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Different Extraction Methods and Solvents for Thymoquinone from *Nigella Sativa* L. Seeds.

Kah Xin Chung* [1], Patricia Lee Yee Wei* [1] and Gabriel Akyirem Akowuah[1]

Faculty of Pharmaceutical Sciences, UCSI University, Kuala Lumpur 56000, Malaysia
*Corresponding author: kahxinchung@gmail.com; & patricia091200@hotmail.my

Abstract

Nigella sativa L., commonly known as black seed, grows natively in the Mediterranean region and Western Asian countries. *Nigella sativa* L. seeds extracts have diverse medicinal properties. The main active constituent, thymoquinone is medicinally very effective against various health-related issues. The objective of this study was to investigate the effects of different extraction methods and solvents on the content of thymoquinone using HPLC. One gram each of powdered black seed was extracted with 20 mL of hexane, ethyl acetate, methanol, ethanol, and water. The mixture was incubated at 4°C in water bath for 2 hours and 4 hours, respectively. The extraction was repeated for hexane and methanol by maceration, percolation, and ultrasonic assisted extraction (UAE). The extracts were centrifuge at 4000 rpm at 4°C for 10 min and filtered. The extracts were then top-up to 20 mL with the respective solvent and analyzed by HPLC. The results in the present study showed that hexane extract by maceration method is the best method to extract the main bioactive component, thymoquinone from *N. sativa* L. seed.

Keywords: *Nigella Sativa* L., Black Seed, Thymoquinone, Extraction Solvents, Extraction Methods, HPLC

Introduction

Nigella sativa L., commonly known as black seed, is one of the oldest domesticated herbs found in some religious and medical text which grows typically natively in the Mediterranean region and Western Asian countries. *N. sativa* L. is the species most investigated for therapeutic purposes among its Ranunculaceae family of flowering plants as it possesses a wide range of medicinal purposes such as antihypertensive, anticancer and immunomodulatory (1). Many bioactive components have been identified in the black seeds. Unsaturated fatty acids, such as arachidic and eicosadienoic, make up most of the fixed oil (1).

The most important active components isolated from the fixed oil are thymoquinone, thymohydroquinone, dithymoquinone, p-cymene, carvacol, 4-terpienol, t-anethol, sesquiterpene longifolene, α-pinene and thymol etc (2). Thymoquinone (TMQ) (Figure:1) has been identified as the most prominent quinine constituent, contributing 54% of the *N. sativa* L. seed oil. TMQ was found pharmacologically beneficial to health such as anti-inflammatory, antioxidant and anti-neoplastic effects both in vitro and in vivo. TMQ also has gastroprotective, hepatoprotective, nephroprotective and neuroprotective effects (3). TMQ is also one of the bioactive natural compounds traditionally used for the treatments of diabetes and hyperlipidaemia as it is proven to reduce blood sugar level. The main mechanism of action is affecting the cellular uptake of hypolipidemic and anti-diabetic properties through activation of adenosine monophosphate kinase in rats and rabbits (4,5).

Several instrumental methods such as colourimetric (6), voltametric (7), flow injection (8), gas and liquid chromatography (9–11), and infrared spectroscopic methods (12,13), have been reported to determine free fatty acid in *N. sativa* L. The ATR-FTIR analysis of free fatty

acid content in *N. sativa* L. seed oil and commercially available oils in the market using spectrophotometry has been reported (14). TMQ content of the seed extracts may vary depending on different extraction methods and solvents. This study aims to investigate the effect of different extraction methods and solvents on the content of TMQ in *N. sativa* L. seeds.

