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ORIGINAL ARTICLE

Ultra-High-Performance Liquid Chromatography-Based Identification and Quantification of Thymoquinone in *Nigella sativa* Extract from Different Geographical Regions

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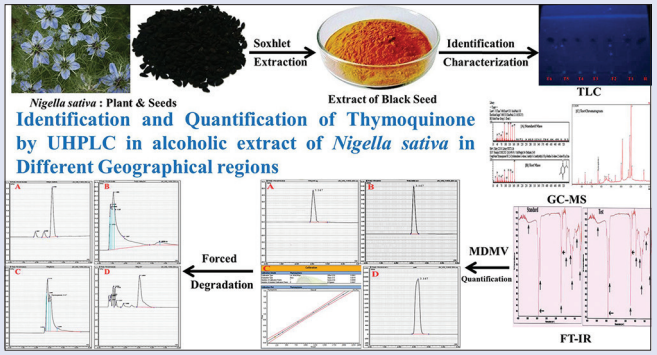
ABSTRACT

Background: Thymoquinone (THQ) is a major bioactive constituent of traditional medicinal plant such as Black seed (*Nigella sativa*, Family: *Ranunculaceae*). **Objective:** The objective of this study was to identify and quantify THQ in Black seeds from different geographical regions and to develop a new ultra-high-performance liquid chromatography-photodiode array detector (UHPLC-PDA) method. **Materials and Methods:** Black seeds were collected in the end of autumn season. The mobile phase was used in this study [Acetonitrile (2 mM): Ammonium Formate at 50:50 proportions, with a flow rate of 0.200 mL min^{-1} and also with Isocratic elution on the Accucoro Vanguish C18 UHPLC column (1.5 µm; 100 × 2.1 mm)]. **Results:** Retention and total run times were 3.147 and 6.0 min, respectively, with injection volume of 5 µL at 254 nm. The method was validated for linearity ($r^2 \geq 0.9987$), accuracy (≥92.90%), and precision (coefficient of variance [CV] ≤2.54%) with a calibration curve range of 100.00–2000.00 ng/mL. THQ was degraded under ultra-violet-light, basic, acidic, and oxidation stress conditions during forced degradation studies. Our developed UHPLC method reported the degradation peaks of the final product of THQ. UHPLC-PDA analysis showed large variation (0.01%–3.03% w/w) of THQ in the samples of different regions in respect to their concentration and occurrence of metabolite. **Conclusions:** Turkey has the maximum quantity of THQ throughout the world. The authors suggested that Turkey is the best region for cultivating Black seed plant of superior variety. One more important study performed, i.e., the gas chromatography and mass spectrometry study, reported THQ (2.863%) to be the primary constituent in the extract of *N. sativa*.

Key words: Black seed (*Nigella sativa* L.), degradation kinetics, method development and validation, thymoquinone, ultra-high-performance liquid chromatography-photodiode array detector

SUMMARY

- Thymoquinone (THQ) is a major bioactive constituent of traditional medicinal plant such as Black seed (*Nigella sativa*, Family: *Ranunculaceae*)
- Eleven samples of *N. sativa* were collected from different geographical regions
- The established new ultra-high-performance liquid chromatography method was shown to be rapid, simple, accurate, selective, sensitive, precise, and consume less time for sample analysis
- Stability/degradation of THQ was also established in different conditions
- The highest and lowest contents of THQ were found to be shown in the sample of Turkey and Sudan, respectively
- Our proposed results could be providing an important input for cultivation of black seed with better management strategies in the future
- It may be useful in the future to fix the exact amount of dose (in black seed extract: THQ) for the treatment of different reported diseases.



Abbreviations used: THQ: Thymoquinone; UHPLC-PDA: Ultra-high-performance liquid chromatography-photodiode array detector; ACN: Acetonitrile; UV: Ultraviolet; GC-MS: Gas chromatography and mass spectrometry; NSO: *Nigella sativa* extracted oil; TLC: Thin-layer chromatography; IR: Infrared spectroscopy; DSC: Differential scanning calorimetry; QC: Quality control; HQC: High-quality control; MQC: Middle-quality control' LQC: Low-quality control; LLOQQC: Lower limit of quality control; US-FDA: United States of Food and Drug Administration; PSS: Primary stock solution; SS: System suitability; LOD: Limit of detection; LOQ: Limit of quantification.

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INTRODUCTION

Nigella sativa is a well-known plant which is used for various purposes and as a flavoring agent in pickles and bread in Middle East, Far East, Indian subcontinent as well as Middle Asia. The common synonyms for *N. sativa* include "Al-Habba Al-Barakah or Al-Habba Al-Sauda" (Arabic name), "black seed" (English name), and "Kalonji" (Urdu). *N. sativa* (Black seed), conventionally or traditionally used to treat headache, anxiety, fever, asthma, diarrhea, and also stroke, is known to be highly

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anti-inflammatory.[1,2] *Nigella sativa* (Black seed) contains maximum amount of phenolic compounds, used as an antioxidant agent[1] and their essential fatty acids also used besides bioactive compounds such as sterols and tocols.[3] Moreover, the yellowish oil contains proteins, reducing sugars, mucilage, amino acids, alkaloids, tannins, organic acids, toxic glucoside, resins, bitter principles, metarbin, crude fiber, glycosidal saponins, vitamins, and minerals.[4] There are so many various oil seeds like Nigella sativa oil (NSO) is particularly utilized in preparations of many formulations that contain phytochemicals with strong antioxidant properties, and also the health benefits.[5,6] Many active compounds have been isolated, identified, and reported so far in different varieties of black seeds. The most important active compounds are thymoquinone (THQ), dithymoquinone, thymohydroquinone, carvacrol, p-cymene, t-anethol, terpineol, α-pinene, sesquiterpene longifolene, and thymol. *N. sativa* also has some other compounds in very least amounts. It has two different types of alkaloids, i.e., isoquinoline alkaloids and indazole or pyrazol alkaloids, which include nigellicine and nigellidine.[7] THQ is an active compound in the crude extracts of NSO, which possesses antioxidant/anti-inflammatory efficacy in models of *in vitro* and *in vivo* investigations as well as asthma, diabetes, encephalomyelitis, neurodegeneration, and carcinogenesis.[5,8] Black seed extracts show so many activities such as antihistaminic, immunopotentiation, hypertensive, antimicrobial, and anti-inflammatory.[9-14] Quinone-based active constituents are responsible for most of these activities.[15,16] Therefore, we selected THQ as an active constituent for geographical distribution of samples (black seed) throughout the world.

