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# Different extraction methods for Thymoquinone from *Nigella sativa* L. seeds and antioxidant activity

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The study aimed to investigate the effects of different extraction methods on the content of thymoquinone using UV-Vis spectroscopy and the antioxidant activities of thymoquinone by measuring the free radical scavenging activity. One gram of powdered *Nigella sativa* L. black seed was added to 20 mL of solvents (hexane and methanol) into a conical flask and mixed. The mixtures were extracted by different methods which were maceration, percolation, and ultrasonic-assisted extraction. In the percolation method, the mixtures were placed in a water bath of 40°C for 4 h whereas, for maceration, the mixtures were left at room temperature for 4 h. On the other hand, the mixtures were replaced in an ultrasonic bath at room temperature for 2 h in ultrasonic-assisted extraction. The extracts were centrifuged at 4000 rpm at 4°C for 10 min and filtered. The extracts were then analyzed qualitatively and quantitatively by a validated method (UV-Vis spectroscopy). On the other hand, the antioxidant activity was measured by DPPH radical scavenging. In UV analysis, the percolation method with methanol extracted the most content (0.9102%) of thymoquinone. The free radical scavenging activity was observed highest (95.6891%) in the methanol extracts by percolation method. The results in the present study show that methanol extract by percolation method is the best method to extract the main bioactive component, thymoquinone from *N. sativa* seed. Furthermore, the *N. sativa* seed extracts exhibit antioxidant activity.

**Keywords:** Black seed, DPPH Assay, Extraction methods, Free radical scavenging activity, Thymoquinone, UV-Vis Spectroscopy

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## Introduction

*Nigella sativa* L, commonly known as black seed, is one of the oldest domesticated herbs found in some religious and medical texts which grow typically natively in the Mediterranean region and West Asian countries[1]. *N. sativa* is the species most investigated for therapeutic purposes in the Ranunculaceae family of flowering plants[2] as it possesses a wide range of medicinal properties such as antihypertensive, anticancer, and immunomodulatory[3].

*N. sativa* L. seeds are widely used in traditional medicine practices such as Unani, Ayurveda, Arabian, Indian and Chinese due to its wide range of advantages to health. Based on the Prophet Mohammed, it narrated that the *N. sativa* L. seeds can be used to treat every ailment except death[2]. In addition, it was mentioned in "The Canon of Medicine" by Ibn Sina, that *N. sativa* L. seeds can assist the body's recovery from fatigue and energy boosting[4]. *N. sativa* L. was described as a valuable herbal remedy in 5th century B.C. of Hippocrates. The seed was used to treat hepatic, digestive disorders, skin rashes, snakebites, scorpion stings, old tumours, and abscesses. It was also used to treat diseases of women and children such as oral thrush and skin conditions[5]. Apart from being used as a herbal remedy in traditional medicine, *N. sativa* L. seeds also used as a spice in curries as *N. sativa* L. seeds give a pungent, bitter taste yet aromatic smell[4].

Thymoquinone (TQ) has been identified as the most prominent quinine constituent, contributing 54% of the *N. sativa* seed oil. TQ was found pharmacologically beneficial to health such as anti-inflammatory[6], antioxidant[7], and anti-neoplastic effects[8] both *in vitro* and *in vivo*[9]. TQ also has gastroprotective, hepatoprotective, nephroprotective, and neuroprotective effects[9].

Several instrumental methods such as colourimetric[10], voltametric[11], flow injection[12], gas and liquid chromatography[13-15], and infrared spectroscopic methods[16-17], have been reported to

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determine free fatty acid (FFA) in *N. sativa*. The attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) analysis of FFA content in *N. sativa* L. seed oil and commercially available oils in the market using spectrophotometry has been reported[18].

Extraction methods of *N. sativa* L. seed oils play a significant role in the pharmacological properties as well as the yield of the extracts. A recent study was carried out to compare effects of different extraction methods on the extracted proportion, antioxidant properties and thermal behaviour of the *N. sativa* L. oil. The seed oil was extracted with supercritical fluid extraction (SFE) and cold press (CP)[19]. The finding reveals that the concentration of TQ presented in oil extracted by SFE method (6.63 mg/mL) is higher than the oil extracted by CP method (1.56 mg/mL)[19]. Other extraction techniques on *N. sativa* L. reported are such as microwave-assisted extraction[20,21] and hydrodistallation[20] have been employed successfully to isolate the major constituents in the *N. sativa* L. seeds.