One of the reported methods to analyze TMQ with different solvent extraction quantitatively is using HPLC. A simple and linear HPLC method is developed to determine the amount of TMQ extracted with different extraction solvents which are benzene, methanol, ethanol, hexane and water. Absorbance spectra analysis of TMQ standard is scanned and used to compare with the absorbance spectra of TMQ extracts with different solvents to confirm the presence of TQ in extract using UV-Visible spectrophotometer. The maximum absorbance is reported to be 254 nm. Hence, the HPLC method developed is also using 254 nm as wavelength detection. The chromatogram of each extraction solvent is then used to analyze TMQ quantitatively and the percentage composition of TMQ is reported and used to compare the solvent extraction. Extraction solvents with higher percentage composition of TMQ indicate that it is a better choice of extraction solvent. The HPLC method developed is then validated from linearity, precision, accuracy, selectivity, and robustness. This will ensure that the method is trustable and reproducible (15).

Another method reported using HPLC to analyze TMQ quantitatively to determine the best organic solvent and extraction method to extract TMQ from blackseed. This study takes into account of the extraction method and extraction solvent of TMQ from blackseed. The extraction technique used in this study are maceration, reflux, Soxhlet and ultrasound assisted extraction (UAE). Each extraction technique uses three different extraction solvents which are methanol, petroleum ether and hexane. HPLC is used for quantitative analysis of extracted TMQ. A calibration curve is generated using five dilutions of standard TMQ to obtain the regression equation and correlation. The regression equation is then used to quantify the amount of TMQ extracted by each extraction method with different extraction solvents respectively. The HPLC method developed is not validated from precision and accuracy. Thus, method validation should be carried out to ensure the quality and reliability of the result shown. However, it cannot be doubted that the extraction method plays a significant role and has a direct link to amount of TMQ extracted and time as well as the cost used (16).

Materials and Methods

Materials and Chemical Reagents

Black seeds were purchased from a local commercial store. Hexane, methanol, ethanol, ethyl acetate, thymoquinone, acetonitrile (HPLC grade) was purchased from Sigma-Aldrich. All other chemicals and solvents were of analytical or HPLC grade.

Solvent Extraction

5 solvents (hexane, methanol, ethanol, ethyl acetate and water) were selected to be the solvent extraction for extracting TMQ from blackseed. One gram of black seed powder was weighed in a conical flask. 20 mL of each solvent extraction were added to the different conical flasks respectively. The mixtures were incubated at 40°C in water bath for 2 h and 4 h, respectively. The extracts were centrifuged at 4000 rpm for 10 mins at 4°C and filtered. The filtrate was made and top-up to 20 mL with the respective solvent and analyzed by HPLC.

Extraction Methods

Conventional techniques

Maceration

Extract one gram of black seed with 20 mL of solvent (hexane and methanol in two different conical flasks) was macerated at room temperature for 4 h. Then, the extract solution was centrifuged at 4000 rpm at 4°C for 10 min and filtered.

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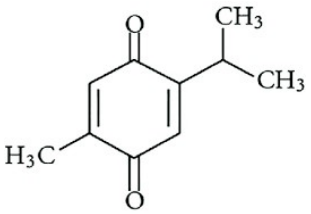

Figure 1 Chemical structure of thymoquinone (TMQ)

Percolation

Extract one gram of black seed with 20 mL of solvent (hexane and methanol in two different conical flask) was heated in a 40°C water bath for 4 h. Then, the extract solution was centrifuged at 4000 rpm at 4°C for 10 min and filtered.

Non-conventional techniques

UAE

One gram of black seed with 20 mL of solvent (hexane and methanol in two different conical flasks) was ultrasonicated for 1 h. Then, the extract solution was centrifuged at 4000 rpm at 4°C for 10 min and filtered.

HPLC Analysis Method Development and Validation

The HPLC analysis was performed using an isocratic elution with C-18 reversed-phase column. The flow rate was 1 mL/min and the injected volume was 20 µL. The thymoquinone peak was detected at 254 nm and quantified by external standard method. The HPLC method was validated based on the ICH guidelines (17). The validation parameters were linearity, sensitivity, precision, and accuracy. All standard and extract solutions were filtered through 0.45 mm membrane filters before being injected (10 µL) into the HPLC system.

