope of standard curves ($p \geq 0.05$).

Limit of detection and limit of quantification

The analysis was carried out at decreasing concentrations to determine the minimal concentration with a signal-to-noise ratio of 3:1. Under the experimental conditions, the LOD value was 0.023 µg/mL whereas LOQ values were 0.07 µg/mL respectively. In addition, the analyte peak of the LOQ samples was identifiable, discrete, and reproducible with accuracy within ≤ 20% and a precision of ≤ 20% (Figures 2 and 3). Table 1 shows the results of statistical analysis of the experimental data, such as the slope, the intercept, and the correlation coefficient obtained by the linear square treatment of the result along with the standard deviation of the slope (S_b) and intercept (S_a).

Recovery

The accuracy of proposed method was calculated by recovery analysis, which afforded recovery of 99.21-99.82% after spiking the additional standard drug solution to the previously analyzed test solution. The % RSD values were found in the range of 1.42-1.97. These results indicated the accuracy of the proposed method.

Precision

The intra-day and inter-day precision values were also studied using different concentration of standard THQ in the range concentration range of calibration curve. Calibration curve and quality control samples were prepared and three replicate measurements were conducted in a period of three days.

The intra-day coefficient of variation values (precision) was in the range of 2.28 to 6.92 where as inter-day coefficient of variation values (precision) was in the range of 1.21 to 7.55 (Table 2). These results indicate that the method has good precision and are within the acceptance limit of ≤ 15% and ≤ 15% for precision and accuracy respectively.

Reproducibility

Reproducibility of the method was checked by obtaining precision of the method in another laboratory using different instruments and analyzed by another person but in the same condition. Both intra-day and inter-day precision was examined in labs. There were no significant differences observed in the % RSD values of intra-day and inter-day precision, which indicates the reproducibility of the method.

Robustness

The robustness of a method describes its ability to withstand small changes in its operating conditions. A method which is unaffected by change in experimental parameters, within the control limits defined in the method protocol, is said to rugged. The small changes made included the flow rate, and composition of mobile phase (Tables 3

Linearity range (µg/mL)	0.07-12
Regression equation	y=788235.3x + 34591.3
Correlation coefficient	0.99833
Slope ± SD	788235.3 ± 11549.3
Intercept ± SD	34591.3 ± 405.71
Slope without intercept ± SD	784997 ± 11234
Standard error of slope	6675.74
Standard error of intercept	234.51
95% confidence interval of slope	759509.6 - 816961.0
95% confidence interval of intercept	33582.2 - 35600.4
Bias of intercept	–0.0261

Table 1: Linear regression data for the calibration curve (n=3).