DPPH radical scavenging test is widely used to evaluate the antioxidant activity of *N. sativa* L[19, 22-23]. A recent study revealed that *N. sativa* L. possess antioxidant property and was able to reduce the DPPH solution to yellow-coloured diphenyl picrylhydrazine[22]. Several studies have reported that the extraction solvents[23] and extraction methods may affect the antioxidant properties using the DPPH assay[19].

Yet, the study of effects of different extraction methods using conventional methods on the TQ content by different studies are limited. Thus, there's a need for alternative extraction methods that are suitable to employ to extract TQ content in *N. sativa* L. easily and effectively. This present study aimed to investigate the effect of different extraction methods on the content of TQ and antioxidant properties in *N. sativa* L. seeds.

## Materials and Methods

### Materials and chemical reagents

*N. sativa* L. seeds were purchased from Sri Murugan Udhayam Sdh. Bhd., Malaysia; Batch no. SMT 0121. HPLC/ACS grade Methanol ($CH_3OH$); Anhui Fulltime, China, Hexane ($CH_3(CH_2)_4CH_3$); Anhui Fulltime, China, 2,22,2-Diphenyl-1-picrylhydrazyl (DPPH); Thermo Fisher Scientific, USA, Thymoquinone, Sigma-Aldrich, China.

### 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.

#### *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.

### Free radical scavenging activity

The antioxidant activity of the *N. sativa* seed was analyzed by studying the scavenging activity of the stable free radical DPPH. DPPH solution was prepared by dissolving 9.75 µg of DPPH in a 250 mL volumetric flask with methanol. 0.20 mL of sample diluted with 0.80 mL methanol was then mixed with 2 mL of DPPH solution. The absorbance of the mixtures was measured at the wavelength of 517 nm. The percentage of DPPH radical scavenging was determined with respective solvents as blank. Ascorbic acid was used as a positive control in this test. The percentage of free radical scavenging activity is calculated by using the following formula[24]:

$$\% \text{ DPPH} = \frac{(\text{Absorbance control} - \text{Absorbance sample})}{(\text{Absorbance control})} \times 100\%$$

### Statistical analysis

A one-way ANOVA test has been employed to determine the significant difference between the amount quantified by UV-Vis spectroscopy based on the absorbance. In addition, a one-way ANOVA test was carried out to determine the significant difference between the DPPH radical scavenging activity and different extraction methods.

## Results and Discussion

UV-Vis spectroscopy is a non-invasive method widely used to study various chemical compounds. In

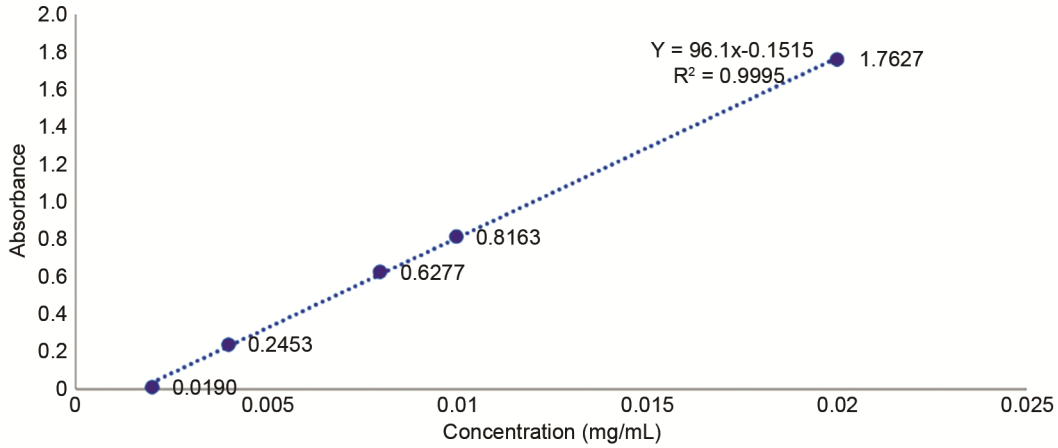


Fig. 1 — Calibration curve for the UV-Vis spectroscopic method.

