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Optimization of Ultrasonic Assisted Extraction Conditions Using Response Surface Methodology and Identification of Thymoquinone from Black Cumin (*Nigella sativa* L.) Seed Extract[§]

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SUMMARY

Research background. Nigella sativa L., commonly known as black cumin, is a medicinal plant renowned for its rich bioactive composition and health-promoting properties. Among its key compounds, thymoquinone has gained significant attention in nutraceutical and pharmaceutical research for its potential to prevent and manage chronic inflammatory conditions and immune dysfunctions. With growing global interest in natural health solutions, this study aims to optimize ultrasonic-assisted extraction (UAE) conditions to maximize thymoquinone yield and characterize the bioactive compounds. By employing UAE and advanced analytical techniques, the research contributes to developing sustainable, bioactive-rich extracts with applications in health and nutrition. The present study aims to optimize the ultrasonic-assisted extraction conditions for bioactive compounds and to identify thymoquinone in the extract of black cumin (*Nigella sativa* L.) seeds.

Experimental approach. In this study, ultrasonic assisted extraction method was employed with response surface methodology (RSM) software, in order to extract the bioactive compounds, including total phenolic content (TPC) and DPPH radical scavenging activity. In order to enhance the

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extraction efficiency of bioactive compounds, the following conditions were determined: mass of seed powder to volume of solvent ratio of 50–100 %, extraction temperature of 30 °C, amplitude of 30–60 % and extraction time of 30–60 min. Black cumin seed extracts were characterized using scanning electron microscopy (SEM), while gas chromatography-mass spectrometry (GC-MS) analysis was conducted to identify thymoquinone. Additionally, Fourier transform infrared (FT-IR) spectroscopy confirmed the presence of thymoquinone and several functional groups, including amines, alkanes, acids, esters, alkyls, and alkenes.

Results and conclusions. Ultrasonic extraction using methanol as a solvent resulted in a higher yield of thymoquinone (28.62 %), identified through GC-MS analysis. The presence of thymoquinone was further confirmed by the functional groups detected in FTIR analysis. Under the specified extraction conditions, total phenolic content (TPC), yield percentage, and DPPH radical scavenging activity increased by approximately 271.03 mg/g GAE, 4.5 %, and 83.06 %, respectively. In addition to thymoquinone, thymohydroquinone was also identified based on its molecular mass, retention time, and peak values. Thymoquinone (TQ), a naturally derived and potent phytochemical, offers a range of therapeutic properties, including immune-enhancing potential.

Novelty and scientific contribution. Thymoquinone is a bioactive compound found in black cumin seeds, known for its potent antioxidant and immunity boosting properties. This research was conducted with the intention of achieving the best possible extraction conditions for bioactive substances. Additionally, the results support the potential of thymoquinone as a therapeutic agent for the management of various health conditions. The novelty lies in the development and optimization of extraction techniques to maximize the yield and bioactivity of thymoquinone, a compound renowned for its robust antioxidant and immune-modulating properties. This work uniquely bridges the gap between traditional uses of black cumin and modern scientific validation, addressing global health priorities. The findings underscore the importance of *Nigella sativa* as a sustainable and natural source of health-promoting compounds, aligning with the increasing demand for plant-based bioactive compounds in preventive healthcare. By characterizing the extraction conditions and demonstrating therapeutic potential of thymoquinone, this study contributes to both the academic literature and practical advancements in functional food and nutraceuticals development.

Keywords: ultrasonic extraction optimization; thymoquinone identification; black cumin; FTIR analysis; GC-MS analysis; antioxidant potential

