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Supercritical CO_2 Extraction and Characterization of Thymoquinone-Rich Nigella sativa L. Seed Oil: A Process Optimization Framework Using RSM

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ABSTRACT: **Background**: Post-pandemic interest in plant-derived bioactive compounds has renewed attention on Nigella sativa L. seeds and thymoquinone (TQ), a principal bioactive constituent. Efficient recovery of TQ-rich N. sativa seed oil is important for its potential applications in functional food and health-related formulations.

Method: Supercritical carbon dioxide (SC-CO_2) extraction was employed to recover N. sativa seed oil and TQ. The effects of extraction pressure, temperature, and seed particle size were investigated, while CO_2 flow rate was maintained at 0.012 kg min^{-1}. Response surface methodology (RSM) was used for process modelling and optimization. The extracted oil was characterized using GC–MS/MS, GC–FID, fatty acid profiling, FTIR, NMR, and SEM.

Result: The highest TQ concentration of 4.06% (w/w) was obtained at 15 MPa, 40 °C, and 0.87 × 10^{-3} m particle size. TQ was confirmed and quantified using GC–MS/MS and GC–FID. The optimized conditions effectively recovered TQ-rich N. sativa seed oil. Chromatographic, spectroscopic, and microscopic analyses further characterized the extracted oil's chemical and structural properties.

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Conclusion: SC-CO_2 extraction combined with RSM provides an effective approach for optimizing TQ-rich N. sativa seed oil recovery and characterization. The integrated extraction and analytical framework offers a systematic basis for process optimization and evaluation of the chemical and structural properties of the resulting oil.

1. INTRODUCTION

In the post-COVID-19 era, growing interest in plant-derived bioactive compounds has accelerated research into natural products with preventive and therapeutic potential. Among these, Nigella sativa L. (black cumin) has received considerable attention owing to its diverse pharmacological properties, primarily attributed to its major bioactive constituent, thymoquinone (TQ) (Islam et al., 2021; Badary et al., 2021; Li et al., 2023; El-Sayed et al., 2023). N. sativa, a medicinal herb belonging to the order Ranunculales, has been extensively used in traditional systems of medicine, including Ayurveda, Unani, Siddha, Tibb-e-Nabawi, and traditional Jewish medicine, for the treatment of a wide range of ailments (Majeed et al., 2021; Rashwan et al., 2023; Khatoon et al., 2024; Alu'datt et al., 2024; Alberts et al., 2024; Dabeer et al., 2022).

The seeds of N. sativa are a rich source of nutritionally and biologically valuable constituents, containing approximately 36–38% fixed oil, 0.4–0.45% volatile oil, 16–19.9% protein, 33.9% carbohydrates, 5.5% dietary fibre, 1.79–3.74% minerals, and about 6% moisture (Ardhi et al., 2024). In addition to its nutritional value, N. sativa has been recognized as a safe natural ingredient, and the U.S. Food and Drug Administration (FDA) has classified it as Generally Recognized As Safe (GRAS) for use as a spice, natural flavouring, colouring, and preservative agent (U.S. Food and Drug Administration, 2019; Kumar et al., 2020; Aggarwal et al., 2024). Increasing consumer preference for clean-label foods and naturally derived additives has further encouraged the food industry to replace synthetic preservatives with plant-based alternatives such as spices and their bioactive extracts (Chatterjee et al., 2025). Consequently, developing efficient, environmentally sustainable extraction technologies to recover high-purity thymoquinone-rich N. sativa seed oil has become an important area of research, with promising applications in the food, nutraceutical, pharmaceutical, and cosmeceutical sectors.

Essential oils extracted from N. sativa seeds are enriched with bioactive compounds such as thymoquinone (TQ), p-cymene, thymohydroquinone (THQ), α-thujene, β-pinene, carvacrol, thymol, limonene, dithymoquinone, 4-terpineol, and sesquiterpene longifolene (Albakry et al., 2022; Dimitrijević et al., 2024). Out of these, TQ is the most abundant, comprising 30-52.5% of the oil (depending on the seed source) (Albakry et al., 2022; Srinivasan, 2018). TQ exhibits therapeutic activities, including antidiabetic, anticancer, anti-inflammatory, analgesic, nephroprotective, pulmonary protective, gastroprotective, and immune-strengthening effects (Chatterjee et al., 2025). TQ efficiently inhibits Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) and SARS-CoV-2, and clinical studies have confirmed its efficacy in reducing COVID-19 symptoms (Ahmad et al., 2013; Bencheqroun et al., 2022). TQ treats neurodegenerative and neuropsychiatric diseases due to its low molecular weight (Derosa et al., 2024). N. sativa seed oil containing 5% TQ has shown a positive impact in allergic rhinitis caused by seasonal allergens (Majeed et al., 2024). Othman et al. (2024) observed that TQ can reduce palmitate-induced inflammation and cell toxicity in macrophages by restoring energy metabolism and balancing cytokine output (Othman et al., 2024). The functional bioactives

of N. sativa oil have substantial commercial value, enabling new product formulations in response to changing consumer demand and adding value to human health.

The present study proposes an efficient method for extracting thymoquinone (TQ)-rich Nigella sativa L. seed oil using supercritical carbon dioxide extraction (SCFE-CO_2). Conventional extraction techniques, including hydro-distillation, Soxhlet extraction, and steam distillation, have been widely used to isolate bioactive compounds from natural products because of their simplicity and effectiveness (Zhang et al., 2018; Bitwell et al., 2023; Mungwari et al., 2025). However, these methods often involve high solvent consumption, prolonged extraction times, thermal degradation of heat-sensitive constituents, and environmental concerns from organic solvent use (Santos et al., 2022; Lanjwani et al., 2025). Consequently, environmentally sustainable ("green") extraction technologies, such as microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), supercritical fluid extraction (SCFE), pulsed electric field (PEF), and ultrasound-assisted extraction (UAE), have gained considerable attention because they minimize the use of hazardous solvents, reduce energy consumption, shorten extraction time, and enhance the recovery of valuable phytochemicals (Bhadra et al., 2017; Nde & Foncha, 2020; Gautam et al., 2021).

Among these techniques, SCFE using carbon dioxide has emerged as one of the most promising alternatives because CO_2 is non-toxic, non-flammable, inexpensive, readily available, and easily recyclable (Uwineza & Waśkiewicz, 2020; Sirohi et al., 2024). In its supercritical state, CO_2 exhibits unique physicochemical properties, combining gas-like viscosity and diffusivity with liquid-like density, thereby providing excellent mass transfer characteristics and solvent power (Budisa & Schulze-Makuch, 2014). Moreover, adjusting extraction pressure and temperature can precisely tune the solvating capacity of supercritical CO_2, enabling selective extraction of target compounds. Its relatively low critical temperature (31.1 °C) makes SCFE particularly suitable for recovering thermolabile bioactive constituents, such as thymoquinone, with minimal thermal degradation (Mukhopadhyay, 2009). In addition, the solvent-free nature of the final product minimises downstream purification requirements, making the process attractive for industrial-scale applications.

Several studies have demonstrated the superiority of SCFE-CO_2 over conventional extraction methods. For instance, Duan et al. (2024) reported that high-pressure SCFE-CO_2 increased astaxanthin recovery from Hematococcus pluvialis by 20–70% compared with ultrasound-assisted and organic solvent extraction methods (Duan et al., 2024). Similarly, Rubio-Rodríguez et al. (2012) showed that SCFE-CO_2 at 25 MPa and 313 K produced omega-3-rich fish oil with lower lipid oxidation and reduced arsenic contamination, while subsequent online fractionation further decreased free fatty acid content relative to conventional extraction techniques (Rubio-Rodríguez et al., 2012). These findings highlight the strong potential of SCFE-CO_2 as a green, efficient technology for selectively extracting high-value, thermolabile phytochemicals such as thymoquinone from N. sativa seeds.

