

## Phenolic Content, Antioxidant Capacity and Antimicrobial Activity of Essential Oil from *Habbatus sauda* Seed

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This paper presents an extraction and antimicrobial study of essential oil obtained from *Nigella sativa* seeds. Extraction using polar solvent resulted in higher recovery of desired bioactive compounds, thymoquinone and thymol. It was found that extraction of antioxidant (i.e. Agidol 7) favours aqueous solvent, i.e. 50% ethanol and 50% methanol. The highest TPC, TFC and antioxidant activities were obtained at power 224W and temperature of 50 °C. This study shows that *N. sativa* seed oil has an effective antibacterial activity from the *in-vitro* studies. The result showed that the essential oil extracted using n-hexane has the highest (30.17%) effectiveness against *E. coli*.

**Key words:** *Nigella sativa*, Ultrasonic assisted extraction, Flavonoid, Thymol, Thymoquinone, Antimicrobial.

*Nigella sativa* is commonly known as habbatus sauda belongs to a botanical family of Rununculaceae. The *N. sativa* seed is rich with medicinal value and has been used as a natural remedy since antiquity, especially by people in the Mediterranean region. Previous research revealed that it contained an abundance of active ingredients useful for anticancer and anti-inflammatory, anti-dermatophyte, asthma, hypertension, diabetes, cough, bronchitis, fever, dizziness and gastrointestinal disturbances.

The first step to recover and purify essential oil from plant materials involves an extraction process. The yield of essential oil is dependent on the solvent used, extraction method and condition<sup>1</sup>. Conventional extractions such as soxhlet extraction and maceration (ME) are normally performed at high temperatures for several hours. In recent years, a better extraction method has been developed such as the ultrasonic-assisted

extraction (UAE), microwave-assisted extraction (MAE) and supercritical extraction. Supercritical extraction is less favorable owing to its energy consumption and higher capital cost. The localize superheating in microwave-assisted extraction induces a rapid temperature rise thus possesses challenge in temperature control. Extraction is a mass transfer process involving solvent transport to the solid phase (inner transport), dissolution of the solutes (solubility) and release of solutes from the solid matrix to the bulk phase (external transport). The UAE technique reduces the inner and external mass transfer limitation and hence increases the yield of extraction. Furthermore, ultrasonic wave can break the cell membranes reducing control of inner mass transport. Therefore, the UAE method was employed in this work. Solvent type plays an important role in essential oil extraction. A combined effect of different extraction methods (ME, and UAE) and varying solvent polarity to the polyphenol extraction from *N. sativa* has never been studied previously, and hence this is one of the objectives of this work.

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## MATERIALS AND METHODS

### Chemicals and Plant Material

The HPLC grade solvents such as the n-hexane, 2-propanol and methanol were purchased from Merck (Darmstadt, Germany). The dimethyl sulfoxide (DMSO), Thymoquinone, 1,1-diphenyl-2-picrylhydrazyl (DPPH), aluminium chloride ( $\text{AlCl}_3$ ), sodium hydroxide (NaOH), sodium nitrate ( $\text{NaNO}_3$ ), Folin-Ciocalteu reagent, gallic acid and quercetin were obtained from Sigma Aldrich (St. Louis, MO). Solvent for extraction such as 95% ethanol (Copens Scientific (M) Sdn Bhd), 99.8% methanol and 40-50% n-hexane (KOFA Chemical (M) Sdn Bhd) was obtained from a local supplier.

### Essential Oil Extraction

The powdered plant material was weighed (25 wt. %) and mixed with solvent in a 250 ml sealed Erlenmeyer flask. UAE was carried out in an ultrasonic bath (JK-DUCH-6210LHC, China) at either 35 or 53 kHz for time ranged from 15 to 120 minutes and temperature was set at range from 30 to 60 °C. Maceration was performed at 50 °C in a stirred vessel using a similar plant material to solvent ratio. The temperature of 50 °C was chosen based on the result from UAE extraction which shows excellent extraction of essential oil at 50 °C. The supernatant was then separated from the residue by vacuum filtration through 0.45 µm nylon membrane.