Methods of extraction of seed oils are an effective factor in the properties of oils. Solvent extraction, for example, is deficient in selectivity and needs extreme heat, which could cause the degradation of the desired components.[17] The cold press extraction is the conventional method for oil extraction. It involves no heat and/or chemicals and this is preferred by consumers concerned about natural and safe food.[18] However, this method affords low yields,[19] and the residual meal contains 10%–12% oil content, which can eventually limit its uses in industries processing food.[20] In this research study, we used Soxhlet apparatus for extraction of Black seed and for evaporation of solvent by rotary evaporator (BUCHI Rotavapor®, Switzerland).[21]

To quantify THQ in nanoformulation as well as different batches from different geographical locations, a fast, specific, and sensitive method is needed. Though various methods such as high-performance liquid chromatography (HPLC), thin-layer chromatography, pulse polarography, and gas chromatography[22-25] have been reported in literature, till date, none of the methods had quantified THQ in black seed extracts obtained from different regions or in any plasma/serum. The current study aims to determine a very sensitive and fast ultra-HPLC (UHPLC) method for determination of THQ in different geographical batches, whereby the added advantages of short retention time and more sensitivity are observed as compared to HPLC.[26] Thus, one more objective of this research study was to develop a fast, sensitive, and very selective UHPLC method that can evaluate the THQ concentration in different regions of extract, bulk drug, nano-formulation-based targeting, *in vitro*: release studies, and *in vivo*: pharmacokinetic studies of THQ in future.

Genetic variability is considered an important factor for varying amounts of active drug obtained from the same plant but with different locations. A number of research reports are available regarding the variability in the amount of active drugs in the same plant but grown and collected at different regions of the world. One such study is reported for analyzing the medicinal value of *Curcuma longa* (turmeric) obtained from different parts in India.[27] Ashraf *et al*. reported different concentrations of the active drug, i.e., curcuminoids in turmeric samples collected from different locations in India. The study concluded a minimum amount of active drug in sample obtained from Erode, i.e., south province as compared to high amount of the drug in samples from Surat, i.e., west province. The change in active drug concentration is suggested due to change in environmental conditions in both provinces.[27] Another study reported the difference in amount of active drug, i.e., gingerol in *Zingiber officinalis* rhizomes collected from different parts of India, due to environmental differences.[28]

N. sativa plant has very high medicinal value, and increasing its demand to encourage the authors will investigate the chromatographic variability of THQ in the different geographical regions. This is a principal documentation for evaluation of THQ in the different geographical regions. Therefore, this study is very important to evaluate the metabolic variability of THQ through UHPLC in the different geographical regions of Black seed collected from all the major geographical zones of Pakistan, India, Turkey, Egypt, Yemen, Syria, Saudi Arabia, etc., The results attained in this study could be valuable in developing strategies for cultivating this important medicinal plant, for large-scale production, and also helpful for determining the exact amount of dose (in black seed extract) to the treatment of different reported diseases.

MATERIALS AND METHODS

Chemicals and reagents

All the samples of *N. sativa* were collected from different geographical regions of world such as India, Pakistan, and Saudi Arabia or also purchased from various retail stores in Dammam City, Saudi Arabia [Table 1] and were identified by pharmacognosist and phytochemist Dr. Rizwan Ahmad and Dr. Mohd Amir, Imam Abdulrahman Bin Faisal University, respectively. HPLC-grade methanol, acetonitrile, ammonium formate, ammonium acetate, and formic acid were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). THQ standard (purity 99.98%) was purchased from Frinton Laboratories, 4204 Sylon Boulevard, Hainesport, New Jersey, 08036, USA. Milli-Q-Water was used in the entire analysis. All the other chemicals used that were of analytical grade were obtained from different commercial sources.

Extraction of black seed by Soxhlet apparatus

There are so many extraction methods and conditions to use in the study of various biologically active constituents of medicinal herbs;[29] all of them contain limitations related to particular substance classes. Therefore, the primary objective of this research study was testing herbal extracts express any beneficial effects in future for preparation of nanoformulation of extract and their comparison with isolated THQ in different diseases, and their since aqueous extracts[30] as well as THQ, the main active ingredient of the volatile oil of *N. sativa*, have been confirmed to reveal cyto- and genotoxic effects,[31] extraction of black seed with ethanol selected and also to evaporate at room temperature that also recover most of the volatile oil defined to contain chemopreventive effects[32] and antioxidant.[1] Soxhlet apparatus was used for the extraction of black seed in which powdered dry plant material (50 g) was immersed in 70 ml of ethanol absolute at 65°C for 6 h. Ethanolic extract was kept at room temperature to evaporate ethanol and the finally collected oily extract was freeze dried.

Extraction yield

The extraction yield was analyzed by the method of Zhang *et al*. In brief, the obtained filtrate was evaporated to dryness using a rotary evaporator under vacuum at 60°C and was then lyophilized in a freeze-dryer to obtain freeze-dried extract.[42] The extraction yield was calculated as a percentage of the raw material using the following formula:

$$\text{Extraction yield } (\%) = \frac{\text{Weight of the freeze-dried extract}}{\text{Weight of the original sample}} \times 100$$

Identification and characterization of thymoquinone

Thin-layer chromatography method for separation of thymoquinone

For separation of THQ, TLC plates (Merck, Darmstadt, Germany) with the following specifications: silica gel 60G, dimensions (4.5 mm × 10 mm), were used, whereas the mobile phase consisted of chloroform:toluene:: 08:02 as shown in Table 1and Figure 1. The method was chosen on the basis of the R_f values for THQ.

Gas chromatography and mass spectrometry test

Gas chromatography and mass spectrometry (GC-MS) test was conducted in a gas chromatograph (Trace GC Ultra gas chromatograph) attached to a TSQ Quantum XLS single quadruple mass spectrometer, both from Shimadzu (GCMS-QP2010 Ultra, Shimadzu, Japan). Analyses were performed with a fused silica Rxi™-5MS column (30 m × 0.25 mm × 1.0 µm), which was employed for all the analyses. The temperature of the oven was raised to 300°C from 60°C at 4°C/min and maintained isothermally for 12.9 min. The temperatures of injector and detector were maintained at 220°C and 240°C, respectively, with the preparation of 100% of samples in methanol. The split mode ratio of 50:1 was applied for the injection of a 1.0 µL sample. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. Other parameters were kept in the same condition in relation to GC analyses. Electron ionization at 70 eV provided the mass spectra with mass scanning done from 33 to 550 amu. Percentage of each component was calculated based on GC peak areas.