Several studies have investigated how supercritical CO_2 extraction (SCFE-CO_2) operating conditions affect the yield and quality of Nigella sativa seed oil. For example, Solati et al. (2012) reported that extraction at 15 MPa, 40 °C, and 120 min produced the highest thymoquinone concentration (4.09 mg mL^{-1}), whereas the maximum oil yield (23.20%) was achieved at 35 MPa, 60 °C, and 120 min (Solati et al., 2012). These findings demonstrate that the optimum conditions for maximizing oil yield and

thymoquinone recovery are not necessarily identical, highlighting the complexity of the extraction process and the need for advanced optimization strategies.

Response surface methodology (RSM) has been widely used to optimize extraction processes because it evaluates the interactive effects of multiple operating variables while reducing the number of experimental trials. Santos et al. (2024) used RSM to optimize anthocyanin extraction from black hollyhock flowers, achieving a total anthocyanin content of 65.7 ± 0.5 mg g^{-1} and an extraction yield of 53 ± 2.5% by optimizing extraction temperature, acid concentration, and solvent-to-feed ratio (Santos et al., 2022). Likewise, Nde & Foncha (2020) used RSM to optimize the extraction of bioactive compounds from hemp leaves by varying extraction time, temperature, and liquid-to-solid ratio, maximizing polyphenol recovery and anti-inflammatory activity (Nde & Foncha, 2020). Furthermore, Sakdasri et al. (2023) reported a thymoquinone concentration of 12 mg mL^{-1} in black cumin seed oil obtained through mechanical pressing (Sakdasri et al., 2023).

The objectives of the study were to investigate the recovery of thymoquinone-rich N. sativa oil by the Super-Critical CO_2 unit, to optimize the process conditions by applying an artificial neural network and particle swarm embedded hybrid approach, to enumerate the influence of several extraction parameters, and also to characterize the properties of the extracted oil and its sustainability.

2. MATERIALS AND METHOD

2.1 Procurement of N. sativa seed and CO_2

N. sativa L. seed was procured from SEED Agritech Pvt. Ltd, Kerala, India (Spice Board of India) approved vendor.CO_2 (>99.9%) was collected from Bharat Oxytech Pvt. Ltd., Haldia, West Bengal, India.

2.2 N. sativa L. seed sample preparation

The particle size of N. sativa seeds was reduced using an electrical grinder for 50 seconds to 1 minute to obtain an optimal size of approximately 0.71×10^{-3} m. After grinding, the finely ground seeds were sieved to achieve a homogeneous particle-size distribution, which is crucial for overall extraction efficiency. The sieving was carried out using a 24-mesh screen.

2.3 Supercritical carbon dioxide extraction process for extracting N. sativa seed oil

Nigella sativa seed oil was extracted using a laboratory-scale supercritical carbon dioxide extraction (SCFE-CO_2) system. The extraction unit comprised a CO_2 cylinder, a chiller, a high-pressure reciprocating pump (maximum pressure capacity: 100 MPa), stainless-steel extraction vessels (operating pressure up to 120 MPa and temperature up to 250°C), two product separator vessels (operating pressure up to 25 MPa and temperature up to 250°C), high-pressure piping, valves, pressure regulators, and an integrated electrical control and instrumentation panel.

Before extraction, 1000 g of ground N. sativa seeds were loaded into the extraction vessel (0.1–2.0 L capacity), which was then securely sealed to prevent leakage and maintain stable operating conditions throughout the extraction process. Carbon dioxide supplied from a gas cylinder at an initial pressure of approximately 60 kg cm^{-2} (~5.9 MPa) was transferred to a holding tank and cooled to −4 °C using a chiller to obtain liquid CO_2. The liquid CO_2 was subsequently pressurized above its critical pressure

(7.39 MPa) using a high-pressure reciprocating pump equipped with a non-return valve. Simultaneously, the extraction vessel temperature was maintained above the critical temperature of CO_2 (31.1 °C) using a thermostatically controlled heating system to establish supercritical conditions.

Under supercritical conditions (pressure >7.39 MPa and temperature >31.1 °C), CO_2 exhibits liquid-like density combined with gas-like diffusivity and low viscosity, resulting in enhanced mass transfer and excellent solvent properties for the extraction of thermolabile bioactive compounds such as thymoquinone (TQ) from N. sativa seeds (Gawron et al., 2021; Ranieri et al., 2024). Throughout the extraction process, the CO_2 flow rate was kept constant at 0.012 kg min^{-1}. We varied extraction pressure and temperature according to the experimental design to evaluate their effects on oil yield, thymoquinone content, and antioxidant activity (1,1-diphenyl-2-picrylhydrazyl radical scavenging assay), while keeping extraction time fixed at 90 min.

Following extraction, the pressure was gradually reduced to approximately 50–60 atm

(~ 5.07–6.08 MPa), allowing separation of the extracted oil from supercritical CO_2 in a two-stage separation system. In the first separator vessel, the pressure reduction caused the dissolved oil to precipitate and accumulate at the bottom of the vessel, whereas gaseous CO_2 exited through the top outlet. The remaining CO_2 stream containing trace oil was then directed to the second separator vessel, where the residual oil was recovered under further reduced pressure. The extracted oil from both separator vessels was combined and stored in airtight amber glass vials at 4 °C for further physicochemical characterization and analysis.

Figure 1 presents a detailed view of the SCFE-CO_2 extraction unit, while **Figure 2** presents the overall process flow for extracting thymoquinone-rich N. sativa seed oil using SCFE-CO_2 technology.

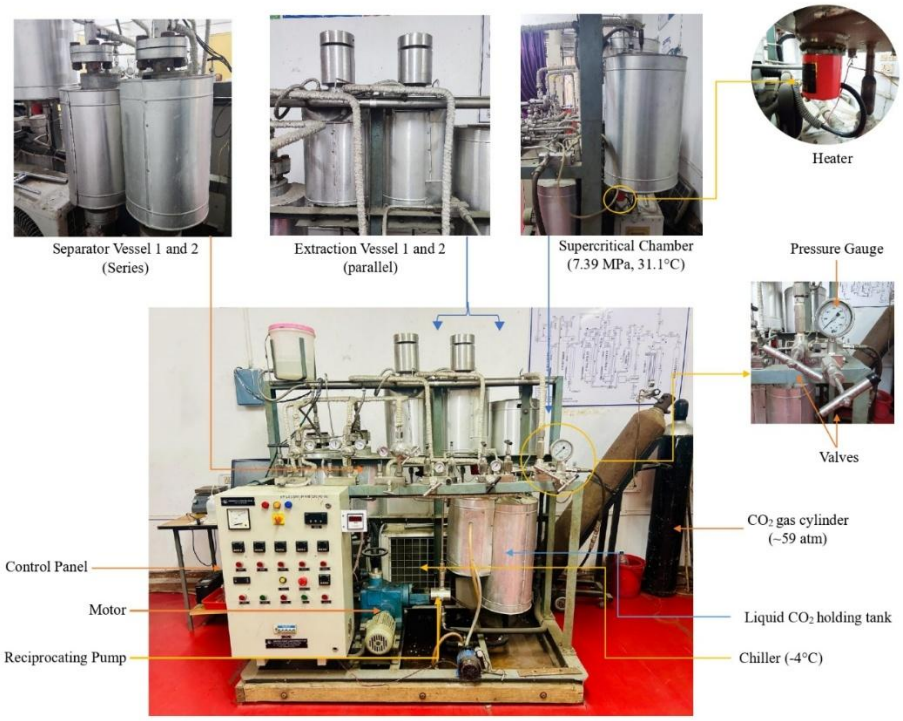


Figure 1. Detailed subsection view of supercritical carbon dioxide extraction unit.