INTRODUCTION

Black cumin (*Nigella sativa* L.), an annual herb belonging to the Ranunculaceae family, is indigenous to the Mediterranean region, Northern Africa, Southern Europe, and Southwest Asia (*1,2*). The western and northern regions of India are the most important areas for the cultivation of black cumin. These regions include Punjab, Himachal Pradesh, Madhya Pradesh, Bihar, West Bengal, Assam, Rajasthan, and Maharashtra (*3*). Black cumin is also known by various names worldwide, including kalonji, kalajeera, nutmeg flower, devil-in-the-bush, black caraway, and Roman coriander (*4*). In 'The Canon of Medicine', Avicenna recognized it as a natural remedy within the "Tibb-e-Nabavi" (Unani-Tibetan system of medicine), highlighting its use in treating a range of ailments 5. According to (*6*), *Nigella sativa* is a bisexual herbaceous plant that grows to a height of 20–90 cm and produces solitary flowers with multiple seeds encased in inflated capsules. Black cumin seeds are rich in bioactive compounds, containing essential oils (0.13–0.39 %), fatty oils (35–50 %), alkaloids, steroids, phenolic compounds, terpenoids, and saponins (*7*). A study by (*8*) found that *Nigella sativa* seeds contain moisture (7.06 %), crude protein (18.67 %), crude fat (45.09 %), crude fiber (7.33 %), ash (4.33 %), and total carbohydrates (17.51 %) on a dry mass basis. Black cumin seeds contain trace amounts of essential minerals, including calcium (2.63 µg/mL), potassium (1.94 µg/mL), zinc (1.24 µg/mL), copper (0.31 µg/mL), and magnesium (0.30 µg/mL) on a dry mass basis (*9*). The primary phytochemicals in black cumin seeds include thymoquinone (TQ), dithymoquinone, thymohydroquinone, carvacrol, p-cymene, sesquiterpene, thymol, 4-terpineol, longifolene, t-anethole, and α-pinene (*10*). Thymoquinone (TQ), a bioactive compound derived from the seeds of *Nigella sativa* (black cumin), has gained significant attention due to its potent antioxidant, anti-inflammatory, anticancer, and antimicrobial properties (*11*). Despite its pungent and bitter aroma, black cumin has been used to flavor a variety of foods, including vegetables, bread, curries, pickles, condiments, and savory dishes (*12,13*). In addition to being a key ingredient in garam masala, black cumin seeds are also one of the five spices in *panch phoran*, a traditional spice blend made from cumin seeds, black mustard seeds, fenugreek seeds, and black cumin seeds (*14,15*).

According to Salehi *et al.* (*16*), nigella seeds have garnered significant interest in the fields of food, cosmetics, and pharmaceuticals. They are known for their therapeutic properties, including the treatment of digestive and respiratory disorders such as asthma (*17*), bronchitis, dysentery, and stomachaches (*18*), as well as their use as an insect repellent, for rheumatism, and in improving kidney and liver function (*19*). Black cumin comprises many substances that are bioactive (*20*), encompassing thymoquinone, which exhibits a range of beneficial effects, such as anticancer,

antioxidant (*21*), antibacterial (*22*), antifungal, anti-inflammatory (*23*), antiviral (*24*), and immune-modulatory properties (*25*).

In this study, the aim was to maximize the amount of total phenolic compounds (TPC), antioxidant activity (DPPH method), and yield percentage from kalonji seed extract by ultrasound assisted extraction. The optimization process was conducted by RSM software, using three independent factors (time, amplitude, and mass of seed powder to volume of solvent ratio): 30–60 min, 30–60 %, and 50–100 %. The best optimal conditions were determined in order to estimate the effective extraction of bioactive compounds and yield percentage.

MATERIALS AND METHODS

Chemicals and reagents

Standard of thymoquinone was purchased from Sigma-Aldrich chemicals private limited (Bangalore, Karnataka, India), HPLC gradient grade methanol and dimethyl sulfoxide (DMSO) were purchased online from Merck (Darmstadt, Germany). Hydrochloric acid (35 %) from Hi-media (Mumbai, Maharashtra, Inidia) Folin-Ciocalteu reagent from Sigma-Aldrich (Bangalore, Karnataka, India), sodium carbonate and aluminum chloride were purchased from Hi-media (Mumbai, Maharashtra, Inidia).

Plant material and extract preparation

During the month of November 2023, mature black cumin seeds of the *Nigella sativa* AN-1 variety were acquired from the Indian Council of Agricultural Research's National Research Centre on Seed Spices (ICAR-NRCSS) in Ajmer, Rajasthan, India. It was necessary to wash the black cumin seeds with deionised water in order to get rid of any debris or seeds that were damaged. After being washed, the seeds were dehydrated by placing them on a drying tray and using hot air at a temperature of 35 °C. After being dried, the seeds were given a fine powdering and then stored at a temperature of -20 °C in deep freezer (Blue Star, CHMK-50), for later use. The moisture percentage of the fresh seed powder, measured in mass of seed powder per volume of solvent, was m/V=(2.19±0.4) %.

Proximate analysis

The analysis of the proximate values for moisture, crude fat, protein, dietary fibre, and ash was carried out in accordance with the standards that have been authorised by the Association of official Analytical Chemists (AOAC), as followed by Khalid *et al.* (*26*). When the total amount of protein, fat, moisture, fibre, and ash in *Nigella sativa* seeds was subtracted from 100, the result was the

amount of carbohydrates that were present in the seeds. The validity of the results was checked by performing each analytical method three times to ensure that it was accurate.