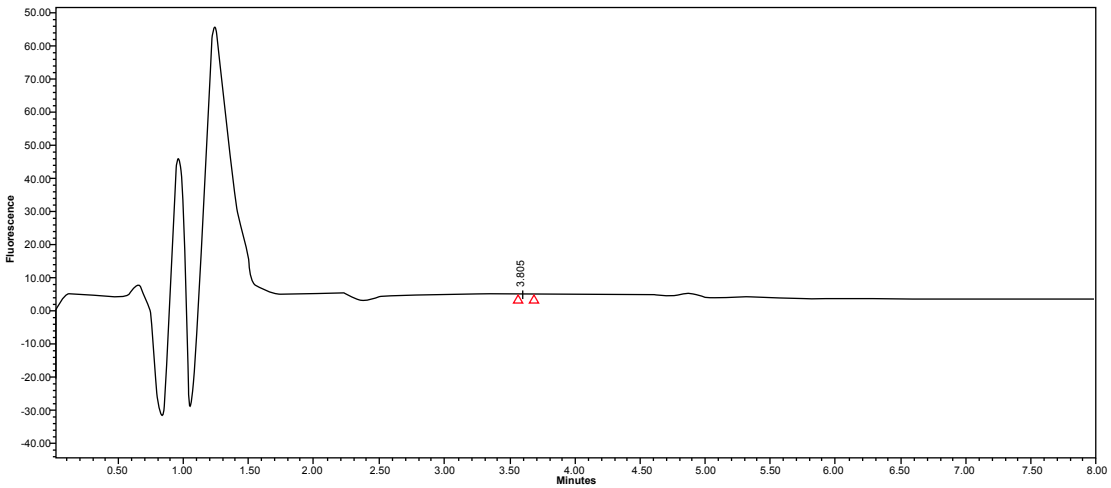

Figure 2: Representative chromatogram of blank sample showing on interference at retention time of THQ.

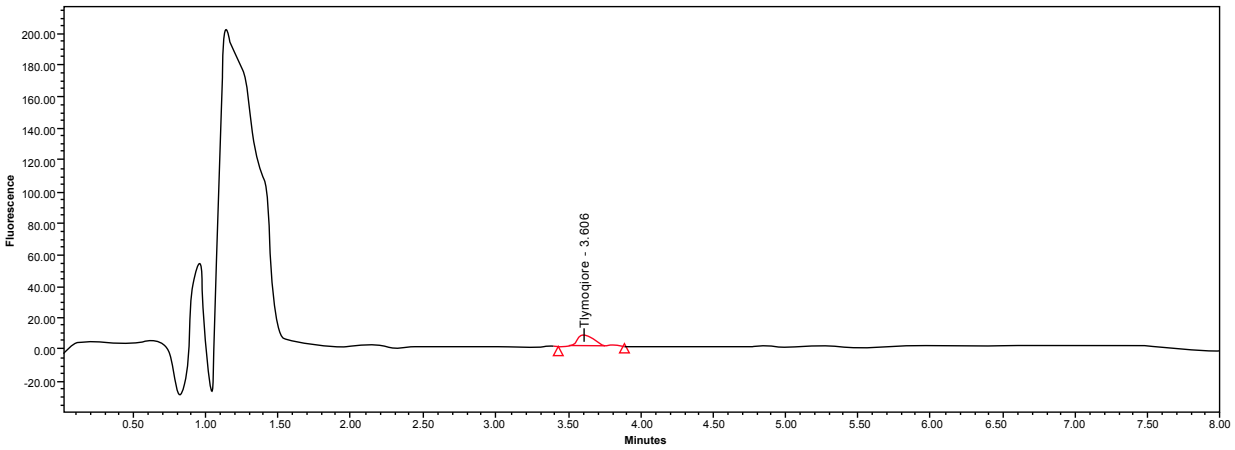

Figure 3: Representative chromatogram of THQ at LOQ level.

Nominal concentration(µg/mL)	Measured concentration Mean ± SD	Standard Error	Precision (RSD)
Intraday[a]			
0.07	0.083 ± 0.005	0.003	6.92
0.24	0.26 ± 0.01	0.005	2.28
3	2.77 ± 0.15	0.088	4.28
10	9.52 ± 0.26	0.152	5.86
Inter-day[b]			
0.07	0.076 ± 0.005	0.003	7.55
0.24	0.26 ± 0.010	0.005	3.85
3	2.78 ± 0.14	0.082	5.10
10	9.75 ± 0.11	0.681	1.21

[a]= based on n=3; [b]= based on n=9

Table 2: Precision of the proposed method.

and 4). It was found that the percentage recoveries of THQ were good under most conditions, and remained unaffected by small changes of experimental conditions.

Application of the method for the quantification of THQ in various extracts and formulations

The peaks of THQ from sample solution were identified by comparing their retention times obtained from the peaks with those of standard. It was observed that there is no any interaction peak of other constituents or excipients detected at retention time of THQ which indicated the suitability of this method for the routine analysis of THQ in marketed formulations (Figure 4). Samples were injected at different level of dilution and calculated concentration showed good precision. Amount of THQ obtained in different extracts and formulation are presented in table 5.