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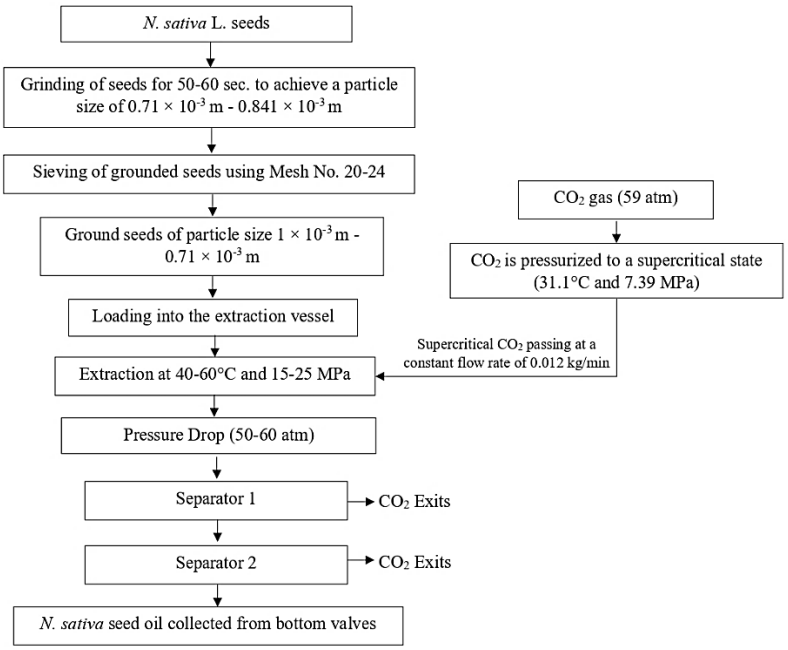


Figure 2. Process flowchart for extracting thymoquinone-rich N. sativa seed oil using supercritical carbon dioxide extraction (SCFE-CO2) technology.

2.4 Experimental design for optimization of process conditions using RSM-FFD

The process optimization was done using a 3^3 full factorial design (FFD) in RSM under the following independent parametric conditions that include pressure (15, 20, and 25 MPa), temperatures (40, 50, and 60°C) and average seed particle size (0.7×10^{-3} m, 0.855×10^{-3} m and 1×10^{-3} m) (Bachkhaznadji et al., 2025). Extraction oil yield% (w/w), TQ% (w/w), and 1,1-diphenyl-2-picrylhydrazyl (DPPH)% were designated as response variables. Coded levels (-1, 0, +1) of independent process variables used in the experimental design are shown in **Table 1.**

Table 1. Independent process variables for the optimization of SCFE-CO2 extracted thymoquinone-rich N. sativa seed oil.

Variables	Symbol	Levels		
		-1	0	1
Temperature (°C)	x_1	40	50	60
Pressure (MPa)	x_2	15	20	25
Particle Size ($\times 10^{-3}$ m)	x_3	0.71	0.841	1

A quadratic model was fitted to the experimental data using multiple regression analysis. Eq (1) shows the second-order polynomial used to describe each dependent variable.

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j}^{k} \beta_{ij} x_i x_j + \epsilon \quad (1)$$

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Here, y is the dependent variable, β_0 is the intercept, β_i are the linear coefficients, x_i & x_j are independent variables (factors), β_{ii} are the quadratic coefficients, β_{ij} represents the interaction coefficients, k represents the quantification of independent variables, and ϵ represents the experimental error. Considering the selected variables, Eq (1) can be written as Eq (2):

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \varepsilon \quad (2)$$

Selected variables x_1, x_2, x_3 stands for the independent process variables: extraction temperature, pressure, and average seed particle size, whereas y denotes the dependent process variable (responses): extraction oil yield% (w/w), TQ% (w/w), and DPPH%. Experimental matrix and response values obtained from FFD are presented in **Table 2.**

Table 2. Responses from different process parameter combinations by full factorial design

Runs	Independent Process Variables			Dependent Response Variables		
	Temperature (°C)	Pressure (MPa)	Particle Size ($\times 10^{-3}$ m)	Yield % (w/w) (g)	TQ (%) (w/w)	DPPH %
	x_1	x_2	x_3	y_1	y_2	y_3
1	60 (H)	20 (M)	1 (H)	18.2 (H)	2.51 (M)	71.21
2	60 (H)	25 (H)	1 (H)	22.5 (M)	1.13 (L)	50.31
3	60 (H)	25 (H)	0.841 (M)	27.8 (H)	1.53 (L)	61.45
4	40 (L)	25 (H)	1 (H)	17.6 (M)	2.01 (L)	66.32
5	60 (H)	25 (H)	0.71 (L)	32.5 (H)	1.73 (L)	58.32
6	40 (L)	25 (H)	0.841 (M)	22 (M)	2.33 (L)	81.34
7	50 (M)	25 (H)	0.841 (M)	29.55 (H)	2.53 (M)	88.77
8	50 (M)	25 (H)	1 (H)	21.11 (M)	2.09 (L)	70.32
9	60 (H)	15 (L)	1 (H)	15.1 (L)	2.39 (L)	75.22
10	50 (M)	20 (M)	0.841 (M)	23.1 (M)	2.77 (M)	91.33
11	60 (H)	20 (M)	0.841 (M)	24.8 (H)	3.1 (H)	92.11
12	50 (M)	20 (M)	1 (H)	17.6 (M)	2.31 (L)	78.12
13	50 (M)	20 (M)	0.71 (L)	30.4 (H)	3.01 (M)	91.99
14	40 (L)	20 (M)	1 (H)	9.3 (L)	2.59 (M)	90.51

15	40 (L)	25 (H)	0.71 (L)	25.6 (H)	2.43 (L)	86.03
16	60 (H)	15 (L)	0.71 (L)	20.5 (M)	3.42 (H)	94.5
17	40 (L)	20 (M)	0.841 (M)	15.9 (L)	3.31 (H)	94.23
18	60 (H)	20 (M)	0.7 (L)	27.8 (H)	3.61 (H)	96.63
19	50 (M)	25 (H)	0.71 (L)	33.2 (H)	2.96 (M)	94.27
20	50 (M)	15 (L)	1 (H)	13.5 (L)	3.04 (M)	94.43
21	40 (L)	15 (L)	0.841 (M)	10.7 (L)	3.4 (H)	95.21
22	50 (M)	15 (L)	0.71 (L)	17.7 (M)	3.85 (H)	98.15
23	60 (H)	15 (L)	0.841 (M)	16.1 (L)	3.1 (M)	92.55
24	50 (M)	15 (L)	0.841 (M)	16.65 (L)	3.48 (H)	95.66
25	40 (L)	15 (L)	1 (H)	9.2 (L)	2.99 (M)	92.23
26	40 (L)	15 (L)	0.71 (L)	15.2 (L)	4.06 (H)	98.41
27	40 (L)	20 (M)	0.71 (L)	18.8 (M)	3.61 (H)	96.76

2.5 Statistical Analysis

Statistical analysis was carried out using analytical tools MINITAB 19 and Design Expert version 23.1. Multiple regression analysis was conducted on experimental data, and the influential model factors were evaluated using Analysis of Variance (ANOVA). Statistical modelling using regression coefficients developed response surface plots.

2.6 GC-MS/MS Analysis

Thymoquinone content in SCFE-CO_2-extracted N. sativa oil was analyzed by GC-MS/MS (SIM Method) (Make and Model: AGILENT-7000 MS & 7890 A-GC), with separation achieved using a DB 5MS column (30 m × 0.25 μm × 0.25 μm). Helium (carrier) and nitrogen (collision) gases were used at 1.0 mL/min. The initial temperature of the oven was 70°C with a 1-minute hold, then increased at 40°C/min up to 180 °C (no hold), followed by a slower increase at 5 °C/min up to 220°C (no hold), and lastly to 285°C at 10°C rise/min with an 8-minute hold. The injection volume was 1 μL/min, performed under splitless mode with an overall run time of 26.25 min. The mass spectrometer was operated with an inlet maintained at 80°C, an interface at 285°C, and an ion at 280°C. The retention time (RT) and mass spectral peak of the sample identified the TQ (Chatterjee et al., 2025).