Extraction procedure

The bioactive compounds from *Nigella sativa* seeds were extracted using an ultrasonic-assisted extraction method. Initially, a laboratory grinder was utilised to ensure that the ripe black cumin seeds were ground into a fine powder. The extraction process was carried out by mixing the powdered seeds with an appropriate solvent (methanol), in a 1:10 ratio of mass of seed powder to volume of solvent (*m/V*), in a 500 mL beaker. Ultrasonic extraction was performed using an ultrasonic probe (Hwasin, Powersonic, Indonesia), (frequency 20 kHz, amplitude 30–60 %), ensuring that the probe was immersed in the seed-solvent suspension. The extraction was carried out at a constant temperature of 32 °C, and the process was timed for varying durations between 30 to 60 min, depending on the experimental design. Ultrasonic energy was utilised to rupture the cell walls and facilitate the release of bioactive chemicals from the seed matrix into the solvent.

Immediately after the extraction procedure was completed, the suspension was filtered through Whatman filter paper in order to remove any solid residues that were present in the combination of liquid extract. After that, the extract was concentrated by evaporating the solvent in a rotary evaporator at a temperature of 45 °C while the pressure was lowered. The resulting crude extract was subjected to freeze-drying at -50 °C in lyophilizer (Freeze Dryer) (Alpha 2-4 LD Plus CHRIST, Germany) for 12 h to remove any remaining moisture and preserve the bioactive compounds. Finally, the dried extract was stored in amber glass vials at 4 °C for further analysis of bioactive compounds, including thymoquinone, TPC, DPPH assay, and yield percentage. For the purpose of ensuring accuracy and reproducibility of the results, each procedure was carried out three times with the intended samples. Ultrasonic-assisted extraction was employed to evaluate thymoquinone content, TPC, DPPH radical scavenging activity, and yield percentage from black cumin seeds. The extraction was carried out at a constant temperature of 32 °C and a frequency of 20 kHz, using a 2.0 cm diameter ultrasonic probe that was immersed in the seed-solvent suspension (*27*). The sample-solvent ratio was maintained at 25 g of black cumin seeds to 250 mL of solvent, placed in a 500 mL beaker. Following the ultrasonic treatment, Whatman filter paper was used to filter the suspension in order to separate the liquid extract from the solid residues remaining in the solution. The solvent was then removed from the sample mixture using a rotary evaporator, set to a temperature of 45 °C, to concentrate the extract. To preserve the bioactive compounds, the raw samples were subjected to freeze-drying at -80 °C for 12 h (*28*). After drying, the samples were

carefully stored in amber glass vials at 4 °C to protect them from light and moisture, ensuring their stability for more thorough investigation.

Determination of DPPH radical scavenging activity

In order to evaluate the DPPH radical scavenging activity, the methodology described by Mohammed *et al.* (*29*) was utilised. The DPPH was dissolved in methanol, which resulted in the preparation of a new stock solution of 0.1 mM stock. To determine the antioxidant capacity of the black cumin seed extracts, 3 mL of the DPPH solution was mixed with 1 mL of the methanolic extracts at varying concentrations. The resulting mixture was thoroughly shaken to ensure uniform distribution and then stored at room temperature (32 °C) in a dark environment to prevent light interference for 30 min. The absorbance of the sample mixtures, together with a control sample, was measured at 517 nm using a UV-VIS spectrophotometer (Thermo Scientific, G10S UV-VIS, USA). This was done after the incubation period had been completed. A comparison was made between the decrease in absorbance of the test samples and the control (*30*), which allowed for the calculation of the DPPH radical scavenging activity. With the help of the following equation, the scavenging capacity of the black cumin seed extracts was expressed as a percentage:

$$\text{DPPH radical scavenging activity} = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}\right) \cdot 100 \qquad /1/$$

where A_{control} represents the absorbance of a methanolic solution containing DPPH, while A_{sample} represents the absorbance of a solution containing DPPH combined with extracts from black cumin seeds.

This equation makes it possible to quantify the extract capacity to neutralise free radicals, which in turn provides insight into the antioxidant potential of the extract.

