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Effect of different extraction solvents on the content of Thymoquinone content of *Nigella sativa* L. seeds using UV-Visible spectroscopy and evaluation of the free radical scavenging activity

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To develop and validate HPLC and UV-visible spectroscopy methods for the determination of Thymoquinone (TMQ) content in different solvent extracts of *N. sativa* L. seed. The evaluation of the free radical scavenging effect of different solvent extracts of TMQ was investigated. Extraction of solvent was done by adding 20 mL of the solvent (hexane, methanol, ethanol, ethyl acetate, and water) to 1 g of blackseed and in a conical flask, respectively. The mixtures were then incubated in a water bath at 40°C for 2 and 4 h, respectively. The mixtures were then centrifuged at 4000 rpm for 10 min at 4°C. The extracts were then quantitatively analyzed using HPLC and UV-visible spectrophotometry. The free radical scavenging activity of TMQ extracts was studied using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The results obtained from HPLC indicate that TMQ extracted using hexane at 4 h (0.075 mg/mL, P <0.05) was significantly higher compared to other extraction solvents used. The results obtained from UV-vis spectroscopy analysis indicate that the methanol extract at 4 h (0.00957 mg/mL, P <0.05) was significantly higher compared to other extraction solvents used. Methanol extracts at 2 h showed the highest free radical scavenging activity (95.8%) compared to other extraction solvents. The results obtained show that hexane extracted the most TMQ at 4 h. Methanol extract demonstrated the highest free radical scavenging activity.

Keywords: DPPH, Extraction solvent, HPLC, Thymoquinone, UV-vis spectroscopy

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Introduction

Medicinal plants have been used since ancient times to treat or prevent disease. The World Health Organization (WHO) states that about 80% of people benefit from a herbal or medicinal plant. Hence, there is a vast number of studies about medicinal plants to scientifically prove the therapeutic properties as well as the safety and toxicity of the medicinal plant. Among the medicinal plant, *Nigella sativa* L. stands out due to its wide range of medicinal properties and rich cultural heritage. *N. sativa* which is more commonly known as blackseed belongs to the family of Ranunculaceae. The flower has about 5 to 10 petals in white or light blue whereas the fruit is swollen capsule with blackseed in it. It has an aromatic smell and tastes bitter[1]. Since ancient civilizations *Nigella sativa* has been used as a traditional medicine to treat many diseases such as fever and cough in the Middle East and Southeast Asia. Black seed is also said to be black cumin in the Holy Bible due to its healing power against a variety of disease. It can be used to treat swollen joints and abscesses by applying it externally to affected areas. This indicates that blackseed has high therapeutic value due to the constituent present in the plant. There are extensive studies about blackseed across five decades and it's been found that a combination of fatty acid, volatile oil and trace element contribute to the therapeutic value of black seed[2]. There are various bioactive compounds found in the blackseed such as thymoquinone (TMQ), thymol, carvacrol, 4-terpineol and a lot more. TMQ is the main bioactive compound obtained from essential oil which is a key factor for the high therapeutic value of blackseed. Among all the studies carried out, the most promising effect of TMQ are its antioxidant, anti-inflammatory, antitumor and antibacterial activities[3,4]. The IUPAC name of TMQ is 2-isopropyl-5methyl-1,4-benzoquinone. It belongs to the class of 1,4-benzoquinones. The molecular formula of TMQ is C10H12O2 and the molecular weight of TQ is 164.204 g/mol. The structure of TMQ consists of 2 double bonds within the ring, two carbonyl groups at position 1 and 4 as

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well as methyl and isopropyl group substituted at position 2 and 5. TMQ is a solid bright yellow compound with a melting point of 49 – 50°C. It is also a light-sensitive compound and is unstable in alkaline PH solution. The pharmacological actions such as antimicrobial, anti-inflammatory and analgesic activity of TMQ is exhibited in the configuration of keto-form[4].