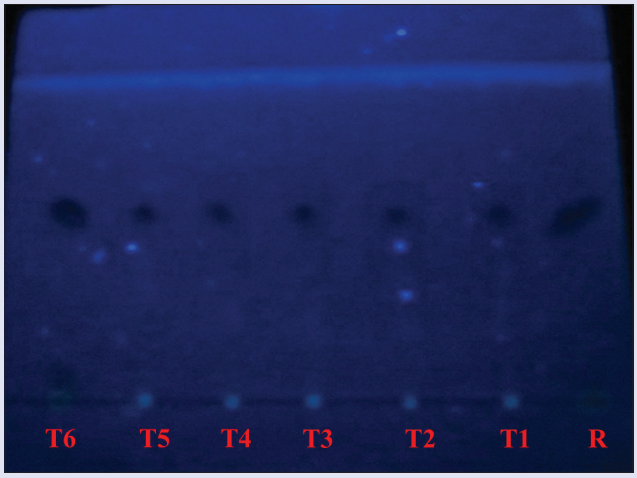

Figure 1: Identification of thymoquinone by thin-layer chromatography plates (mobile phase:chloroform:toluene::08:02) in the R: Standard thymoquinone, T1–T6 represent different extracts of countries as per Table 1 (NS1–NS7)

Infrared spectroscopy

NICOLET iS50 Fourier transform-infrared spectroscopy (FT-IR) spectrophotometer (Thermo Fisher Scientific, 5225 Verona Road, Madison, WI 53711, USA) was used to record the IR spectrum for samples. In detail, each extract (0.1 mg) + 100 mg KBr was mixed and pressed to make a pellet and further subjected to analysis at wavelengths, i.e., 400–4000 cm^{-1}.

Thermal behavior differential scanning calorimetry

Thermal properties of NSO extracted by Soxhlet extraction were examined by differential scanning calorimeter (DSC) using DSC 214 Polyma (NETZSCH-Wittelsbacherstraße 42, 95100 Selb, Germany). Weighing of the oil samples was done (9–10 mg) straight into a DSC-pan (SFI-Aluminium, ASC214, NETZSCH-Wittelsbacherstraße 42, 95100 Selb, Germany). An empty aluminum pan, hermetically sealed, served as the reference. The samples were heated from 20°C with a speed of 10°K/min to 300°C. The process was performed again in the same manner and recording of the DSC thermograph results was done during the second-time melting. The DSC manufacturer's software (Special Library of NETZSCH-Wittelsbacherstraße 42, 95100 Selb, Germany) was employed to analyze the data of the heat flow, and the exact heat of the oil samples was calculated. Results were derived by averaging triplicate samples.

Method development and their validation

Determination of thymoquinone by ultra-high-performance liquid chromatography-photodiode array detector

Thermo Scientific™ Vanquish™ UHPLC system (Thermo Scientific, Germany), made up of a binary solvent delivery system along with photodiode array detector (Chromeleon (c) Dionex Version 7.2.2.6890, Germany), was used to perform UHPLC. For chromatographic separation, the tools used with specifications are as follows; Accucore™ Vanquish™ column with specifications, i.e., C18 (1.5 µm; 100 mm × 2.1 mm), degassed mobile phase of HPLC-grade solvent, i.e., acetonitrile (ACN): 2 mM ammonium formate (50:50 v/v) with isocratic elution, flow rate of mobile phase (0.200 ml/min) as well as injection sample volume of 5 µl as injected at every run. The total run time was 6.0 min [Table 1 and Figure 2] with software Chromeleon (c) Dionex Version 7.2.2.6890.

Table 1: Geographical source, thin-layer chromatography profile, and percentage yield of black seed samples used in the study

Code number	Cultivation regions (provinces)	Source	R_f values of different alcoholic extracts by TLC (mobile phase: Chloroform: Toluene::08:02)	Percentage yield±SD
NS1	Sri Lanka	Exported from Sri Lanka	0.66	15.46±0.71
NS2	India (Uttar Pradesh)	Herbal garden, Integral University, Lucknow, Uttar Pradesh	0.67	16.03±0.55
NS3	India (Delhi)	Herbal Garden, Jamia Hamdard, New Delhi	0.67	15.92±0.51
NS3	Turkey	Exported from Turkey	0.65	14.31±0.55
NS4	Egypt	Exported from Egypt	0.68	13.14±0.42
NS5	Ethiopia	Exported from Ethiopia	0.64	14.16±0.58
NS6	Saudi Arabia	Local farmer, Qassim, Saudi Arabia	0.69	17.38±0.49
NS7	Pakistan	Herbal garden, Hamdard University, Karachi, Pakistan	0.67	16.54±0.61
NS8	Syria	Exported from Syria	0.68	16.11±0.48
NS9	Yemen	Exported from Yemen	0.67	15.32±0.46
NS10	Afghanistan	Exported from Afghanistan	0.66	17.04±0.66
NS11	Sudan	Exported from Sudan	0.67	15.96±0.58