2.7 GC-FID Analysis

GC-FID (Model: Agilent 7890B) method was executed via an HP-5MS ultra inert capillary column of 30 m × 0.25 μm × 0.25 μm dimensions, having a temperature range varying between -60°C and up to 350°C with nitrogen as the carrier gas (flow: 2mL/min), enabling the FFA to elute from the column. The FID temperature was fixed at 250°C, and the initial oven temperature was set to 100°C, then ramped to 250°C at 20°C/min with a 2 min hold time.

2.8 FTIR Analysis

An Agilent Cary630 FTIR Spectrometer equipped with Attenuated Total Reflectance (ATR) was used to obtain FTIR Spectra. ATR enables quick, precise measurement of minute samples with high sensitivity. For analysis, single 1µL drops of the oil sample were placed on the ATR surface at 24°C (ambient temperature). The FTIR spectrum was acquired with 32 scans at 4 cm^{-1} resolution over the spectral region of 600 cm^{-1} to 4000 cm^{-1}. An air spectrum, renewed after each scan, was used to correct the background of all spectra. Between different oil samples (standard and SCFE-CO_2 extracted N. sativa seed oil), hexane was used to clean the plates, and the spectra were recorded as % transmittance.

2.9 ^{1}H NMR and ^{13}C NMR spectroscopy

^{1}H and ^{13}C NMR analysis were conducted with a Joel YH 400 MHz (Tokyo, Japan) NMR spectrometer, functioning at 100 MHz for ^{13}C and 400 MHz for 1H at 25°C temperature. The pulse widths for protons and carbon were measured at 10.15 and 8.14 µs, respectively. Chemical shift (δ) values are expressed in Parts Per Million (ppm) relative to the tetramethylsilane signal. Spectrum recording in magnitude mode was carried out using 10 mg of the sample dissolved in Deuterated Dimethyl Sulfoxide (DMSO-d6). High-resolution ^{1}H and ^{13}C NMR spectra were obtained by collecting approximately 16-32 and 500-5000 scans, respectively, with a recycle time of 2-3 seconds. All samples were scanned from 0-10 ppm for 1H and 0-200 ppm for 13C NMR. The computer memory allocated for data accumulation was 4 kB. The spectral data were processed by applying sine bell window functions, unshifted in F1 dimension and π/4 shifted in F2 dimension.

2.10 Fatty acid (FA) composition analysis

FA analysis of N. sativa seed oil was determined by following the AOAC official method 969.33 against the standard. Saponification breaks down glycerides and phospholipids, releasing FAs, which are converted to their methyl esters using boron trifluoride (BF3) as a catalyst. 100µL of the oil sample was kept for reflection by adding 10 mL of 0.5 molar methanolic NaOH in a 250 mL round-bottom flask for 15 minutes at 60-70°C, followed by adding 10 mL of 14% BF3-MeOH solution and refluxing for 10 minutes at 60-70°C. Then, the RB flask was left to cool to ambient temperature, 10 mL of hexane was added, and the mixture was refluxed for 1 minute. The sample collected in a 50 mL tube was centrifuged for 2 minutes at 4000 rpm. The supernatant layer is collected and filtered using a 0.22 µ filter. The filtrate was fed into the GC-FID (Model: Agilent 7890B) to analyze the fatty acid methyl ester (FAME) of the extracted N. sativa oil with the DB-Fast FAME column (30 m × 250 µm × 0.25 µm dimensions). The initial flow rate was 1 mL/min, with a post-run of 0.5 mL/min. The initial temperature was 50°C and increased to 194°C at 30°C/min with a 3.5 min hold, then increased to 240°C at 5°C/min with a 1 min hold. Individual FAMEs were recognized by contrasting their RTs with those of FAME standards treated under identical conditions (Gawron et al., 2021).

2.11 Scanning Electron Microscopy (SEM) Analysis

The morphological characteristics of N. sativa seeds and the defatted seeds obtained after SCFE-CO_2 extraction were examined using a Carl Zeiss Gemini SEM 360 scanning electron microscope (Germany). The samples were analyzed at magnifications of 500× and 1000×. Before imaging, the samples were affixed to metal stubs with double-sided adhesive carbon tape and subse-

quently sputter-coated with a thin layer of gold under vacuum. The observations were carried out at an accelerating voltage of 5 kV.

2.12 Determination of Radical Scavenging Activity (RSA) of TQ in N. sativa oil

The antioxidant activity was evaluated using the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay, a spectrophotometric method based on radical neutralisation. In this assay, antioxidant compounds donate hydrogen atoms, reducing DPPH in methanol to its non-radical form. The oil was dissolved in dimethyl sulfoxide to prepare the sample solution. It was then mixed with the DPPH solution and incubated in the dark for 30 minutes. The optical density (OD) was measured at 715 nm.

The method relies on monitoring the color transition of DPPH, where its deep purple color diminishes to yellow upon reduction. The results were expressed as percentage radical scavenging activity (%RSA) using Eq (3).

$$\% \, RSA = \frac{\text{Control OD} - \text{Oil sample OD}}{\text{Control OD}} \times 100 \quad (3)$$

1. RESULTS

1.1 Results obtained from the RSM-FFD

Based on the experimental design, we performed 27 experiments (Table 2). The range of N. sativa oil yield was 9.2% to 33.2%, thymoquinone content 1.13% to 4.06%, and DPPH 50.31% to 98.41%. We used regression analysis to fit a second-order polynomial equation to the experimental data. The ANOVA results show the adequacy of the fitted model and the significance of the experimental factors for all responses. **Tables 3, 4, and 5** represent the ANOVA results for the responses, namely yield, TQ, and DPPH, respectively. The fitted models were significant for the three responses. The coefficient of determination (R^2) values was observed to be 0.96, 0.87, and 0.85, respectively. All three factors significantly affected the responses.

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Table 3. ANOVA of yield

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	14.44	9	1.60	44.57	< 0.0001	significant
A-Temperature	2.81	1	2.81	78.04	< 0.0001	
B- Pressure	6.56	1	6.56	182.29	< 0.0001	
C-Particle Size	4.05	1	4.05	112.39	< 0.0001	
AB	0.0170	1	0.0170	0.4720	0.5013	
AC	0.0162	1	0.0162	0.4510	0.5109	
BC	0.0812	1	0.0812	2.26	0.1513	
A^2	0.7546	1	0.7546	20.97	0.0003	
B^2	0.0094	1	0.0094	0.2610	0.6160	
C^2	0.0020	1	0.0020	0.0563	0.8153	
Residual	0.6119	17	0.0360			
Cor Total	15.05	26				

Table 4. ANOVA of thymoquinone

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.07	3	0.3557	29.63	< 0.0001	significant
A-Temperature	0.1108	1	0.1108	9.23	0.0058	
B- Pressure	0.6575	1	0.6575	54.77	< 0.0001	
C-Particle Size	0.3000	1	0.3000	25.00	< 0.0001	
Residual	0.2761	23	0.0120			
Cor Total	1.34	26				

Table 5. ANOVA of DPPH

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	13.64	9	1.52	10.94	< 0.0001	significant

A-Temperature	2.22	1	2.22	16.06	0.0009
B- Pressure	5.75	1	5.75	41.52	< 0.0001
C-Particle Size	2.52	1	2.52	18.20	0.0005
AB	0.5642	1	0.5642	4.08	0.0596
AC	0.1433	1	0.1433	1.04	0.3232
BC	0.1855	1	0.1855	1.34	0.2631
A^2	0.8052	1	0.8052	5.82	0.0275
B^2	0.8037	1	0.8037	5.81	0.0276
C^2	0.2850	1	0.2850	2.06	0.1695
Residual	2.35	17	0.1385		
Cor Total	15.99	26			

Figure 3 illustrates the effects of extraction factors on the responses. The figures also show similar effects across all responses. As pressure increases, the density of the supercritical fluid and its solvent power increase, resulting in higher extraction yield. Increasing temperature provides a dual competitive effect by increasing solvent pressure on the yield. Additionally, high tem-perature induces thermal degradation, oxidation, and polyphenol breakdown. This reduces thymoquinone (TQ) and DPPH. On the other hand, the negative effect of pressure could be attributed to the channeling and dilution effects on the sample during the extraction process. The particle size showed negative effects

due to the mass transfer barrier, reduced surface area, internal cell inaccessibility, and slow kinetics during the extraction process.