UV-Vis spectroscopy, the chemical components absorb light in the visible region. This results in the excitation of the electrons to a higher energy level (from grounded state to excited state). However, the excitation state of the electrons is not stable, which eventually undergoes de-excitation (from the excited state to the grounded state). The absorption of radiation depends on the intensity of light, concentration, and thickness of the compounds which follows the Beer-Lambert Law[25-26].

**Method development**

The standard solution of TQ was scanned between 200 to 400 nm in the spectroscopy to determine the proper wavelength. Based on the UV spectrum obtained, a peak was observed at the wavelength of 243.08 nm. However, studies have reported that TQ gave a prominent peak at a wavelength around 254 – 257 nm[22-23]. Thus, the extracts were scanned at 254 nm.

**Method validation**

The calibration curve (Fig. 1) was generated with five working standards from 0.002 to 0.020 mg/mL by plotting the absorbance of the TQ standard against their concentration, respectively. The precision (Table 1) of the UV-Vis spectroscopy was determined by intra-day and inter-day. Inter-day precision was measured in four replicates in one day, while inter-day precision was measured in one replicate for four consecutive days. The RSD% values of intraday at TQ concentrations (0.008 and 0.020 mg/mL) were low. In contrast, the RSD% values for inter-day at TQ concentration (0.008 and 0.020 mg/mL) were relatively high. Higher values of RSD% reflected the low precision of the UV-Vis spectroscopy. The acceptable RSD% value for impurities in analytical chemistry is up to 10%[27–29]. The RSD% of precision in the UV method is lower than 10%. Thus, the method is said to be precisive.

Table 1 — Method validation of UV-Vis spectroscopic method

| Parameters | |
|---|---|
| Calibration range | 0.002–0.020 mg/mL |
| Detection wavelength | 254 nm |
| Regression equation | Y = 96.1003X + 0.1515 |
| Linearity ($R^2$) | 0.9995 |
| LOD | 0.0005 |
| LOQ | 0.0015 |
| Precision (n=5) | |
| Intraday (%RSD) | |
| 0.008 mg/mL | 0.2720 |
| 0.020 mg/mL | 0.0264 |
| Interday (%RSD) | |
| 0.008 mg/mL | 4.3456 |
| 0.020 mg/mL | 7.8734 |
| Recovery (%) | |
| 0.020 mg/mL | 99.07 |

The accuracy of the HPLC method (Table 1) was determined based on the percentage recovery of the spiked standard TQ into the analyte solution. Exactly 0.020 mg/mL of TQ standard was spiked into the sample solution. A percentage recovery of 99.0718% was obtained. The acceptable criterion of mean recovery is 80 to 120%. Hence, the percentage recovery of the UV-Vis spectroscopy falls within the acceptable range.

**Quantification of TQ in the *N. sativa* seed extract based on UV-Vis spectrometer**

Based on the results obtained (Table 2), the methanol extract by the percolation method showed the highest percentage yield of TQ content (0.9102%)

Table 2 — Quantification of TQ in *N. sativa* L. based on different extraction methods

| Extraction methods | Solvents | Percentage yield (%) |
|---|---|---|
| Percolation | Hexane | 0.7244[a] |
| | Methanol | 0.9102[b] |
| UAE | Hexane | 0.4332[c,e] |
| | Methanol | 0.4771[d] |
| Maceration | Hexane | 0.4278[c,e] |
| | Methanol | 0.5010[f] |

[a-f]Different alphabet in row (e.g. 0.7224[a]; 0.9102[b]) indicates a significant difference ($P$ <0.05).

Table 3 — Free radical-scavenging activity of thymoquinone in standard solution, DPPH solution and ascorbic acid as controls

| Solvent | Mean absorbance | SD | Free radical-scavenging activity (%) |
|---|---|---|---|
| DPPH | 0.7029 | 0.0011 | 0.0000 |
| Thymoquinone | 0.4851 | 0.0020 | 30.9779 |
| Ascorbic acid | 0.0184 | 0.0006 | 97.3869 |

compared to the other two methods. However, the UV-Vis spectroscopy method is not highly specific to the isolation of TQ content. At the detection wavelength of 254 nm which was implemented in this method, other chemical components such as thymol, thymohydroquinone, dithymoquinone, and other impurities might contribute to the increment of the absorbance.