SD: Standard deviation; TLC: Thin-layer chromatography

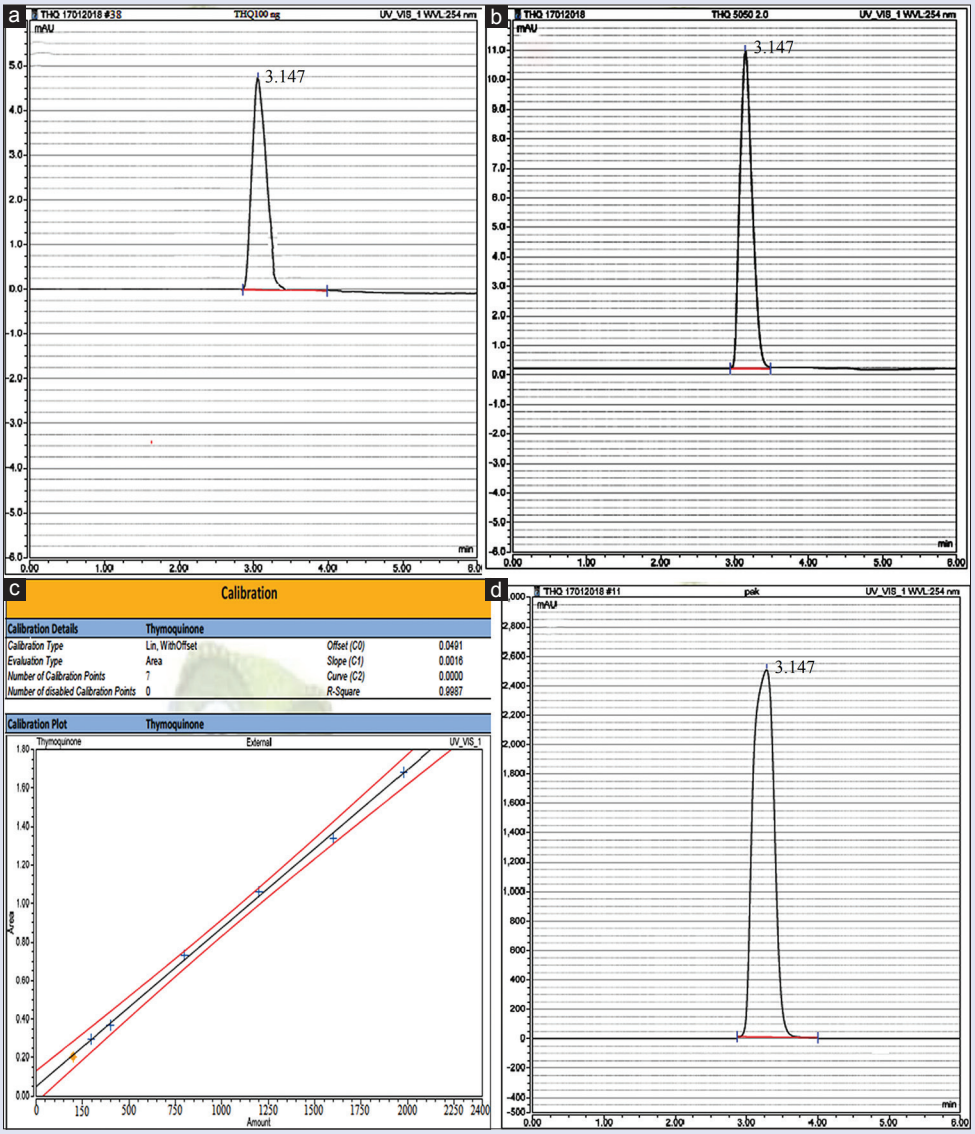

Figure 2: Typical chromatograms of lower limit of quantification thymoquinone (a), standard thymoquinone (b) with their calibration curve (c) (100–2000 ng/mL), and typical chromatogram thymoquinone from extracted black seed (d)

Preparation of samples

Eleven different fresh black seeds (*N. sativa* L.) were collected from different geographical regions, powdered, and sieved (60 numbers); after that, they were collected as uniform powder. For the sample preparation analysis, 50 g of powdered black seeds was refluxed with ethanol for 5 h at a temperature of 65°C. All different extracted samples were filtered using Whatman #4 filter and a glass funnel and evaporated to dryness on a first BUCHI rotary evaporator and after that with water bath followed by freeze dryer. The obtained extract was dissolved in methanol and transferred into UHPLC injection vials for UHPLC–PDA analysis.

Calibration standards and quality control sample preparation

The stock solution of THQ (1.0 mg/mL concentration) was prepared by dissolving 1.0 mg of the THQ and completed up to a final volume of 1 mL in HPLC-grade methanol and after that sonicated at 37°C for 10 min. All the dilutions were made step by step through the main stock in methanol and were filtered by 0.22-µm membrane filter before UHPLC analysis. Seven calibration curve THQ standard points were prepared in methanol to produce a concentration curve (CC) range of 100.00–2000.00 ng/mL (100, 200, 400, 800, 1200, 1600, and 2000.00 ng/mL). However, three levels of quality control (QC) samples were prepared as follows: 1600 ng/mL (high QC [HQC]), 800 ng/mL (middle QC [MQC]), 400.0 ng/mL (low QC [LQC]), and 101.00 ng/mL (lower limit of QC [LLOQQC]). All the samples were stored at a temperature range of 2°C–8°C until use.

Validation of ultra-high-performance liquid chromatography method

According to the United States of Food and Drug Administration guidelines, the developed method was validated.[33] Calibration curve THQ standard points were prepared in methanol to produce a CC range of 100.00–2000.00 ng/mL (100, 200, 400, 800, 1200, 1600, and 2000.00 ng/mL). Concentration of analytes versus peak area was used for plotting the calibration curves using ($1/x^2$) linear least square regression of THQ. The lowest concentration of the calibration curve is the lower limit of quantification (LLOQ), i.e., for the calculation of acceptable accuracy and precision results. LLOQ and limit of detection (LOD) for THQ were calculated at a signal-to-noise ratio of 10:1 and 3:1, respectively, through

injecting a series of many dilutions with standard concentrations. The recovery of THQ was accomplished at LLOQQC, LQC, MQC, and HQC levels. Estimation of intraday precision and accuracy and their replicate analysis of black seed samples was accomplished on the same day. Each run has one calibration curve and also six points of LLOQQC, LQC, MQC, and HQC samples. Six precision and accuracy batches (on 3 consecutive validation days) were analyzed for interday precision and accuracy. The intra- and inter-assay accuracy (%recovery) was calculated as: %recovery = (mean determined concentration/nominal concentration) ×100. The intra- and inter-assay precision (%relative standard deviation [%RSD]) of the method was calculated from the mean measured concentrations as follows: %RSD = (standard deviation of mean measured concentration/mean measured concentration) × 100.

RESULTS

Percentage yields on different geographical regions of extracts (Soxhlet method)

Extraction yield is calculated by extract weight; it is the ratio of the extract weight to the sample weight. Percentage yields ranged from 13.14% to 17.38% Soxhlet extraction with ethanol [Table 1].

Identification and characterization of thymoquinone in the alcoholic extract of black seed

Thin-layer chromatography methods for the identification of thymoquinone in the different extracts

In the extracted black seed were identified by TLC with specifications as silica gel (10 × 20 cm) plates (E-Merck, Darmstadt, Germany), developing solvent system: (mobile phase: chloroform: toluene: 08:02) and seen as dark brown spots. The range of R_f values for all the extracts found was 0.64–0.69 for THQ [Figure 1 and Table 1].