### GC-MS Analysis of Essential Oil

Analysis of the volatile oil: The GC-MS analysis was performed with a quadruple GC-MS system, capillary column (30 m x 0.25 mm; 0.25 µm film thickness). The carrier gas was helium and column head pressure of 15 psi yielding a linear flow rate of 0.8 m/min. The split ratio was 1: 10 and the initial column temperature was held at 200 °C for 15 min and then raised at 10 °C/min and maintained at 260 °C until all components had eluted. The components were identified by matching their mass spectra in the NIST 05 library and their retention indices were compared with literature values.

### Total Phenolic Content (TPC)

Total phenolic contents were assayed using Folin-Ciocalteu reagent, following Singleton's method. An aliquot (0.125 ml) of a suitable diluted extract was added to 0.5ml of ultrapure water and 0.125 ml of the Folin-Ciocalteu

reagent. The mixture was vortex for 3 minutes, before adding 1.25 ml of 7%  $\text{Na}_2\text{CO}_3$  solution. The solution was adjusted with ultrapure water to a final volume of 3ml and mixed thoroughly. After leave in dark for 90 minutes at 25 °C, the absorbance versus prepared blank was read at 760nm. Total phenolic contents of seeds (two replicates per treatment) were expressed as mg gallic acid equivalent per gram (mg GAE/ 100g) through calibration curve with gallic acid. The calibration curve range was 50-400 mg/ml ( $r^2 = 0.99$ ). All samples were performed in two replicates.

### Total Flavonoid Content (TFC)

Total flavonoid content was measured according colorimetric assay. A 250µl diluted extract was mixed with 75µl  $\text{NaNO}_2$  (5%). After 6 min vortex, 150µl of 10%  $\text{AlCl}_3$  and 500µl of NaOH (1M) were added to the mixture. Finally, the mixture was adjusted to 2.5ml with ultrapure water. The absorbance versus prepared blank was read at 510nm. Total flavonoid contents of seeds (two replicate per treatment) were expressed as mg quercetin equivalents per gram (mg QE/ 100g) through the calibration curve quercetin. The calibration curve range was 50-500mg/ml.

### DPPH Assay

The electron donation ability of the obtained extracts and essential oils was measured by bleaching of the purple coloured solution of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) according to the method of Hanato et al. [3]. Diluted essential oil prepared in methanol were added to 0.5ml of a 0.2 mmol/l DPPH methanolic solution. The mixture was shaken vigorously and left standing in dark at room temperature for 30 minutes. The absorbance of the resulting solution was then measured at 517 nm after 30 minutes. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_1)/A_0] \times 100$$

where  $A_0$  is the absorbance of the control at 30 minutes, and  $A_1$  is the absorbance of the sample at 30 minutes.

### Antimicrobial Test

The essential oil from *N. sativa* was individually tested against *Escherichia Coli* (ATCC 8739). The species was obtained from ISO17025 certified lab at Central Laboratory, Universiti Malaysia Pahang. During this investigation, the culture was maintained in

cryovials at -20°C. Broth subcultures were prepared by inoculating, with one single colony from a plate, into a test tube containing 10 ml of sterile nutrient broth (Biolife, Italy). After inoculation, the tubes were incubated overnight at 37 °C until it turbid. The antibacterial and antifungal activity of *N. sativa* essential oils was evaluated using the agar diffusion method. The test was performed using sterile petri dishes (100 mm diameter) with 100 microliters of prepared culture spread on the surface of sterile Mueller–Hinton agar medium (25 ml, pH 7). A sterile filter paper (Whatman No. 1, 6 mm diameter) was immediately impregnated with 10 ul of *N. sativa* essential oil and aseptically placed on the surface of the agar plate that previously inoculated with a sterile microbial suspension (one microorganism per petri dish). Spread plates were then kept at ambient temperature for 30 min to allow diffusion of extracts prior to incubation. After 20 min standing, the plate was turned upside down and incubated at 37 °C for 24 h. All petri dishes were sealed with sterile laboratory films to avoid eventual evaporation of the test samples. The microbial growth on plates was visualized directly on the plate, captured using a digital camera for further processing. The inhibition area was obtained by processing the image as binary using ImageJ software. The percentage of microbial inhibition was calculated using the following equation:

$$\text{Microbial inhibition (\%)} = |Ac \cdot As| / (Ac) \times 100$$

where Ac and As are the areas of the control dish solution without and microbial solutions with *N. sativa* essential oil, respectively.