**Free radical scavenging activity**

Free radicals are relatively harmful elements, especially to the biological components of the human body[30]. It has been linked to many health-related issues and degenerative diseases such as cancer. The antioxidant property of the black seed has been studied extensively as it plays a vital role in health promotion[30]. The DPPH method is a rapid and convenient method to evaluate the antioxidant activity of chemical compounds. The scavenging activity of the extracts was evaluated by the ability of the black seed to act as an electron donor and was measured spectrophotometrically[22,30].

In this study, the absorbance of the solutions was measured at the wavelength of 517 nm and the free radical scavenging activity (FRSA) was determined with the respective solvents as blank (Table 3). Ascorbic acid was used as a positive control in this test.

Based on Fig. 2, the FRSA is affected by the extraction methods and solvents. The methanol extract by the percolation method shows the highest percentage of FRSA (95.6891%) among other extracts. When the same method was employed such as percolation, the methanol extract had higher FRSA than the hexane extract. Recent studies reported that polar extraction solvents would result in higher FRSA[31-32]. The DPPH radical scavenges radicals by four different mechanisms: Transfer of hydrogen atom, proton-coupled electron-transfer, sequential proton-loss electron transfer, and electron-transfer proton-loss with solvent. In alcoholic solvents such as methanol in the present study, hydrogen bonds are formed between the bioactive constituents in the extracts and DPPH[33]. On the other hand, there is a significant difference ($P$ <0.05) between the FRSA values (Table 4) when the same extraction solvent, but a different extraction method was employed. For instance, the hexane extract by percolation method was relatively higher (30.1290%) compared to the other two extraction methods (Maceration: 15.2566% > Percolation: 7.9342%).

Table 4 — Free radical-scavenging activity of Thymoquinone in black seed based on different extraction methods

| Extraction methods | Solvent | Mean absorbance | SD | Free radical-scavenging activity (%) |
|---|---|---|---|---|
| Percolation | Hexane | 0.4911 | 0.0007 | 30.1290[a] |
| | Methanol | 0.0303 | 0.0011 | 95.6891[b] |
| UAE | Hexane | 0.0614 | 0.0005 | 7.9342[c] |
| | Methanol | 0.6471 | 0.0007 | 91.2596[d] |
| Maceration | Hexane | 0.0936 | 0.0004 | 15.2566[e] |
| | Methanol | 0.5956 | 0.0011 | 86.6879[f] |

[a-f] Different alphabet in row (e.g.30.1290[a]; 95.6891[b]) indicates significant difference ($P$ <0.05).

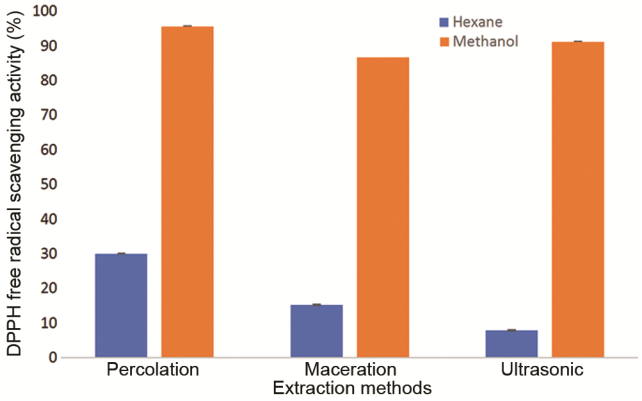


Fig. 2 — Free radical scavenging activity of extracts by different extraction methods.

## Conclusion

In the present study, the investigation of the effect of different extraction methods on thymoquinone content was successfully employed by using UV-Vis

spectroscopy. Among three different extraction methods, percolation was found to be the best method for extracting thymoquinone (0.9102%) in *N. sativa* seed based on the UV-Vis spectroscopic method. The thymoquinone content in *N. sativa* determined by UV-Vis spectroscopy was significantly different for the maceration, percolation, and ultrasonic extraction methods. The free radical scavenging activity was observed highest (95.6891%) in methanol extracts by percolation method.

The quantitative analysis of thymoquinone content in the extracts using the UV-Vis spectroscopic method was not satisfactory as seen in the results. Hence, different types of analysis methods such as High-Performance Liquid Chromatography (HPLC), Liquid Chromatography – Mass Spectroscopy (LC-MS) analysis, can be employed. Besides, the quantitative analysis using FTIR can be performed to determine the effect of different extraction methods on thymoquinone content in the extracts.

## Conflict of interest

The authors declare that there are no conflicts of interest.

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