Gas chromatography and mass spectrometry studies

The results showed 19 compounds in the black seed extract. The major component of the black seed extract was THQ (2.863%), rest of them are not useful to this study such as l-(+)-ascorbic acid 2,6-dihexadecanoate (4.828%), hexadecanoic acid, ethyl ester (12.379%), (Z, Z)-9,12-octadecadienoic acid (19.018%), 10,13-eicosadienoic acid, and methyl ester (38.209%) [Table 2 and Figure 3]. The THQ was identified and confirmed through the alcoholic extract of black seed [Figure 3a and b].

Infrared spectroscopy

The results from IR spectra [Figure 4] revealed that alcoholic extract of black seed matches with that of the standard THQ samples. Figure 4 reveals FT-IR spectrum of dried alcoholic extract at mid-IR region (4000–400 cm^{-1}). In this study, the IR spectrum of alcoholic extract of black seed showed very close features of absorption bands identical to the most common triglyceride molecules with certain fatty acids [Table 3].

Thermal behavior of Nigella sativa oil

The curve of NSO showed a plain thermogram, with one peak possessing features such as onset temperature at 204.69°C, melting enthalpy at 44.01 J/g, and melting peak at 239.06°C [Figure 5]. The weight loss occurred in black seed oil in the percentage of 34.3 at 251.61°C [Figure 5].

Method development and their validation of ultra-high-performance liquid chromatography-photodiode array detector for thymoquinone

The UHPLC-PDA chromatograms for THQ as shown in Figure 2 demonstrate the selectivity of the method. THQ recovery was determined

Table 2: Gas chromatography and mass spectrometry identification of chemical composition of extracted black seeds

Compound name	RT	Peak area	Peak area (%)	MW
Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-	5.509	310,665	0.193	220
Glycerin	5.672	3,001,293	1.865	92
B-Pinene	6.045	158,374	0.098	136
Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	6.045	158,374	0.098	136
3-Carene	6.257	96,308	0.060	136
O-Cymene	6.333	4,284,328	2.662	134
D-Limonene	6.377	274,450	0.171	136
Gamma.-Terpinene	6.565	434,690	0.270	136
cis-4-methoxy thujane	6.84	162,970	0.101	168
1,2,3,4-Butanetetrol, [S-(R*, R*)]-	6.94	179,209	0.111	90
trans-4-methoxy thujane	6.99	993,449	0.617	168
Thymoquinone	7.658	4,607,681	2.863	164
2-Cyclopenten-1-one, 3-methyl-2-(2,4-pentadienyl)-, (Z)-	7.777	587,054	0.365	162
Phenol, 2-methyl-5-(1-methylethyl)-	7.828	911,710	0.566	150
1,3-Propanediol, 2-(hydroxymethyl)-2-nitro-	8.341	1,386,219	0.861	151
1,2,4-Methenoazulene, decahydro-1,5,5,8a-tetramethyl-, [1S-(1.alpha.,2. alpha.,3a.beta.,4.alpha.,8a.beta.,9R*)]-		424,397	0.264	204
Dihydroartemisinin, 10-O-(t-butyloxy)-		385,513	0.240	182
t-Butylhydroquinone	8.739	1,986,729	1.234	166
2,5,5,8a-Tetramethyl-6,7,8,8a-tetrahydro-5H-chromen-3-one	8.801	629,644	0.391	220
1,5,9,9-Tetramethyl-2-oxatricyclo[6.4.0.0 (4,8)]dodecane	9.021	357,880	0.222	265
2,6-Octadiene, 1-(1-ethoxyethoxy)-3,7-dimethyl-	9.416	2,066,541	1.284	226
l-(+)-Ascorbic acid 2,6-dihexadecanoate	10.026	7,769,925	4.828	652
Hexadecanoic acid, ethyl ester	10.148	19,923,439	12.379	284
7-Hexadecenal, (Z)-	10.763	4,110,210	2.554	196
(Z, Z)-9,12-Octadecadienoic acid	10.9	30,607,692	19.018	266
10,13-Eicosadienoic acid, methyl ester	11.028	61,494,257	38.209	308
Octadecanoic acid, ethyl ester	11.107	6,672,630	4.146	312
10,13-Eicosadienoic acid, methyl ester	12.335	6,967,722	4.329	322
Total		160,943,353	100.00	

RT: Retention time; MW: Molecular weight

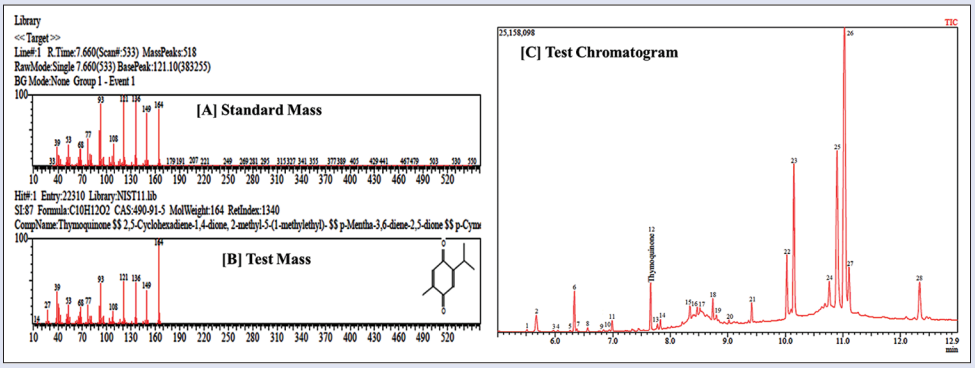

Figure 3: Gas chromatography and mass spectrometry chromatography analysis of alcoholic extract of *Nigella sativa* extracts by Soxhlet extraction method