Thymoquinone (TMQ) is one of the most significant essential oils identified in blackseed. TMQ is said to have antioxidant activity due to supporting various antioxidant enzymes. This is because TMQ is the neutralizer of free radicals or free oxygen species such as peroxidase which in turn increases the effect of glutathione peroxidase which will reduce hydrogen peroxidase to water, glutathione reductase. This will synthesize reduced glutathione which acts as a scavenger for oxidant species and glutathione S-transferase which will detoxify oxidative stress which in turn protects against oxidant toxicity. Other than supporting various antioxidant enzymes, TMQ is also said to have anti-inflammatory activities by playing a vital role in the arachidonic acid pathway cascade by inhibiting cyclooxygenase (COX) and 5-lipoxygenase[5]. COX is further classified according to the isoform into COX-1 and COX-2 and the distribution of COX-1 and COX-2 differ where COX-1 will be highly distributed in the stomach, kidney and platelets whereas COX-2 will be highly distributed throughout the body. COX is responsible for converting arachidonic acid to prostaglandins (PGs) which is a lipid modulator that will induce an inflammatory response. TMQ will inhibit COX enzyme which reduces the production of PGs and hence reduce inflammation[6]. 5-lipoxygenase on the other hand is involved in the biosynthesis of leukotrienes which is an inflammatory mediator. Leukotrienes will increase the recruitment and retention of inflammatory cells at the targeted site by decreasing vascular permeability. Inhibition of 5-lipoxygenase by TMQ will reduce the production of leukotrienes thus bringing an anti-inflammatory effect[7]. Thus, this study is to determine the effect of different extraction solvents on the TMQ content of blackseed and to evaluate the free radicle scavenging effect of different solvent extracts.

Rationale of study

The main active constituents of *N. sativa* seeds extracts are TMQ. The varying polarity of extraction solvents might change the content of TMQ in different solvent extracts, thus affecting the radical scavenging activity of the extract.

Materials and Methods

Chemical preparation

The stock TMQ solution (5 mg/mL) was prepared by weighing 50.0 mg of TMQ and dissolving it in 10 mL of methanol. The stock TMQ was wrapped with aluminium foil and stored in the freezer prior to use. The DPPH solution (0.1 mM) was prepared by weighing 9.75 mg of DPPH and dissolving it in 250 mL of methanol. The DPPH solution prepared was wrapped with aluminium foil prior to use. The ascorbic acid solution (5 mg/mL) was prepared by weighing 50.0 mg of ascorbic acid and dissolving it in 10 mL of methanol.

Solvent extraction

Black cumin seed (UDHAYAM) was purchased from Sri Murugan, India. One gram of blackseed was weighed in a conical flask. Exactly 20 mL of hexane, methanol, ethanol, ethyl acetate, and water were added to the conical flask respectively and were incubated at 40°C in a water bath for 2 and 4 h respectively. The extracts were then transferred to a 15 mL centrifuge tube and centrifuged at 4000 rpm for 10 min at 4°C. The centrifuged mixtures were then filtered using filter paper in another 15 mL centrifuge tube and stored in the freezer prior to use.

UV analysis

UV double beam spectrometer was used to scan the wavelength of TMQ standard from 200 nm up to 400 nm. This was to determine the maximum wavelength of absorbance value of TMQ for use as detection wavelength in the UV analysis method. Validation of the method from linearity, sensitivity, precision and accuracy was done. The absorbance reading was obtained in quintuplicate, and it was used to calculate the concentration of TMQ extracts for each extract. The result was reported as an average quantity and expressed by RSD.