Statistical Analysis

Statistical analysis was performed with SPSS software (version 23) using One-way ANOVA test to determine the significant

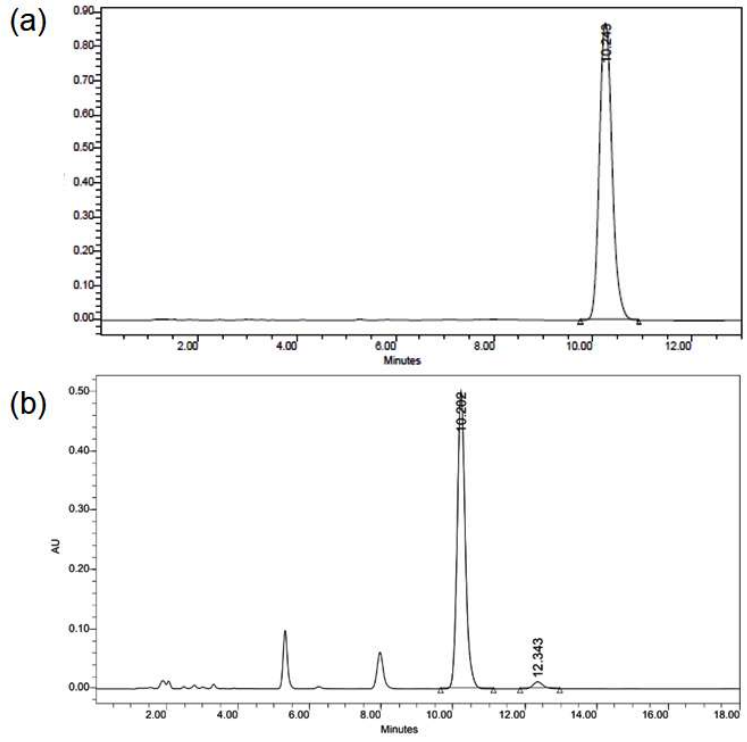

Figure: 2. HPLC chromatogram of (a) thymoquinone standard (b) methanol extract

difference. The level of significant were set at $P < 0.05$.

Results and Discussion

Method Development

HPLC is widely used in pharmaceutical analysis since it has high efficiency, high sensitivity, and high resolution. Most of the HPLC separations are carried out in a reversed phase in which the solutes form hydrophobic interactions with the stationary phase to retain the solutes. Thus, the polar solutes will elute first, followed by the lower polarity (18).

The HPLC method developed was based on the optimum mobile phase of acetonitrile and water in the ratio of (50:50 v/v) and detection at 254 nm. The detection wavelength of 254 nm was selected based on the UV scan of TMQ standard solution and literature review reports of HPLC analysis of TMQ in black seed (19,20). HPLC separates the molecules based on the analyte's solubility hence different mobile phase compositions of acetonitrile and water were studied (21). The optimum mobile phase composition for better resolution of separation of thymoquinone in the extract was acetonitrile: water, 50:50 v/v. Figure 2 shows the typical chromatogram of authentic standard of TMQ and N. sativa extract. The HPLC method developed was validated for linearity, precision, and accuracy to ensure reproducibility and suitability of the method.

Linearity and Sensitivity

Stock solution of 1 mg/mL of TMQ standard was prepared. Serial dilutions were prepared in the concentration range of 0.02 - 0.40 mg/mL of the 1 mg/mL stock solution of TMQ. A linear calibration curve was established by plotting the peak area against concentration. Figure 3 shows the calibration curve generated from a linear range of 0.02 mg/mL to 0.40 mg/mL. The r^2 value obtained was 0.9992, indicating a good relationship between the concentration of TQ standard and the peak area. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the area and the slope obtained from the calibration curve of TQ standards (12). The values of LOD and LOQ are 0.015 mg/mL and 0.04 mg/mL, respectively.