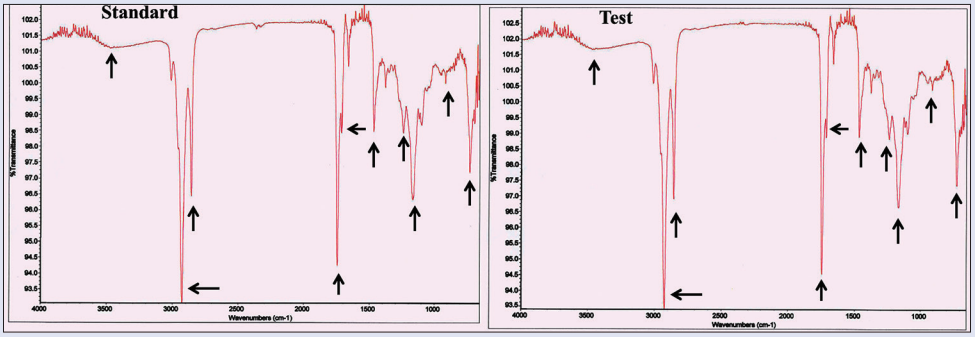

Figure 4: Fourier transform-infrared spectroscopy spectra of *Nigella sativa* extract scanned at 4000–400 cm^{-1}; Soxhlet extraction infrared-spectra with standard infrared spectra

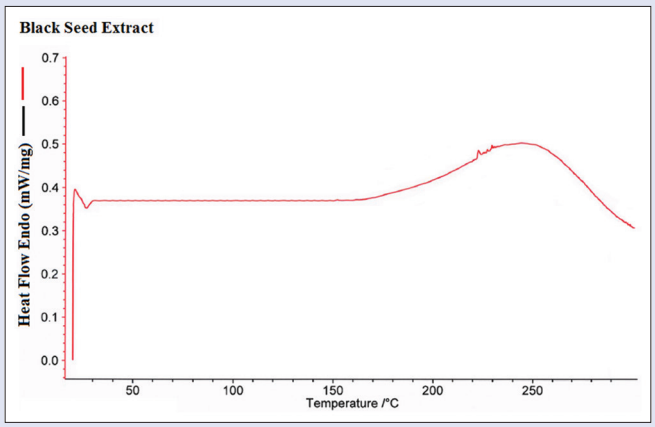

Figure 5: Differential scanning calorimetry thermal behavior of extracted *Nigella sativa* oil by Soxhlet extraction method

by comparing the peak areas of all samples that were prespiked with analytes (THQ) at LLOQQC, LQC, MQC, and HQC levels, with peak areas of analytes showing >99% extraction of samples at all concentration levels, whereas the method found was linear ($r^2 > 0.9987$) (sample range: 100.00–2000.00 ng/mL) [Figure 2c]. No significant interferences were notified at the RT of analytes. Intra- and inter-batch percentage CV for all QC levels of THQ, i.e., between 0.89 and 2.54, whereas intra- and inter-batch accuracy, i.e., within the range of 92.90%–98.07%, for all QC levels of THQ, are shown in Table 4. The extracted compounds were characterized using GC-MS spectra [Table 2 and Figure 3], FT-IR spectra [Table 3 and Figure 4], and LC-PDA analysis [Figure 2a and b].

Table 3: The main peaks in the Fourier transform-infrared spectroscopy spectrum of alcoholic extract of *Nigella sativa* extracted by Soxhlet method, matched with thymoquinone standard

Peak (cm^{-1})	Functional group
3482	Primary amines (-NH_2 groups)
2923.30, 2855.38	C-H stretching vibration (aliphatic) (CH_3)
1721	C=O stretching vibration (ester)
1458.08, 1367	C-H bending vibration (aliphatic) (CH_2)
1165.07	C-O stretching vibration (ester)
936,717	trans-CH=CH

THQ was further analyzed through GC-MS to get mass spectra as presented in Figures 3b, i.e., molecular ions of high-resolution mass spectra for THQ (λ_{max} 254 nm). Above all, the parameters have proved that the alcoholic extract of black seed possesses THQ.

The UPLC-PDA analysis showed the contents of THQ in *N. sativa* extract in the range of 1.44 ± 0.21–4.48 ± 0.28 (μg/g) [Table 5].

Linearity

The CC for THQ showed linearity (concentration range: 100.00–2500.00 ng/mL) with $r^2 \geq 0.9987$, precision (i.e., %CV, 0.89%–2.54%), and accuracy (92.90%–98.07%) [Figure 2].

Accuracy and precision

No significant interferences were noticed at the RT of analytes from any endogenous peak in any batch. The representative chromatogram as shown in Figure 2a and b (i.e., LLOQ and standard THQ) exhibits selectivity of the method. Mean THQ recovery (spiked $n = 6$) as observed at different QC level was 98.84 ± 0.91% for LLOQQC, 99.07 ± 0.92% for LQC, 99.18% ± 1.61% for MQC, and 99.52% ± 1.71% for HQC. Similarly,

Table 4: Precision and accuracy data for thymoquinone

QC ID	Theoretical content (ng/mL)	Intra-batch			Inter-batch			Percentage recovery
		Mean concentration observed (ng/mL)	Accuracy[a] (%)	CV[b] (%)	Mean concentration observed (ng/mL)	Accuracy[a] (%)	CV[b] (%)	
LLOQQC	101.00	95.61±1.19	94.66	1.24	94.38±1.71	93.45	1.81	98.84±0.91
LQC	400.00	381.61±8.15	95.40	2.14	371.58±9.43	92.90	2.54	99.07±0.92
MQC	800.00	771.69±11.38	96.46	1.47	766.48±12.52	95.81	1.63	99.18±1.61
HQC	1600.00	1569.19±13.89	98.07	0.89	1557.10±14.91	97.32	0.96	99.52±1.71

Values (mean±SD) are derived from six replicates, [a]Accuracy (%)=Mean value of [(mean observed concentration)/(theoretical concentration)] ×100; [b]Precision (%): Coefficient of variance (percentage)=SD divided by mean concentration found×100. LLOQQC: Lower limit of quality control; LQC: Low-quality control; MQC: Middle-quality control; HQC: High-quality control; SD: Standard deviation; QC: Quality control

Table 5: Quantity of thymoquinone in *Nigella sativa* extract (*n*=6)

Samples	Amount (µg/g)
Sri Lanka	2.61±0.49
India (Uttar Pradesh)	3.59±0.64
India (Delhi)	3.48±0.42
Turkey	4.48±0.28
Egypt	4.31±0.26
Ethiopia	3.06±0.56
Saudi Arabia	2.34±0.56
Pakistan	2.54±0.31
Syria	1.49±0.38
Yemen	1.45±0.41
Afghanistan	2.16±0.49
Sudan	1.44±0.21

the intra- and inter-batch precision, i.e., %CV, as well as intra- and inter-batch accuracy for THQ samples was in the ranges of 0.89%–2.54% and 92.90%–98.07%, respectively, as shown in Table 4.