Free radical scavenging activity

Exactly 200 µL of methanol and 800 µL of sample extracts were added to a test tube. A solution of 0.1 mM DPPH solution was prepared and 2mL of DPPH solution was added to the test tube as well. The steps above were repeated with different solvent extractions of TMQ and then vortexed. The reaction mixture was wrapped with aluminium foil and let to stand for 60 min in a dark place. The absorbance of TMQ

standard solutions, ascorbic acid solution and a mixture of sample extracts were measured at 517 nm with respective solvent as blank. The percentage of free radical scavenging activity was calculated using the formula:

$$\frac{(\text{Absorbance of control} - \text{Absorbance of standard})}{\text{Absorbance of control}} \times 100$$

Results and Discussion

The wavelength used was determined by the wavelength scanning from 200 to 400 nm. The highest peak for detection of TMQ standard solution was 254 nm using UV spectrophotometry. It works by obtaining the absorbance spectra of a compound and correlating it to the quantity of compound present. A wavelength is selected, and the beam will emit light energy and this light energy will pass through a monochromator. The main function of a monochromator is to allow a certain wavelength of light to pass through it. Then, the light will get to the sample and the remaining light will be detected by the detector. The main advantage of using UV-Visible spectrophotometry in quantitative analysis is to save time and cost. It is also easier to carry out compared to using HPLC for quantitative analysis[8]. Quantitative analysis of TMQ can also be done using UV-Visible spectrophotometry either with a single beam or double beam. The wavelength scanned that covers UV and visible region (200 nm – 800 nm) will be carried out to determine the max absorbance of TMQ. Method to quantify TMQ should be developed and validated to ensure the method developed is reliable. The absorbance value of set working standards of TMQ with known concentration is used to plot a graph (Fig. 1). The correlation coefficient of the standard curve should not be lesser than 0.995. The method is then validated from accuracy and precision. The main limitation of using UV-Visible spectrophotometry is that TMQ is a light and heat-sensitive compound. Hence, this will lead to a decrease accuracy of the data[9].

Linearity

The linearity of the UV method was evaluated using 5 working standards of TMQ solutions (0.002, 0.004, 0.008, 0.010 and 0.020 mg/mL) that were prepared by diluting the stock TMQ solution. The R2 obtained was 0.9995. The LOD and LOQ were calculated to be 0.0005 mg/mL and 0.0015 mg/mL respectively using the formula mentioned in method (Table 1).

Precision

The precision of the method was evaluated using 0.008 and 0.02 mg/mL of standard TMQ solutions. The reported intra-day RSD were 0.2720 and 0.0264% respectively (Table 2) and the interday RSD were reported to be 3.9632 and 8.3865% respectively (Table 3). The percentage of RSD value should be lesser than 2% to meet the acceptance criteria[10].

The percentage recovery was used to evaluate the accuracy of the UV method. The percentage recovery was reported to be 99.07% (Table 4) which falls within the acceptable range of 80 to 120%[10-11].

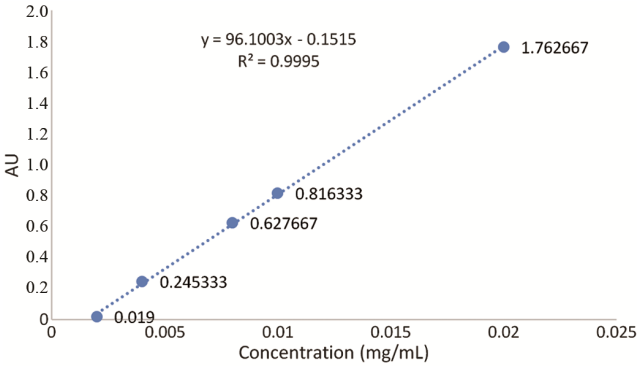

Fig. 1 — Calibration curve for the UV method.