### Statistical Analysis

Each experiment was repeated in triplicates. Analysis of variance (ANOVA) was

performed by using the data analysis tools in Microsoft Excel 2010, and a least significant difference (LSD) test was used to compare the means with a confidence interval of 95%.

## RESULTS AND DISCUSSION

### Influence of Solvent Type and Extraction Method on Essential Oil Yield from *N. sativa*

Gas chromatography mass spectrometry (GC-MS) analysis of the essential oil showed a significant amount of thymoquinone (0.6~0.8%), thymol (0.09~0.15%), agidol 7 (0.33~6.44%), p-cymene (0.31~0.57%), and (E)-<sup>2</sup>-Ocimene (0.11~0.13%). It was found that extraction of antioxidant (Agidol 7) favours aqueous solvent, i.e. 50% ethanol and 50% methanol (Fig. 1). The highest yield of essential oil was obtained using 50% ethanol followed by n-hexane, while the lowest is 50% methanol. Extraction using ethanol shows highest simultaneous extraction of TFC (2.47 µg QE/g DW), TPC (0.23 mg GAE/g DW) and antioxidant activities (60%). Methanol has a higher yield of TPC (0.38 mg GAE/g DW) and antioxidant activities (67%) but very low TFC (0.25 µg QE/g DW). Extraction using n-hexane, 50% methanol and 50% ethanol is not notable, thus, ethanol was employed for the remainder of this work. It was found that there is no significant difference at P < 0.05 on the effect of residence time and ultrasonic frequency after 30 minutes. However, the highest TPC, TFC and antioxidant activities were obtained at power 224W and temperature of 50 °C (Fig. 2). Extraction at the higher temperature (60 °C) is not an improvement due to thermal degradation of bioactive compounds. It was found that UAE is better than ME as it can provide much higher extraction of TFC, TPC and antioxidant within 30 minutes compared to 4 hours for ME.

**Table 1.** GC-MS analysis of essential oil from *Nigella Sativa* seeds

VOC	n-Hexane	Ethanol	Methanol	50% Ethanol	50% Methanol	Harzallah <i>et al</i> <sup>2</sup>
p-Cymene	0.57	0.43	0.31	ND	ND	49.48
(E)- <sup>2</sup> -Ocimene	0.13	ND	0.11	ND	ND	ND
Thymoquinone	0.8	0.6	0.63	ND	ND	0.79
Thymol	0.09	0.14	0.15	ND	ND	ND
Agidol 7 (AO 425)	0.33	0.45	1.14	6.44	2.43	ND
Sitosterol	0.05	0.07	0.07	ND	ND	ND

### Assessment on Antimicrobial Properties of *N. Sativa* Oil

Fig. 3 shows excellent antimicrobial properties of the essential oil extracted from *N. sativa*, which were studied using *E. coli* ATCC8739. The inhibition area was obtained by processing the image as binary using ImageJ software. The

highest inhibition effect of 30.17% was achieved using oil extracted using hexane, meanwhile ethanol extracted oil also showed some (7.25%) antimicrobial activities. Oil obtained using ethanol showed lower antimicrobial inhibition due to lower concentration of thymoquinone as opposed to those obtained using n-hexane.