Robustness

System suitability (SS) for %CV as well as THQ recovery was evaluated through changes in different parameters such as mobile phase ratio for ACN: 2 mM ammonium formate (49.8:50.2%, 50:50%, 50.2:49.8%, v/v/v), pH of the mobile phase (5.8, 6.1, and 6.2), and flow rate (0.19, 0.20, and 0.21 mL/min). In the results, a low value for %CV of THQ (0.51%–3.54%) as shown in Table 6a proves the robustness of the method.

Ruggedness

A complete batch of THQ, using different analysts and columns alongwith different sets of solutions, was processed and analyzed further for precision and accuracy in order to determine ruggedness. The mean as observed for %accuracy as well as %correlation between drug variance ($n = 6$), as shown in Table 6b, revealed a range of 95.10%–98.32% and 1.05%–2.77%, respectively.

Limit of detection and limit of quantification

The dilution of spiked BH with standard THQ (signal-to-noise reaches 3 and 10) resulted a value of 50 ng/mL (LOD) and 100.00 ng/mL (limit of quantification).

Stability study

The stability study for primary stock solution (PSS) and SS THQ solutions was performed at 4°C. PSS and SS are identified stable ≤1 month. All QC standards (low, medium, and high) were exhibited with stability ≤8 h for postpreparative stability study.

Forced degradation study

Forced degradation studies were performed for identifying the stability of the developed method. THQ was exposed for four stress conditions (UV light, oxidation, base, and acid). THQ experienced very fast degradation in the basic conditions. The chromatogram of THQ totally disappeared when heated up to 80°C for 15 min in 0.1 M NaOH solution. The major degradation chromatogram was eluted at 1.080 min in the basic conditions [Figure 6b]. Mild degradation was shown for THQ when heated at 80°C under acid conditions. Two additional peaks were observed in the acid-degraded sample at 1.897 and 2.550 min [Figure 6a]. The acid- and base-treated samples showed different retention time; RT values of both acid and base products were different to each other. Degradation products' elution time was less than that of THQ standard; this means degradation products exhibit high polarity than THQ standard.

There are a number of degradation peaks shown in the exposure of UV samples of THQ. Although all the degradation product peaks were well determined from THQ [Figure 6d]. Four degradation product peaks were observed in oxidative stress conditions; all of them resolved and identified from THQ [Figure 6c]. However, the recovery of THQ from oxidative stress and UV was found to be 42.18% and 72.16%, respectively. On the basis of our results, observation revealed that THQ is more sensitive for both the oxidation and photolysis conditions. All the peaks of THQ homogeneity were verified by photo diode array detector (PDA) spectral analysis. The peak purity was higher than the threshold value of 9.66 in all cases. Therefore, by the advantage of this study, the developed method was reported as a stability-indicating method to determine the THQ concentration. Separated degradant peaks of THQ are most important task for future prospect.

DISCUSSION

The established UHPLC method was used for the quantification of THQ in different extracts of *N. sativa* collected from the different geographical regions. THQ (standard) eluted out as a separate peak at 3.147 min. The analysis of THQ was attained on λ_{max} 254 nm which satisfactory results for proposed method. Previously developed analytical methods, i.e., HPLC-UV for the identification and quantification of THQ, have various drawbacks; for example, poor resolution, poor separation, and complex solvent mixtures. These reported methods are also not rapid and selective has complication over gradient elution is very necessary. This research has developed a validated method with linearity range in between 100.00 and 2000.00 ng/mL, which constructs the method most appropriate for trace quantification of THQ. UHPLC-PDA technique provide to improve quality of results, i.e., increased detection limits and also chromatographic resolution with higher sensitivity. The benefits of our developed method over previously developed methods are the short time analysis (6.0 min), high sensitivity 100 ng/mL for THQ, and simple extraction procedure. This research study proposed percentage of THQ in different extracts of *N. sativa,* in which the highest and lowest amounts of THQ were found to be shown in the sample of Turkey and Sudan, respectively [Table 5].

As per the previously reported data on the nutritional value of *N. sativa* seed, it is observed that, besides the high oil content, black seed also contains various sources of bioactive compounds and minerals.

Table 6: Robustness of the method for thymoquinone

(a) Robustness (%)			
Conditions	**LQC (400.0 ng/mL)**	**MQC (800.0 ng/mL)**	**HQC (1600.0 ng/mL)**
Mobile phase (ACN: 2 mM ammonium formate [50:50, v/v])			
Negative level (49.8:50.2, *n*=3)	369.94±13.09 (3.54)	771.64±13.28 (1.72)	1564.11±13.46 (0.86)
Zero level (50:50, *n*=3)	377.28±11.11 (2.94)	779.34±12.79 (1.64)	1576.67±11.48 (0.73)
Positive level (50.2:49.8, *n*=3)	385.37±12.08 (3.13)	779.49±11.38 (1.46)	1582.69±12.14 (0.77)
Flow rate (0.200 ml/min)			
Negative level (0.19, *n*=3)	372.89±10.29 (2.76)	778.17±11.75 (1.51)	1565.11±15.96 (1.02)
Zero level (0.20, *n*=3)	377.59±11.49 (3.04)	788.49±13.78 (1.75)	1579.16±16.76 (1.06)
Positive level (0.21, *n*=3)	382.19±11.16 (2.92)	759.89±13.67 (1.80)	1566.73±15.48 (0.99)
pH of mobile phase (pH=6.0)			
Negative level (5.8, *n*=3)	375.18±11.08 (2.95)	768.46±11.62 (1.51)	1572.45±17.49 (1.11)
Zero level (6.1, *n*=3)	381.67±10.13 (2.65)	779.46±12.49 (1.60)	1580.48±16.07 (1.02)
Positive level (6.3, *n*=3)	385.16±12.16 (3.16)	781.15±3.98 (0.51)	1572.44±14.48 (0.92)
(b) Ruggedness (%)			
QC ID	**Theoretical content (ng/mL)**	**Mean concentration observed (ng/mL)**	**Accuracy[a] (%)**
LOQQC	101.00	96.99±2.69 (2.77)	96.03
LQC	400.00	380.39±11.03 (2.90)	95.10
MQC	800.00	778.29±14.41 (1.85)	97.29
HQC	1600.00	1573.17±16.55 (1.05)	98.32