Table 1 — Linearity, LOD and LOQ value by UV-VIS spectrophotometric method

Parameters	Thymoquinone
Linear range (mg/mL)	0.002–0.02
Calibration equation	Y = 96.1003X + 0.1515
Correlation coefficient	0.9995
LOD (mg/mL)	0.0005
LOQ (mg/mL)	0.0015

Table 2 — Intra-day precision results of UV-VIS spectrophotometric method

Thymoquinone	Intra-day precision (n=4)	
Concentration (mg/mL)	Mean±SD (Absorbance)	Intra-day RSD (%)
0.008	0.8260±0.0022	0.2720
0.020	2.0754±0.0005	0.0264

Table 3 — Inter-day precision results of UV-Vis spectrophotometric method

Thymoquinone	Inter-day precision (n=4)	
Concentration (mg/mL)	Mean±SD (Absorbance)	Inter-day RSD (%)
0.008	0.8040±0.0319	3.9632
0.020	1.9385±0.1626	8.3865

Table 4 — Recovery results of UV-Vis spectrophotometric method

Thymoquinone	Recovery (n=5)			
Added concentration (mg/mL)	Mean absorbance	SD	RSD (%)	Recovery (%)
0.020	1.8650	0.0173	0.9287	99.0718

Quantification of TMQ extracts

The quantification of TMQ extracts was calculated using the absorbance reading from UV-VIS spectroscopy at 254 nm (Table 5-7). The wavelength of 254 nm is absorbed by many molecules containing certain chromophores. The absorbance reading is directly proportional to the concentration of the TMQ extracts using the Beer-Lambert Law. However, this method is not specific to detecting TMQ as there are other phytochemicals in black seed that contain chromophores that absorb the wavelength of 254 nm. Therefore, methanol is reported to be the highest extraction solvent of TMQ due to extracting phytochemicals other than TMQ[12-13].

Free radical scavenging activity

DPPH assay was used to evaluate the free-radical scavenging activity of TMQ extracted with different solvents (Table 8). The stable radicle form DPPH will react with a molecule or compound with a weak A-H bonding. This reduces DPPH which leads to changes from violet to a yellow colour. Then, the absorbance

Table 5 — Quantification of TMQ in blackseed with different solvent extraction for 2 h

Thymoquinone			Quantification (n=5)		
Solvent	Absorbance	Concentration calculated (mg/mL)	Mean±SD (mg/mL)	RSD (%)	Amount quantified (mg/g)
Hexane	0.5180	0.0070	0.00701±0.00004	0.5701	7.0083
	0.5270	0.0071			
	0.5250	0.0070			
	0.5210	0.0070			
	0.5190	0.0070			
Methanol	0.4120	0.0059	0.00586±0.00002	0.1487	5.8553
	0.4110	0.0059			
	0.4100	0.0058			
	0.4110	0.0059			
	0.4120	0.0059			
Ethanol	0.4400	0.0062	0.00616±0.00002	0.3020	6.1634
	0.4420	0.0062			
	0.4420	0.0062			
	0.4420	0.0062			
	0.4380	0.0061			
Water	0.0720	0.0023	0.00233±0.00001	0.2446	2.3299
	0.0730	0.0023			
	0.0730	0.0023			
	0.0720	0.0023			
	0.0720	0.0023			
Ethyl acetate	0.1840	0.0035	0.00349±0.00001	0.1635	3.4870
	0.1840	0.0035			
	0.1840	0.0035			
	0.1830	0.0035			
	0.1830	0.0035			

Table 6 — Quantification of TMQ in blackseed with different solvent extraction for 4 h

Thymoquinone			Quantification (n=5)		
Solvent	Mean absorbance	Concentration calculated (mg/mL)	Mean±SD (mg/mL)	RSD (%)	Amount quantified (mg/g)
Hexane	0.5420	0.0072	0.00724±0.00002	0.2979	7.2435
	0.5450	0.0072			
	0.5470	0.0073			
	0.5460	0.0073			
	0.5430	0.0072			
Methanol	0.7260	0.0091	0.00910±0.00002	0.2045	9.1019
	0.7240	0.0091			
	0.7220	0.0091			
	0.7220	0.0091			
	0.7220	0.0091			

(*Contd.*)

Table 6 — Quantification of TMQ in blackseed with different solvent extraction for 4 h (*Contd*.)