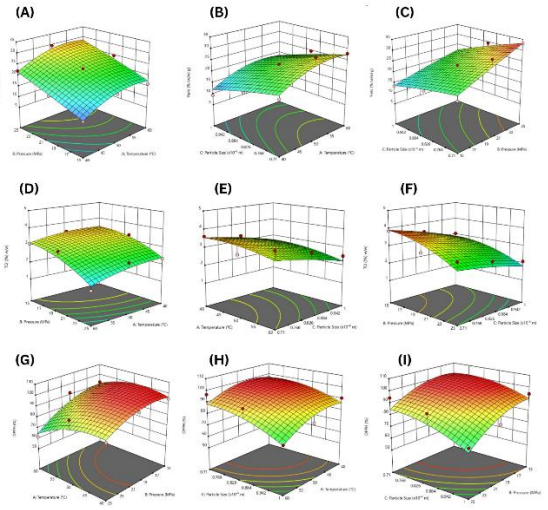


Fig 3. 3D response surface plots (a-c), Contour plots (d-f), Box-Cox plots (g-i), and Residuals vs Run graphs (j-l) of yield, thymoquinone, and DPPH indicate the model's behaviour and performance.

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1.2 Quantitative Analysis of TQ via the GC-MS/MS

GC–MS/MS operating in Single Ion Monitoring (SIM) mode provided a robust quantification method, as evidenced by the scan data shown in **Figure 4** At a retention time of 5.093 minutes, 4.06% TQ was detected from the oil sample, obtained under conditions of 40 °C temperature, 15 MPa pressure, and a particle size of 0.71 × 10^{-3} m, against the pure TQ standard.

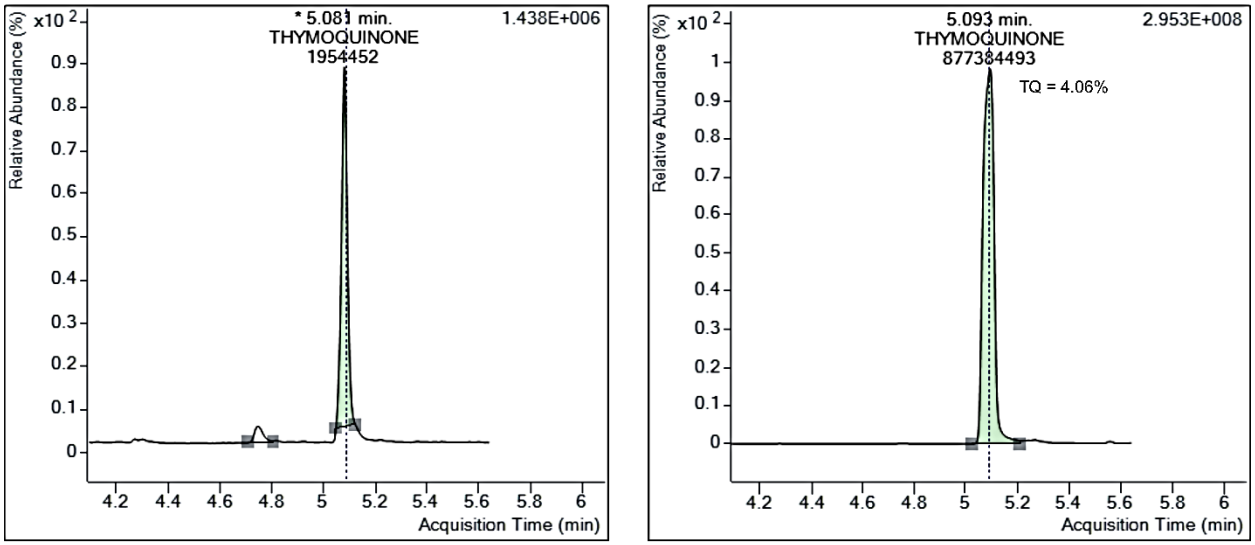


Fig 4: GC-MS/MS (SIM mode) analysis result of thymoquinone content in (a) standard thymoquinone, and (b) thymoquinone in SCFE-CO_2 extracted *N. sativa* oil with extraction conditions at 15 MPa, 40°C, and 0.71 × 10^{-3} m particle size.

1.3 Quantitative Analysis of TQ via the GC-FID

A better quantification result, supported by the GC-FID chromatogram in **Figure 5**, showed TQ of 40739.86 mg/kg, at 5.094 min, which is equivalent to 4.07% (w/w) in the SCFE-CO_2-extracted N. sativa oil sample at conditions 40°C temperature, 15MPa pressure, and 0.71 × 10^{-3} m particle size against pure TQ standard.

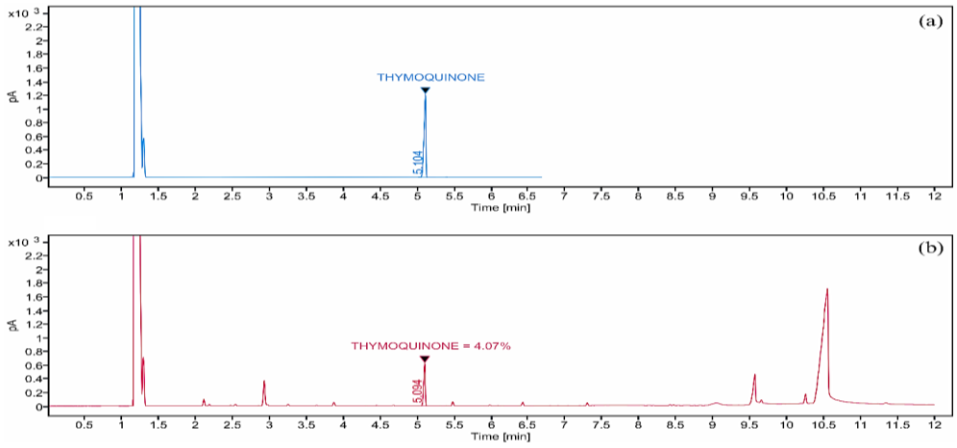
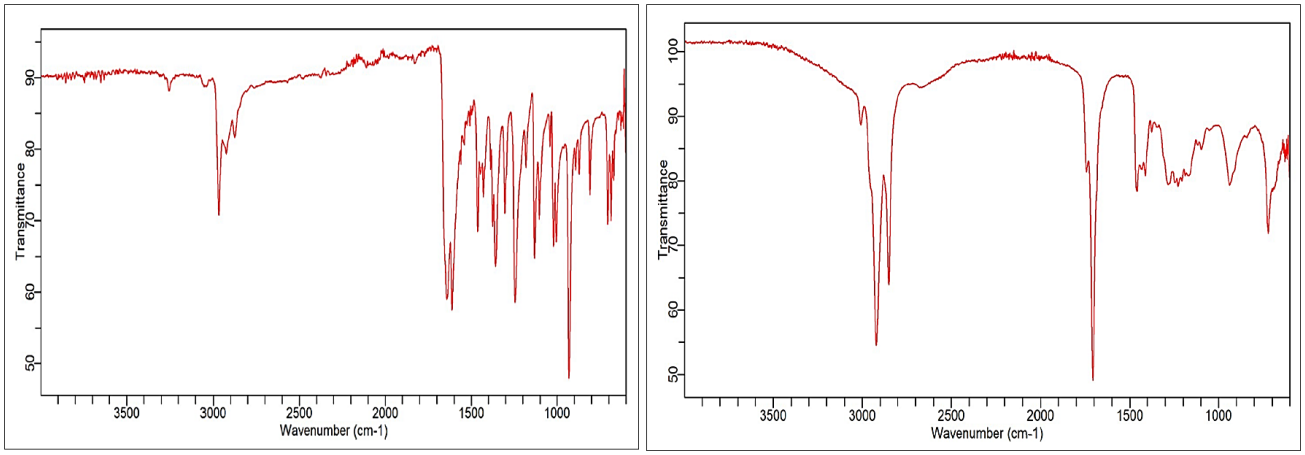


Fig 5. GC-FID chromatograms of (a) standard thymoquinone and (b) thymoquinone in SCFE-CO_2 extracted *N. sativa* oil sample, validating the estimation of optimum TQ content of 4.06% w/w in *N. sativa* oil with extraction conditions at 15 MPa, 40°C, and 0.71 × 10^{-3} m particle size.