Determination of total phenolic content (TPC)

The Folin-Ciocalteu method was utilised as mentioned by Mohammed *et al.* (*29*) in order to determine the total phenolic content (TPC) of the black cumin seed extracts. This was done in line with the aforementioned reference. The production of a new stock solution of standard gallic acid was done in order to generate a calibration curve. In each and every analysis, one millilitre of the appropriately diluted extract was combined with half a millilitre of the Folin-Ciocalteu reagent that had been diluted tenfold (1:10 *V/V*). Each tube received 2 mL of a 20 % sodium carbonate (Na_2CO_3) solution after five minutes of incubation. The final volume was adjusted to 10 mL using distilled water. To enable complete reaction development, the sample combinations were then allowed to sit at room

temperature for an hour in a light-restricted setting. Following the incubation period, a UV-VIS spectrophotometer (Thermo Scientific, G10S UV-VIS, USA) was used to detect the sample absorbance at 760 nm. For the purpose of producing a calibration curve, gallic acid (mg) was used as the reference. The total phenolic content was expressed as mL of gallic acid equivalents (GAE) per one hundred grammes of dry sample mass. To ensure precision and reproducibility, all samples were analyzed in triplicates, and the results were reported as the mean value±standard deviation. This method provides an effective means of quantifying the phenolic compounds present in the black cumin seed extracts, which are known for their antioxidant activity.

Scanning electron microscopy image analysis

Scanning electron microscopy (SEM) was utilized to examine the morphological changes in black cumin seed matrices after ultrasonic-assisted extraction. A mass of 1.0 mg of sample was meticulously affixed to aluminium stubs and sputter-coated with a tiny gold layer to improve conductivity. High-resolution imaging was conducted at an appropriate accelerating voltage to capture detailed structural features. SEM analysis provided high-definition images, enabling the visualization of cell wall integrity, surface morphology, and structural disruptions induced by ultrasonic treatment. This analysis was instrumental in evaluating the degree of cellular disintegration, thereby validating the efficiency of the extraction process.

Gas chromatography-mass spectrometry analysis

The Shimadzu QP 2010 Ultra GC-MS apparatus, which was made in Japan, was utilised in order to conduct the analysis on the methanolic extract. This particular capillary column, known as the Rxi®-5 Sil MS column, was employed by the gas chromatograph (GC) in order to perform its analytic functions. The length of the column was twenty meters, its internal diameter (ID) was 18 mL, and the thickness of the film was 18 µm. After the sample had been diluted, it was injected using the standard mechanism, and the flow rate of helium gas was maintained at 1 mL per minute throughout the process. For a period of 5 min, the oven was preheated to a temperature of 70 ºC. An electron energy of 70 eV and an ion source temperature of 200 ºC are utilised in the process of mass spectrometry. According to the results of the GC-MS analysis performed on the seed extracts, the presence of thymoquinone was established.

Fourier transform infrared spectroscopy (FT-IR) characterization

The spectra of thymoquinone were recorded using FT-IR technique (Spectrum BX-II, Perkin Elmer). The spectra of thymoquinone displayed distinct peaks of varying intensity, suggesting the

presence of important functional groups such as C=O, C-H, -CH2, -CH3, C=C, and C-O. This suggests that thymoquinone was well incorporated into the black cumin seed extract. A strong band at 2951.86 cm $^{-1}$ indicates C-H stretching of tertiary carbons group.

Experimental design and statistical analysis

Statistical analysis was conducted using Design-Expert v. 13.0 software (*31*), a widely used tool for statistical design and analysis. A central composite design (CCD), which is a sort of response surface methodology (RSM), was utilised in this investigation in order to optimise the extraction process of black cumin seed extract into a more efficient manner. The primary variables under investigation were extraction time (ranging from 30 to 60 min), ultrasonic amplitude (30–60 %), and mass of seed powder to volume of solvent ratio (50–100 %). The optimization aimed to assess their effects on three key responses: total phenolic content (response 1), antioxidant activity using the DPPH method (response 2), and yield percentage (response 3). To ensure reliable and accurate results, each experiment was performed with three repetitions, and twenty different iterations of the experiment were carried out in total, systematically varying the factors within the defined ranges. This approach enabled the determination of the most effective conditions for maximizing the desired outcomes. For the purpose of ensuring the dependability and repeatability of the findings, the experiments were carried out three times simultaneously. In order to provide a clear depiction of the variability and precision of the measurements. The mean and the standard deviation were the two measures that were used to report the data that was collected from each experiment. For the purpose of optimising the experimental settings and analysing the impacts of various factors on the answers, statistical analysis was performed with the help of the Design-Expert v. 13.0 (*31*), which was utilised. In order to determine whether or not the differences that were discovered between the experimental groups were meaningful from a statistical point of view, a one-way analysis of variance (ANOVA) was carried out. When the p-value was found to be less than 0.05 ($p<0.05$), it was decided that the differences were statistically significant.