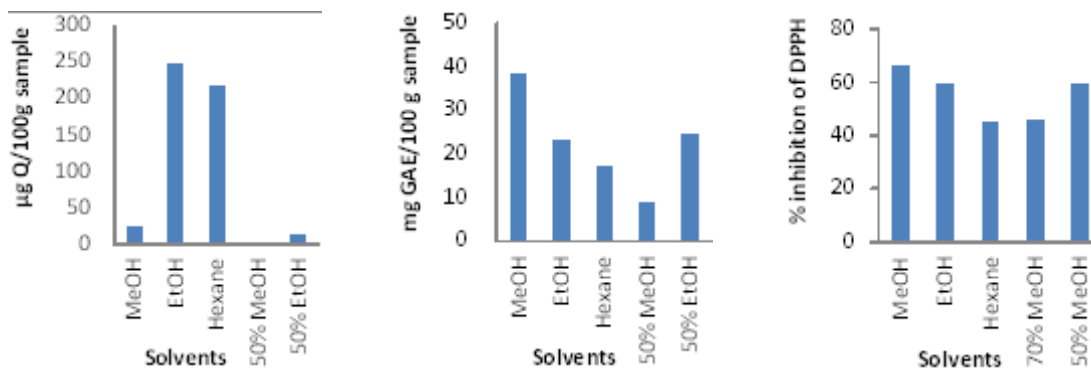


Fig. 1. Effect of solvent to TFC, TPC and antioxidant extraction from *N. sativa*

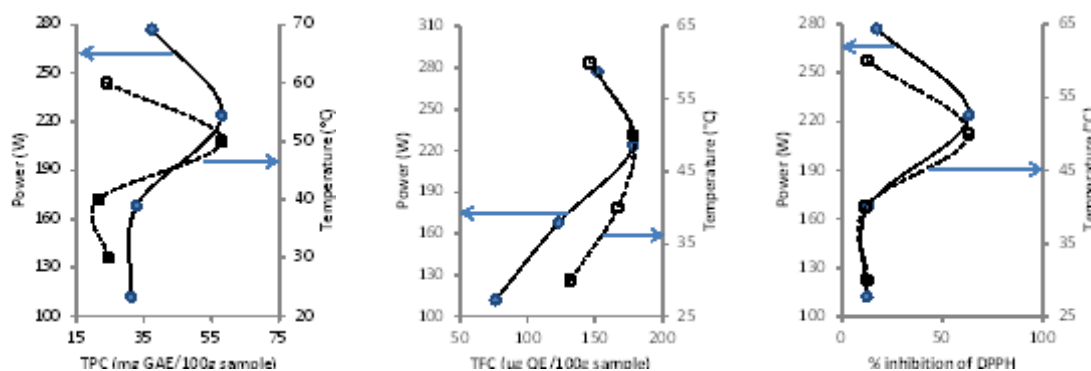


Fig. 2. Effect of power and temperature to TFC, TPC and antioxidant extraction from *N. sativa*

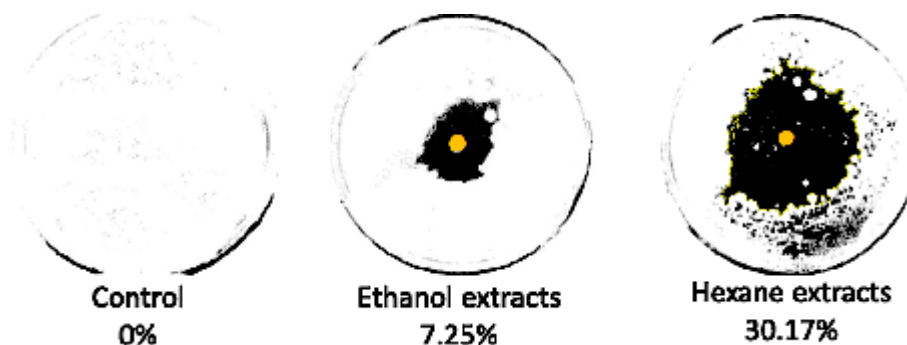


Fig. 3. Antimicrobial properties of essential oil from *N. sativa*

## CONCLUSIONS

The essential oil yield depends on the solvent used for the extraction process. The highest thymoquinone content (0.8%) was obtained using n-hexane extracts while the highest thymol concentration (0.15%) was obtained using methanol extracts. It was found that extraction of antioxidant (Agidol 7) favours aqueous solvent, i.e. 50% ethanol and 50% methanol. The highest TPC, TFC and antioxidant activities were obtained at power 224W and temperature of 50 °C. This study shows that *N. sativa* seed oil has effective antibacterial activities in *in-vitro* studies. The result showed that the essential oil obtained from ethanol and hexane extracts are effective against *E. coli*. Essential oil extracted using hexane is more potent (30.17% inhibition) as opposed to those obtained using ethanol (7.25% inhibition).

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