1.4 FTIR analysis to show the existence of TQ through crucial groups in SCFE-CO_2 extracted N. sativa oil

N. sativa seed oil contains several bioactive compounds, among which TQ is the most prominent due to its conformational flexibility. Previously, thymoquinone was estimated using Gas Chromatography (GC-MS/MS) and GC-FID analysis. Additionally, FTIR spectra revealed the presence of distinct sharp, strong, and weak peaks corresponding to crucial functional groups such as C-H, -CH2, -CH3, N. sativa seed oil carbonyl (C=O), ether (C-O), and alkene (C=C) through the peaks, suggesting the presence of TQ with respect to standard TQ in **Figure 6.**



(a) (b)

Fig 6: FTIR spectrum of (a) standard thymoquinone and (b) thymoquinone in SCFE-CO_2 extracted *N. sativa* oil sample scanned at 600 cm^{-1} – 4000 cm^{-1}.

1.1 Qualitative determination using ^{1}H NMR and ^{13}C NMR spectroscopy

^{1}H NMR spectra as shown in **Figure 7** represent (a) pure thymoquinone (TQ), (b) thymoquinone in SCFE-CO_2 extracted N. sativa seed oil sample, and (c) stack overlay of pure TQ and TQ in SCFE-CO_2 extracted N. sativa seed oil sample. The chemical shift for the aromatic protons of thymoquinone falls within the (δ) 6-7 ppm range, which is identical to that observed for the thymoquinone in the SCFE-CO_2 extracted N. sativa seed oil sample, indicating their averages are equivalent. In the upfield region, where aliphatic protons typically appear **Table 6**, entries (3-5), the values range from (δ) 1-3 ppm, with both samples showing the same average. This proton experiment confirms the presence of thymoquinone in the SCFE-CO_2-extracted N. sativa seed oil sample.

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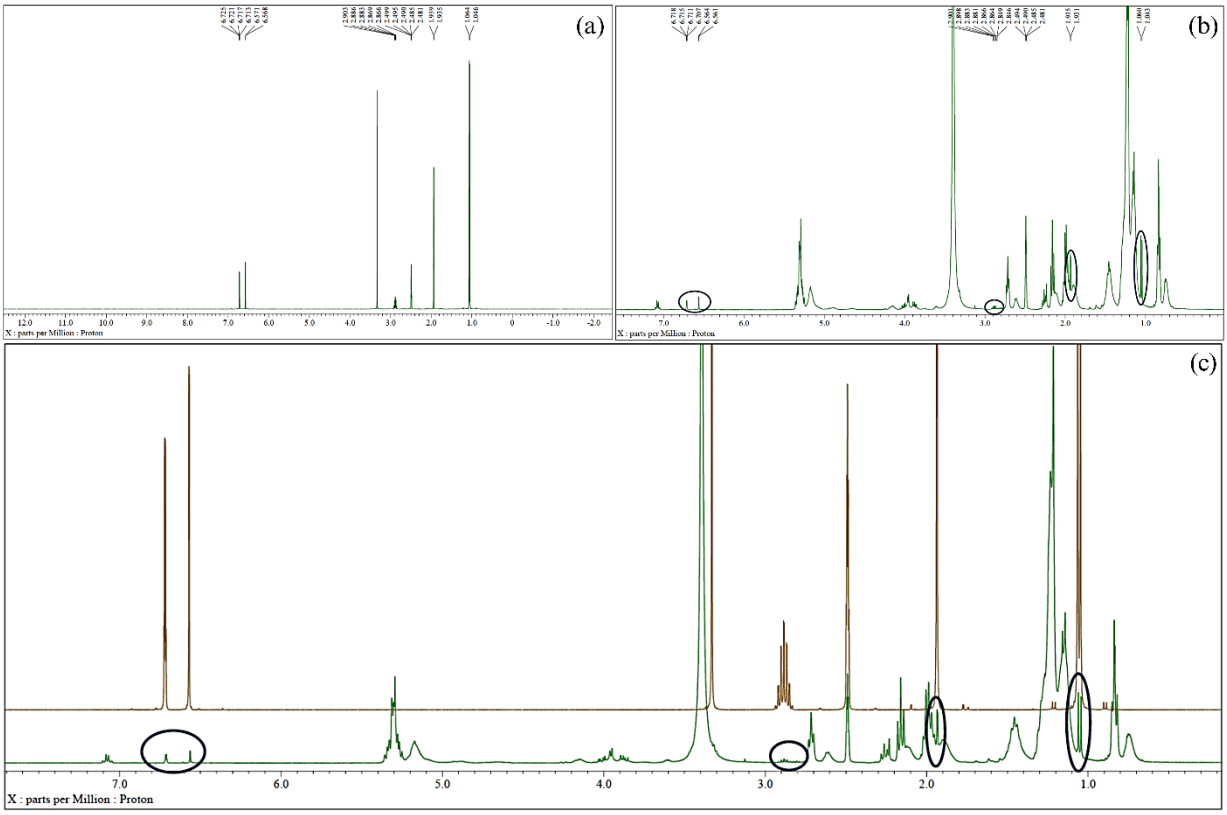


Fig 7. 1H NMR spectra of (a) pure thymoquinone (TQ), (b) thymoquinone in SCFE-CO2 extracted N. sativa oil sample, and (c) stack overlay of pure TQ and TQ in SCFE-CO2 extracted N. sativa oil sample. The aromatic proton chemical shifts (δ 6–7 ppm) and aliphatic proton shifts (δ 1–3 ppm) observed in both spectra are consistent, indicating the presence of thymoquinone in the SCFE-CO2 extracted N. sativa oil sample.

Table 6: 1H NMR chemical shifts of pure thymoquinone and thymoquinone in SCFE-CO2 extracted N. sativa oil sample

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Sr. No.	^{1}H Chemical Shift of TQ (ppm)	^{1}H Chemical Shift of TQ in SCFE-CO_2 extracted *N. sativa* oil sample (ppm)	Average of chemical shift TQ (ppm)	Average of chemical shift TQ in SCFE-CO_2 extracted *N. sativa* oil sample (ppm)
1	6.725, 6.721, 6.717, 6.713	6.718, 6.715, 6.711, 6.707	6.712	6.719
2	6.571, 6.568	6.564, 6.561	6.569	6.562
3	2.937, 2.935, 2.920, 2.917, 2.903, 2.900, 2.886, 2.883, 2.869, 2.866, 2.851, 2.849, 2.834, 2.831	2.918, 2.915, 2.901, 2.898, 2.883, 2.881, 2.866, 2.864, 2.849, 2.846	2.88	2.88
4	1.939, 1.935	1.935, 1.931	1.937	1.933
5	1.064, 1.046	1.060, 1.043	1.055	1.051

Figure 8 shows the 13C NMR spectra of (a) pure thymoquinone (TQ), (b) thymoquinone in SCFE-CO_2 extracted N. sativa seed oil sample, and (c) stack overlay of pure TQ and TQ in SCFE-CO_2 extracted N. sativa seed oil sample. The chemical shift for the aromatic carbon of thymoquinone, as illustrated in **Figure 8.**