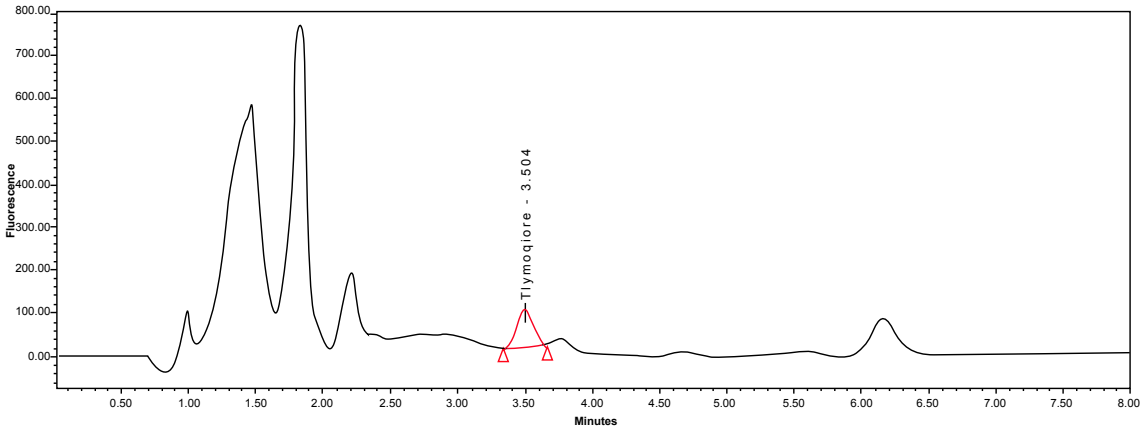

Figure 4: Representative chromatogram of THQ obtained after injection of 25µl concentration level of 1.4 gm/25 ml of Baraka Capsule.

Mobile phase composition 20-mM KH_2PO_4 buffer- Acetonitrile		Nominal concentration (µg/mL)	Measured concentration (Mean ± SD)	%RSD
Original	Modified			
60:40	58:42	0.24	0.25 ± 0.026	10.58
		10	11.25 ± 0.24	2.19
	60:40	0.24	0.27 ± 0.01	6.41
		10	10.15 ± 0.61	6.04
	62:38	0.24	0.26 ± 0.01	3.85

Table 3: Robustness of the method by changing the mobile phase ratio.

Flow rate (mL/min)		Nominal Concentration (µg/mL)	Measured Concentration (Mean ± SD)	%RSD
Original	Modified			
1	0.9	0.24	0.22 ± 0.021	7.6
		10	9.56 ± 0.18	3.87
	1	0.24	0.25 ± 0.017	6.72
		10	10.56 ± 0.41	2.64
	1.1	0.24	0.26 ± 0.026	5.25
		10	10.88 ± 0.83	3.76

Table 4: Robustness of the method by changing the flow rate.

Extracts & Formulations	Calculated concentration Mean ± SD (µg/ml)	%RSD
Methanolic Extracts	5.19 ± 0.43	8.37
Petrolium Extracts	2.23 ± 0.29	13.17
Nigella Setiva Oil	5.56 ± 3.17	5.69
Baraka Capsule	3.61 ± 2.39	6.64
Black seed Rub	2.00 ± 0.11	5.27

Table 5: Measured concentration of THQ in different extracts and formulations (µg/ml).

Conclusion

In conclusion, a reliable HPLC florescence method with high sensitivity, robust, reproducible and short elution time was developed for the determination of THQ in extracts and various formulations. This method was validated for its selectivity, accuracy as recovery, precision and robustness and was successfully applied for the estimation of THQ in various extracts and formulations.

Acknowledgement

Authors are grateful to the Research Center of the College of Pharmacy and the Deanship of the Scientific Research of the King Saud University for their funding and support.