Precision

The system suitability of the method was evaluated by intra-day and inter-day precision in triplicates. The standard solutions were analysed by HPLC for 4 times/day and 1

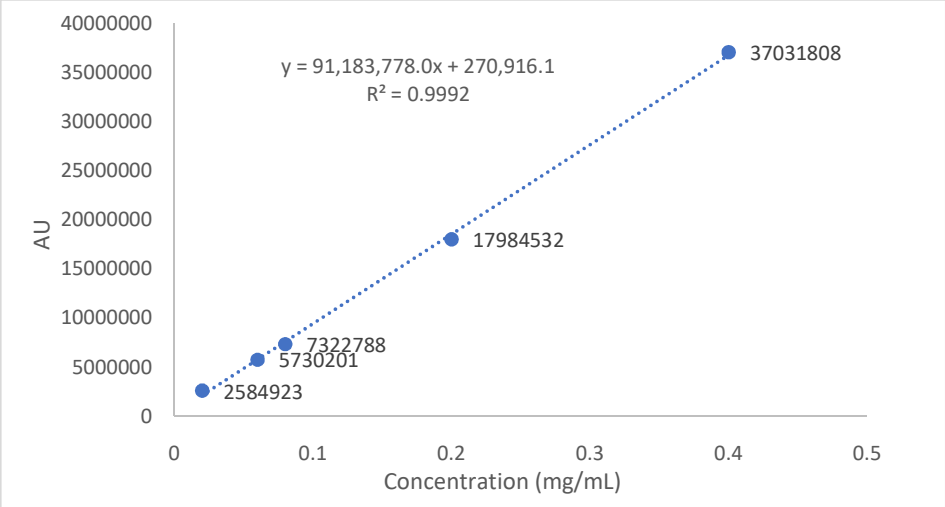

Figure 3 Calibration curve for the HPLC method

Different Extraction Methods and Solvents

time/day for four consecutive days. The resulting peak areas were used to calculate standard deviation and the relative standard deviation. The precision of the HPLC method developed was reported in % RSD at 0.08 mg/mL and 0.40 mg/mL to be 2.7 % and 3.4% for intra-day and 7.9 % and 8.9% for inter-day respectively. Acceptable precision was revealed by relative standard deviation data. The relative standard deviation of TMQ was less than 3.5% for intraday precision (Table 1) and less than 9.0% for interday precision (Table 1)

Accuracy

The accuracy of the HPLC method was determined based on the percentage recovery of the spiked standard TQ into the analyte solution. 0.12 mg/mL of TMQ standard was spiked into the sample solution. The percentage recovery of 92.0% was obtained. (Table 2) Hence the percentage recovery of the HPLC method falls within the acceptable range (17,22,23).

Quantification of TMQ in different solvent extracts

The TMQ content in the extracts obtained was quantified based on the peak area using linear regression data (Table 1). The concentration of TMQ was expressed in gram per dry weight of black seed. The results obtained for the quantification of TMQ at 2 h and 4 h of extraction are shown in (Table 3). The highest amount quantified of TMQ (1.501 mg/g) from blackseed was obtained with the extraction of hexane for 4 h as compared to the other solvents. This is ascribed to the high solubility and intensity of interaction of TMQ in matrix with hexane. TMQ is a non-polar monoterpenoid ketone and therefore, more soluble in a non-polar solvent such as hexane. It is apparent from the results of the present study that TMQ content of the blackseed extracts varies with respect to the solvent used for the extraction. Though high temperatures may decrease viscosity and surface tension to allow good penetration of the solvent into the sample matrix, the extraction temperature of 40°C was selected for this study. This is due to the non-polar nature of TMQ to avoid its decomposition at high temperatures. The effects of other parameters such as extraction time and extraction methods were also studied in this research. It was observed that hexane at extraction at 4 h has higher TMQ content as compared to extraction time of 2 h.

Quantification of TMQ in extracts of different extraction methods

The effects of different extraction methods on the TMQ content of the *N. sativa* were investigated by using the hexane and method for non-polar and polar extraction solvents. Methanol was selected as extraction solvent for the polar solvents because it showed the highest content of TMQ at 4 h of extraction as compared to ethanol and water. Based on the results obtained (Table 4), the maceration method with hexane yielded the highest amount of TMQ (1.530 ±0.002 mg/g) however, the content was not significantly different ($P > 0.05$) by percolation using hexane (1.501 ± 0.002 mg/g). Percolation and maceration are efficient extractions methods for bioactive compounds from plants. Percolation is a continuous process which is suitable for both non-polar and polar compounds at controlled temperature. Maceration is an appropriate method for labile bioactive plants constituents.