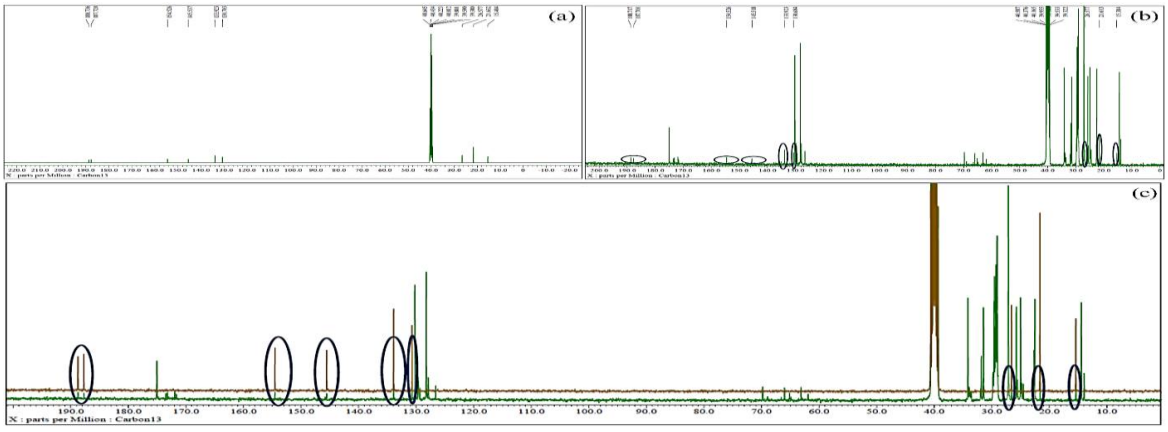


Fig 8. 13C NMR spectra of (a) pure thymoquinone (TQ), (b) thymoquinone in SCFE-CO2 extracted N. sativa oil sample, and (c) stack overlay of pure TQ and TQ in SCFE-CO2 extracted N. sativa oil sample. The aromatic carbon chemical shifts (δ 130–190 ppm) and aliphatic proton shifts (δ 15–26 ppm) observed in both spectra are identical, confirming the presence of thymoquinone in the N. sativa oil sample.

Entries 1- 6 are observed between (δ) 130-190 ppm, which is consistent with thymoquinone in the SCFE-CO_2-extracted N. sativa seed oil sample, showing identical ppm values in both samples. The region indicating aliphatic carbon, typically found in the upfield region (**Table 7**, entries 7-9), ranges from (δ) 15-26 ppm, and both samples show the same ppm values. This carbon analysis confirms the presence of thymoquinone in the SCFE-CO_2-extracted N. sativa seed oil sample.

Table 7: ^{13}C NMR chemical shifts of pure thymoquinone and thymoquinone in SCFE-CO_2 extracted *N. sativa* oil sample

Sr. No.	^{13}C Chemical Shift of TQ in ppm	^{13}C Chemical Shift of TQ in SCFE-CO_2 extracted *N. sativa* oil sample in ppm
1	188.736	188.717
2	187.720	187.701
3	154.526	154.526
4	145.537	145.518
5	133.923	133.923
6	130.703	130.684
7	26.577	26.577
8	21.632	21.613

1.2 Fatty acid composition of SCFE-CO_2 extracted N. sativa seed oil

Table 8 shows the FA composition of SCFE-CO_2-extracted N. sativa seed oil with RT, where the conjugated linoleic acid (C18:2 n-6) was 47.32%, saturated fatty acid (SFA) 25.48%, polyunsaturated fatty acid (PUFA) 49.67%, monounsaturated fatty acid (MUFA) 24.85%, ω-6 49.67%, and ω-9 24.85%. **Figure 9** shows the chromatogram of FA composition in (a) standard thymoquinone and (b) N. sativa seed oil extracted through SCFE-CO_2.

Table 8. Fatty acid composition of SCFE-CO_2 extracted *N. sativa* seed oil

FAs	Nomenclature	RT	Content (%)
C18:0	Methyl stearate	9.336	4.43
C16:0	Methyl palmitate	7.748	21.05
SFA			25.48
C18:2 ω-6	Methyl linoleate	10.181	47.32

C20:2 ω-6	Methyl cis-11,14-eicosadienoate	12.37	2.35
PUFA			49.67
C18:1 ω-9	Methyl cis-9 oleate	9.634	24.85
MUFA			24.85
Trans Fat			0.0
C18:2 n-6	Conjugated linoleic acid		47.32

FA: Fatty acid; SFA: Saturated fatty acid; MUFA: Monounsaturated fatty acid; PUFA: Polyunsaturated fatty acid; RT: Retention time

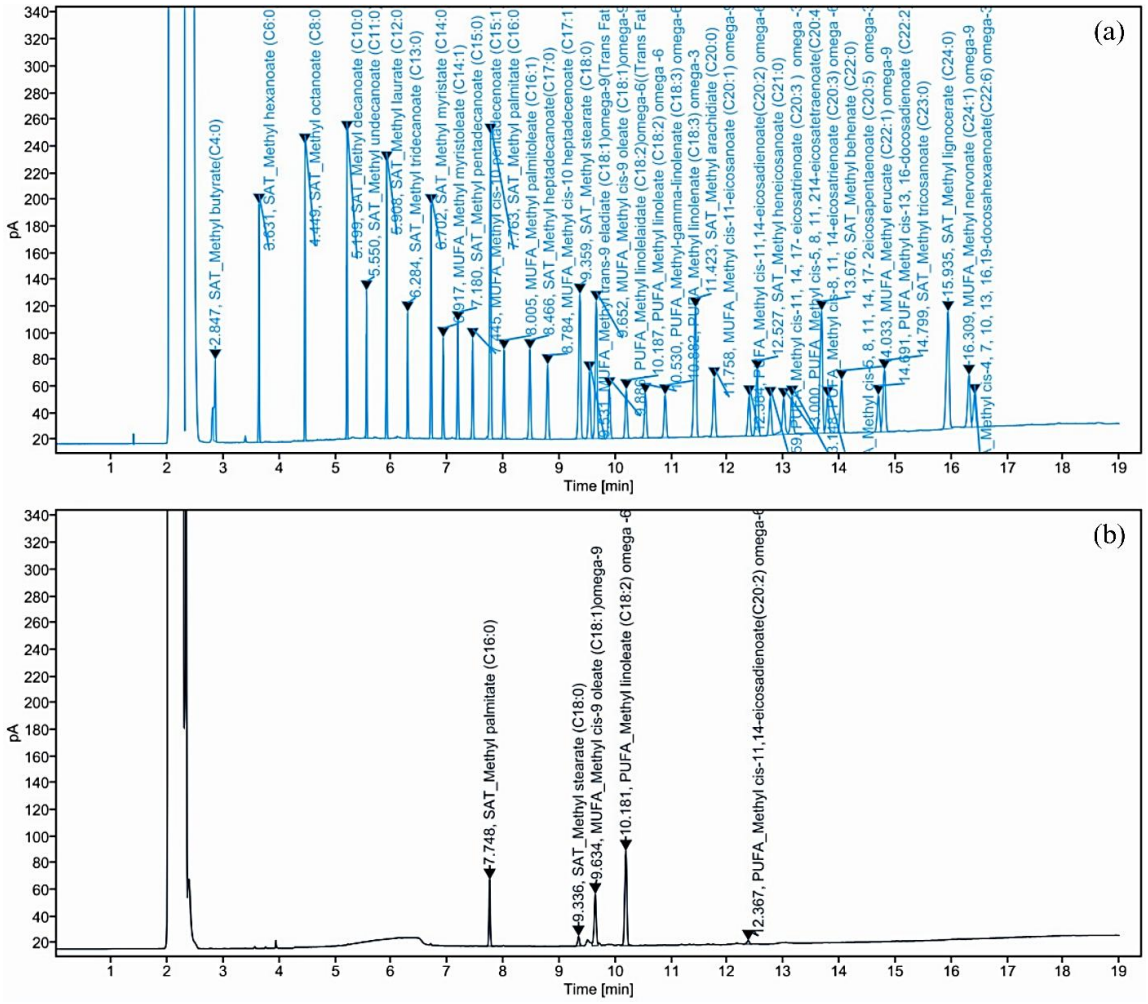


Fig 9. Chromatogram of fatty acid composition in (a) standard thymoquinone and (b) SCFE-CO_2 extracted *N. sativa* seed oil**.**

1.3 SEM Analysis of N. sativa Seeds Before and After SCFE-CO_2 Extraction

The surface morphology was examined at magnifications of 500× and 1000×. The micrographs in **Figure 10 (a & b)** show a compact, well-organized cellular architecture in untreated N. sativa seeds. In contrast, images in **Figure 10 (c & d)** reveal disrupted cell wall integrity and an uneven, irregular surface in the defatted seeds after SCFE-CO_2 extraction, indicating efficient

recovery of oil and associated bioactive constituents.