Thymoquinone	Quantification (n=5)				
Solvent	Mean absorbance	Concentration calculated (mg/mL)	Mean±SD (mg/mL)	RSD (%)	Amount quantified (mg/g)
Ethanol	0.7710	0.0096	0.00957±0.00003	0.3132	9.5734
	0.7660	0.0095			
	0.7720	0.0096			
	0.7660	0.0095			
	0.7670	0.0096			
Water	0.0730	0.0023	0.002328±0.000005	0.2446	2.3278
	0.0720	0.0023			
	0.0720	0.0023			
	0.0720	0.0023			
	0.0720	0.0023			
Ethyl acetate	0.2100	0.0038	0.00376±0.00007		
	0.2010	0.0037		1.8588	3.7638
	0.2100	0.0038			
	0.2200	0.0039			
	0.2100	0.0038			

Table 7 — Concentration of TMQ in different solvent extraction for 2 and 4 h

Thymoquinone	Quantification (n=5)	
Solvent	Mean peak area	
	2 h	4 h
Hexane	0.5220^{ac}	0.5446^{ac}
Methanol	0.4112^{b}	0.7232^{b}
Ethanol	0.4408^{c}	0.7684^{c}
Ethyl acetate	0.1836^{d}	0.2101^{d}
Water	0.0724^{e}	0.0722^{e}

Different alphabet indicates significant difference (P<0.05).

Table 8 — Comparison of the free radical-scavenging activity of controls used and TMQ extracts used

Free radical-scavenging activity of control used	
Solvents	Free radical-scavenging activity (%)
DPPH	0.0000
Thymoquinone	30.9779
Ascorbic acid	97.3869

Free radical-scavenging activity of extracts		
Solvents	Free radical scavenging activity (%)	
	2 h	4 h
Hexane	33.5057^{a,,}	30.1290^{a},,
Methanol	95.7650^{b,,}	95.6891^{b,c}
Ethanol	71.2606^{c,,}	94.9208^{c,b}
Water	56.7581^{d,e}	62.2641^{d,,}
Ethyl acetate	48.7765^{e,d}	47.4249^{e,,}

Different alphabet indicates significant difference (P <0.05).

readings were taken at 517 nm with ascorbic acid as the positive control of the DPPH assay. Based on the result reported, methanol has the highest free radical scavenging activity (95.77%) compared to other extraction solvents. This is because methanol is a universal polar solvent that extracts more polar phytochemicals like terpenoids and flavonoids from black seed. Fig. 1 showed that polar extraction solvents like methanol, ethanol and water have higher free radical scavenging activities compared to hexane and ethyl acetate which is less polar. Hence, TMQ is not the only bioactive compound that contributes to the free radical scavenging activity[14].

Conclusion

In this study, the UV method develop is validated from linearity, precision, and accuracy. All of the criteria validated lies in the acceptable range. The effect of extraction solvent on thymoquinone extraction from blackseed and evaluation of free radical scavenging activity were successfully investigated. Based on the investigation, methanol extracted the most TMQ from blackseed based on the quantified by using the UV-Vis spectrophotometry method. The quantification of TMQ with different extraction solvents shows that it is significantly different from each other. Thus, the choice of extraction solvent will play a vital role in extracting thymoquinone. The main concern of this study was the stability of thymoquinone and the non-specificity of UV-Vis spectroscopy in detecting thymoquinone. The stability of thymoquinone could be maintained with proper storage due to its light and heat-sensitive properties of thymoquinone. Other methods of analysis such as Liquid Chromatography–Mass Spectrometry (LC-MS) and Fourier-Transform Infrared Spectroscopy (FTIR) could be considered for the analysis of thymoquinone in *N. sativa* extracts for future studies.

Conflict of interest

The authors have no conflict of interest.

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