The content of TMQ in hexane by maceration and percolation methods were significantly different ($p < 0.05$) ultrasonic extraction method. This may be due to the rapture of cell structure of the black seed which eventually exposes the TMQ to react with the solvent. [19]. Ultrasonic-assisted extraction (UAE) is cost effective method of extraction sin it requires less time, energy, and flexibility of using low temperature for extraction. The high intensity sound of UAE enables the extraction of bioactive constituents from plant materials. Acoustic cavitation generated by UAE can rupture plant tissues leading to large contact area for efficient interaction between solvent and the plant tissue for better extraction. However, the acoustic energy generates heat

Table 1: Intra-day and Inter-day Precision results

Intra-day Precision (n=4)				
Concentration (mg/mL)	RT Mean ± SD (min)	Intra-day RSD (%)	Mean ± SD (Peak area)	Intra-day RSD (%)
0.08	10.09 ± 0.05	0.4	6833670.0 ± 187524.2	2.7
0.40	10.12 ± 0.01	0.4	33703380.5 ± 112125.5	3.3
Inter-day Precision (n=4)				
Concentration (mg/mL)	RT Mean ± SD (min)	Inter-day RSD (%)	Mean ± SD (Peak area)	Inter-day RSD (%)
0.08	10.6 ± 0.5	4.3	2431835.0 ± 182921.9	7.7
0.40	10.6 ± 0.4	4.1	7778039.7 ± 662861.6	8.9

Table 2: Recovery Results of HPLC method

Thymoquinone		Recovery (n=3)		
Added Concentration (mg/mL)	Peak Area	Mean ± SD	RSD (%)	Recovery (%)
0.12	15611560	14932433 ± 6528234	4.4	92.0

Table 3: The content of TMQ in Different solvent extracts at 2 h and 4 h

Solvent	Extraction time	
	Amount quantified in 2 h (mg/g)	Amount quantified in 4 h (mg/g)
Hexane	1.155 ± 0.002 [a]	1.501 ± 0.003 [b]
Methanol	1.120 ± 0.002 [a]	1.347 ± 0.004 [b]
Ethanol	1.314± 0.006 [a]	1.220 ±0.010 [a]
Water	0.009± 0.003 [a]	1.091 ± 0.004 [b]
Ethyl Acetate	1.352 ±0.003 [a]	1.357 ± 0.002 [a]

[a] Different alphabet in the same row indicates significant difference (P < 0.05), n=3

Table 4: Quantification of Thymoquinone in blackseed based on different extraction methods

Method	Extraction solvent	
	Amount Quantified in hexane (mg/g)	Amount Quantified in methanol (mg/g)
Percolation	1.501 ± 0.002 [a]	1.347 ± 0.003 [a]
UAE	1.208 ± 0.002 [a]	0.770 ± 0.001 [b]
Maceration	1.530 ±0.002 [a]	1.270 ± 0.007 [a]

[a]Different alphabet in a column indicates significant difference (P < 0.05), n=3

which may destroy low molecular weight secondary metabolites from plant if the optimum extraction time is not determine prior to UAE (20-22).

Conclusion

In the present study, the effect of extraction methods and solvents on thymoquinone content from blackseed were successfully investigated by HPLC. Based on the investigation, hexane extracted the most TMQ from blackseed based on the quantified amount by using HPLC. Among the three different extraction methods, maceration was found to be the best method for extracting thymoquinone in *N. sativa* L. seed based on HPLC assay. The HPLC quantification results obtained by percolation and ultrasonic extractions were not significantly different from maceration method. Thus, the optimum choice of extraction method and solvent is important in obtaining the highest yield of TMQ from black seed.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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