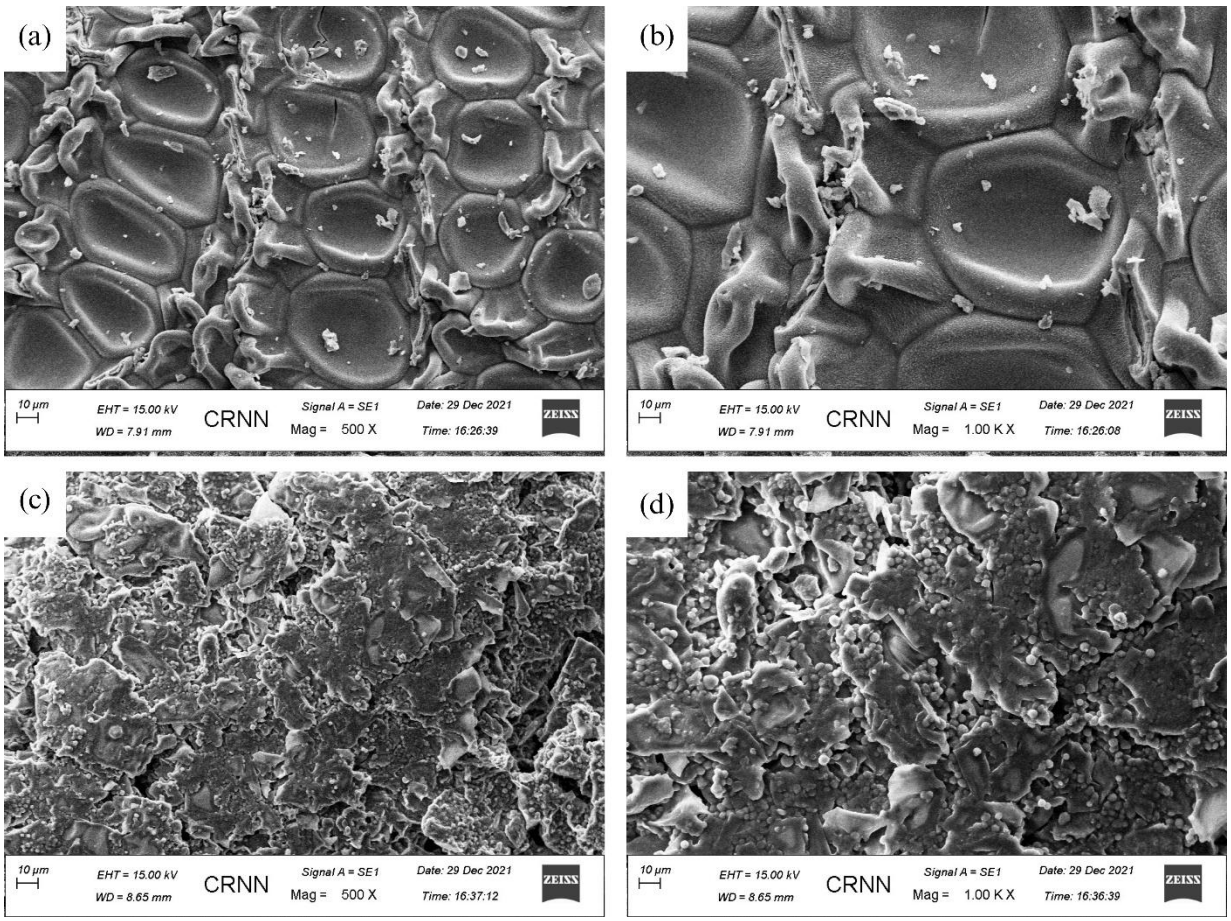


Fig 10. Microstructural study using Scanning Electron Microscopy (SEM) at 500X and 1KX magnification of (a & b) *N. sativa* seed and (c & d) post SCFE-CO_2 extracted defatted *N. sativa* seed.

1.4 RSA activity of SCFE-CO_2 extracted N. sativa seed oil

The antioxidant potential of N. sativa seed oil was assessed through DPPH radical scavenging activity (DPPH-RSA). The oil showed a substantially reduced optical density (0.025) compared with the standard (1.576), indicating strong neutralisation of DPPH radicals. This corresponds to a radical scavenging activity of 98.41%, as determined using Eq. (3), confirming the strong antioxidant potential of N. sativa seed oil.

2. DISCUSSION

This study demonstrated an efficient and sustainable approach for extracting thymoquinone-rich *Nigella sativa* L. seed oil using supercritical carbon dioxide extraction (SCFE-CO_2). The process enabled the recovery of both seed oil and thymoquinone under relatively mild operating conditions, which is advantageous for preserving thermolabile phytochemicals and other bioactive constituents.

Compared with conventional extraction techniques such as hydro-distillation, Soxhlet extraction, and steam distillation, SCFE-CO_2 offers several advantages, including solvent-free extraction, high selectivity, reduced thermal degradation of heat-sensitive phytochemicals and bioactives, lower environmental impact, and convenient solvent removal through CO_2 recycling. These characteristics make SCFE-CO_2 an attractive and potentially scalable technology for recovering high-value natural products.

The optimized extraction conditions for maximum oil yield were 20 MPa, 55 °C, and an average particle size of 0.71×10^{-3} m, resulting in a maximum *N. sativa* seed oil yield of 34.24% (w/w). In contrast, the highest thymoquinone content of 4.06% (w/w) was obtained at 15 MPa, 40 °C, and an average particle size of 0.87×10^{-3} m. The difference between the optimum conditions for oil yield and thymoquinone recovery indicates that extraction parameters influence overall oil recovery and selective enrichment of individual bioactive constituents differently. The lower temperature associated with maximum thymoquinone recovery may also help minimize thermal degradation of this heat-sensitive compound.

Comprehensive physicochemical characterization confirmed the quality and chemical characteristics of the extracted oil. The high DPPH radical scavenging activity of 98.41% further demonstrated the extracted oil's strong antioxidant potential, likely associated with thymoquinone and other bioactive constituents.

Overall, SCFE-CO_2 provides an effective and sustainable extraction platform for producing *N. sativa* seed oil enriched with thymoquinone. The optimized process and comprehensive characterization demonstrate its potential to obtain high-value, bioactive-rich oil for applications in food, nutraceutical, pharmaceutical, and cosmeceutical formulations.

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AUTHOR CONTRIBUTIONS

G.C. conceived the study, developed the methodology, conducted the investigation, curated the data, prepared the visualizations, and wrote the original draft. S.C. contributed to optimization studies, artificial neural network modelling, swarm intelligence analysis, and data interpretation. A.R. contributed to the experimental work, drafting of the manuscript, and participated in writing, reviewing, and editing the final version. A.K.S. contributed to the study's conceptualisation and provided critical review and editing of the manuscript. A.S. contributed to the conceptualization of the study and provided critical review and editing of the manuscript. All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST

The authors declare no competing interests.

ETHICS STATEMENT

This research did not involve human participants, animal subjects or the collection of primary data, and ethical approval was therefore not required.

DATA AVAILABILITY

The data will be made available upon reasonable request.

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