References

1. El-Fatatry HM (1975) Isolation and structure assignment of an antimicrobial principle from the volatile oil of *Nigella sativa* L. seeds. Pharmazie 30: 109-111.
2. El-Kadi A, Kandil O (1986) Effect of *Nigella sativa* (the black seed) on immunity. Bulletin of Islamic Medicine. Proceedings of the 4th International Conference on Islamic Medicine, Kuwait pp 344-348.
3. Gali-Muhtasib H, Roessner A, Schneider-Stock R (2006) Thymoquinone: a promising anti-cancer drug from natural sources. Int J Biochem Cell Biol 38: 1249-1253.
4. Khader M, Bresgen N, Eckl PM (2009) *In vitro* toxicological properties of thymoquinone. Food Chem Toxicol 47: 129-133.
5. Gheita TA, Kenawy SA (2012) Effectiveness of *Nigella sativa* oil in the management of rheumatoid arthritis patients: a placebo controlled study. Phytother Res 26: 1246-1248.

6. Basem Shomar (2012) Major and trace elements in *Nigella sativa* provide a potential mechanism for its healing effects. Journal of Medicinal Plants Research 6: 4836-4843.
7. Thabrew MI, Mitry RR, Morsy MA, Hughes RD (2005) Cytotoxic effects of a decoction of *Nigella sativa*, Hemidesmus indicus and Smilax glabra on human hepatoma HepG2 cells. Life Sci 77: 1319-1330.
8. Farah IO, Begum RA (2003) Effect of *Nigella sativa* (N. sativa L.) and oxidative stress on the survival pattern of MCF-7 breast cancer cells. Biomed Sci Instrum 39: 359-364.
9. Shafi G, Munshi A, Hasan TN, Alshatwi AA, Jyothy A, et al. (2009) Induction of apoptosis in HeLa cells by chloroform fraction of seed extracts of *Nigella sativa*. Cancer Cell Int 9: 29.
10. Khan N, Sultana S (2005) Inhibition of two stage renal carcinogenesis, oxidative damage and hyperproliferative response by *Nigella sativa*. Eur J Cancer Prev 14: 159-168.
11. Ghosheh OA, Houdi AA, Crooks PA (1999) High performance liquid chromatographic analysis of the pharmacologically active quinones and related compounds in the oil of the black seed (*Nigella sativa* L.). J Pharm Biomed Anal 19: 757-762.
12. Hajhashemi V, Ghannadi A, Jafarabadi H (2004) Black cumin seed essential oil, as a potent analgesic and antiinflammatory drug. Phytother Res 18: 195-199.
13. Burits M, Bucar F (2000) Antioxidant activity of *Nigella sativa* essential oil. Phytother Res 14: 323-328.
14. Ali BH, Blunden G (2003) Pharmacological and toxicological properties of *Nigella sativa*. Phytother Res 17: 299-305.
15. Velho-Pereira RM, Barhate CR, Kulkarni SR, Jagtap AG (2011) Validated high-performance thin-layer chromatographic method for the quantification of thymoquinone in *Nigella sativa* extracts and formulations. Phytochem Anal 22: 367-373.
16. Michelitsch A, Rittmannsberger A (2003) A simple differential pulse polarographic method for the determination of thymoquinone in black seed oil. Phytochem Anal 14: 224-227.
17. Hadad GM, Salam RA, Soliman RM, Mesbah MK (2012) High-performance liquid chromatography quantification of principal antioxidants in black seed (*Nigella sativa* L.) phytopharmaceuticals. J AOAC Int 95: 1043-1047.
18. Pathan SA, Jain GK, Zaidi SM, Akhter S, Vohora D, et al. (2011) Stability-indicating ultra-performance liquid chromatography method for the estimation of thymoquinone and its application in biopharmaceutical studies. Biomed Chromatogr 25: 613-620.
19. International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use: Harmonised Triplicate Guideline on Validation of Analytical Procedures: Methodology, Recommended for Adoption at Step 4 of the ICH Process on November 1996 by the ICH Steering Committee, IFPMA, Switzerland.