Values (mean±SD) are derived from six replicates: [a]Accuracy (%) = Mean value of ([mean observed concentration]/[theoretical concentration]) × 100; [b]Precision (%): Coefficient of variance (percentage)=SD divided by mean concentration found×100; Theoretical contents; LOQQC: 101.00 ng/mL, LQC: 400.00 ng/mL; MQC: 800.0 ng/mL; and HQC: 1600.0 ng/mL. LLOQQC: Lower limit of quality control; LQC: Low-quality control; MQC: Middle-quality control; HQC: High-quality control; SD: Standard deviation; ACN: Acetonitrile

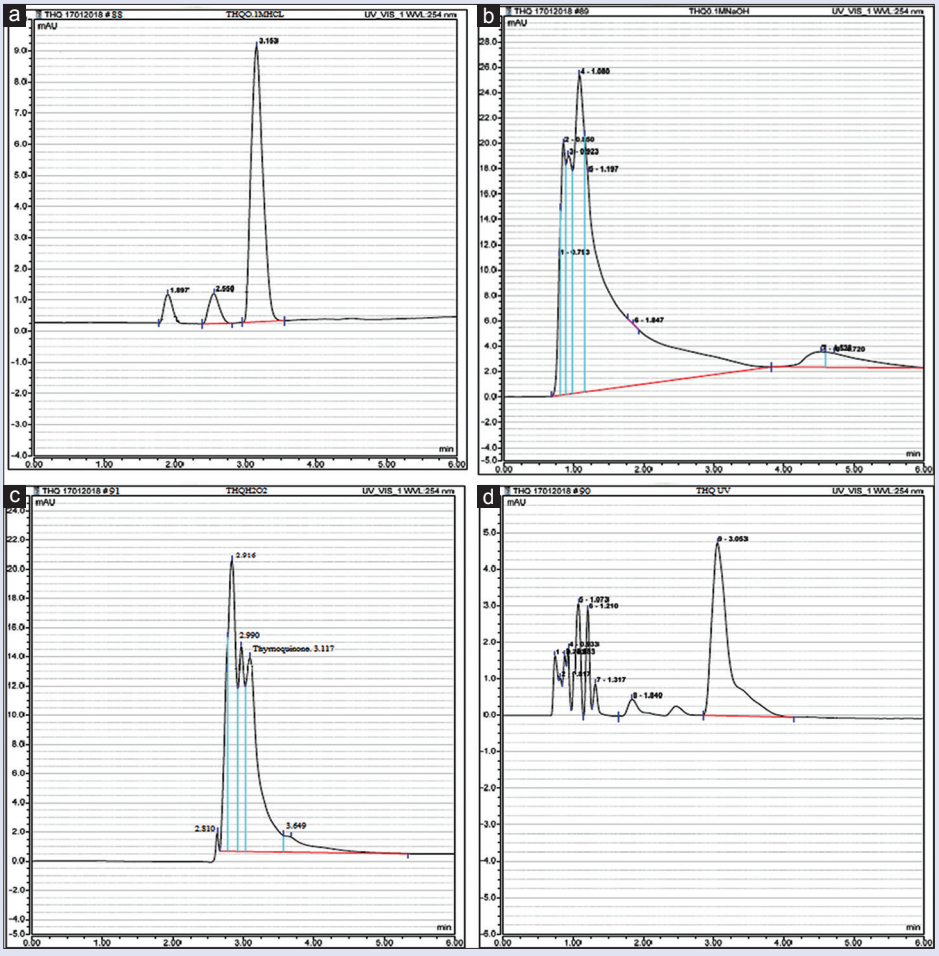

Figure 6: Ultra-high-performance liquid chromatography-chromatograms of acidic- (a), basic- (b), oxidation [H_2O_2]- (c), and ultraviolet-induced (d) forced degradation of thymoquinone

Compounds were recognized through comparison of their retention indices and mass obtained in GC-MS chromatogram [Figures 3b and c] with those of GC-MS library data stored in the computer library and Adams libraries spectra[34-36] and enumerated along with molecular weight, retention time, and peak area. Burits and Bucar *et al's*. method for the extraction of essential oil used light petroleum followed by steam distilling of the extract.[1] They indicated that their essential oil possessed THQ as the main component, while in this study, THQ is the major component of the Soxhlet extraction method.

As per the previously reported data, the high content of THQ was found by the extraction of Soxhlet extraction method in comparison of other previous reported methods such as cold press and supercritical fluid extraction.[34,37] In fact, the cold press gives low yields, and usually the extraction process is time consuming[19] which may also affect the concentration of the target compounds from NSO quantity. THQ is a phytochemical compound of NSO, which is interesting to study as it contributes to the oil's general stability and potential health benefits.[38] It has also been shown to be the reason for the biological antioxidant activity of NSO and also for the majority of the beneficial health effects related to the seeds and oils.[39]

DSC offers evidence of the additional specific heat across a broad temperature range.[40] Any endothermic or exothermic event is noted as a peak in the chart, and its area is in proportion to the enthalpy achieved or lost, respectively. Figure 5 shows the DSC melting curve for NSO extracted by Soxhlet extraction method. The melting curves of these seed oils show more endothermic peaks and shoulders. In general, NSO shows similar DSC melting point and profiles higher than 120°C temperature regions. This result concurs with the published data.[41] The onset temperature and peak value of the black seed oil compared to previously reported literature by Mohammed *et al*., 2016, are also more similar.[34] Thus, it is clear that the thermal stability of the *N. sativa* seed oils is not significantly affected by any type of extraction method, either cold press extraction or supercritical fluid extraction, with no weight loss observed before 150°C, and all the extraction oils have the same functional groups.

CONCLUSIONS

The concentration of THQ variability could be due to the changes in different environmental conditions all over the world. The highest and lowest amounts of THQ were found to be shown in the sample of Turkey and Sudan, respectively [Table 5]. The established method was shown to be rapid, simple, accurate, selective, sensitive, and precise. The stability study is indicated for the selectivity of the method. Developed method reported as a very less time for sample analysis and their samples preparation technique is very simple and reproducible. In the future, our proposed results could be providing an important input for cultivation of black seeds with better management strategies. Turkey region is the most desirable cultivation region for the greater production of black seeds at large scale.

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Conflicts of interest

There are no conflicts of interest.

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