RESULTS AND DISCUSSION

Model fitting and RSM analysis

The effectiveness of ultrasonic extraction is significantly influenced by various experimental parameters, including extraction time, ultrasonic amplitude, and ratio of mass of seed powder to volume of methanol. Table 1 provides a detailed summary of the measured responses across 20 experimental runs, designed using central composite design (CCD), to evaluate the impact of these

factors. It is well-established that parameters such as the duration of ultrasonication, the intensity of the ultrasonic waves, and the ratio of mass of seed powder to volume of solvent (methanol, in this case) play a crucial role in determining the efficiency of bioactive compound extraction, including antioxidant activity, total phenolic content (TPC), and overall yield from seed extracts. For black cumin seed extracts, the results showed that the DPPH radical scavenging activity, TPC as GAE, and yield reached values of 83.06 %, 339.85 mg/g and 4.5 %, respectively. These findings emphasize the critical influence of extraction conditions on the recovery of bioactive compounds. Specifically, the optimization of ultrasonic-assisted extraction (UAE) parameters was shown to significantly enhance the phenolic antioxidant recovery. This underscores the importance of fine-tuning factors like ultrasonication time, amplitude, and mass of seed powder to volume of solvent to maximize the yield and antioxidant potential of black cumin seed extracts. These results further suggest that careful adjustment of ultrasonic extraction parameters can greatly improve the efficiency of extracting valuable compounds such as thymoquinone and other phenolic antioxidants from black cumin seeds. As such, the study highlights the need for optimization in the UAE process to achieve the best possible outcomes in terms of both yield and antioxidant activity.

Scanning electron microscopy (SEM)

Fig. 1 illustrates the morphological changes observed in black cumin seed extracts following the ultrasonic-assisted extraction process. At 40× magnification, as shown in Fig. 1a, the surface of the seed matrix appeared relatively smooth, with minimal disruption. This suggests that the ultrasonic extraction did not cause significant surface damage at lower magnification. However, when the sample was further magnified at 1000× (Fig. 1b), the structural changes became more evident. The ultrasonic treatment induced noticeable surface cracking and fragmentation, resulting in an irregular, porous structure. The detailed cellular images clearly show that the ultrasonic waves caused significant disruption to the cell walls, leading to the formation of cracks and voids in the matrix. This structural disintegration indicates that sonication effectively breaks down the cell walls, increasing the surface area and enhancing the release of bioactive compounds. The images at higher magnification provide compelling evidence that the ultrasonic treatment leads to maximum cell disruption and a loss of structural integrity. These findings suggest that the ultrasonic-assisted extraction process is highly efficient in promoting the release of valuable compounds from black cumin seeds. The increased porosity and cellular damage are likely contributing factors to the higher recovery of bioactive compounds such as thymoquinone, phenolic compounds, and other antioxidants. Therefore, it can

be concluded that sonicated samples yield the maximum amount of bioactive compounds, enhancing the overall extraction efficiency.

Gas chromatography-mass spectrometry (GC-MS)

The GC-MS chromatogram of the methanol extract from black cumin seeds revealed the presence of thymoquinone and thymohydroquinone. Thymoquinone (TQ) was the primary volatile component, contributing 28.62 %, while thymohydroquinone were tentatively characterized in extracts from black cumin seeds (Fig. 2). In a C18 stationary phase, the gas chromatogram showed two main peaks that were separated after 59 min of run time. The most prevalent compound is thymohydroquinone ($C_{10}H_{14}0_2$) and thymoquinone ($C_{10}H_{12}0_2$). These compounds were described using accurate m/z 166.22 and 164.20 mass fragmentation patterns that produced ion peaks (*32*).

Fourier transfrorm infrared spectroscopy (FTIR)

The FT-IR spectrum of the methanol solvent extract Fig. 3 displays a broad absorption band at 3400.92 cm^{-1}, indicating the presence of N–H stretching vibrations. The peaks observed between 3000 and 2800 cm^{-1} correspond to the stretching vibrations of isopropyl and -CH_3 groups in thymoquinone derivatives. Specifically, the peak at 2853.87 cm^{-1} represents the symmetric stretching of the three methyl groups, while the peak at 2923.68 cm^{-1} is associated with C-H stretching in the tertiary carbon of the isopropyl group. Research by Sopyan *et al.* (*33*) confirms that thymoquinone exhibits distinct FTIR absorption bands at 2924 cm^{-1} and 2854 cm^{-1}, further supporting these peak assignments for thymoquinone derivatives. These findings are consistent with the results reported by (*34*). Additional bands corresponding to C–O stretching vibrations, associated with alcohols, esters, and ethers, were observed in the range of 1112.94 cm^{-1} to 780.06 cm^{-1}.

Response surface methodology (RSM)

In ultrasonic-assisted extraction, a face-centred central composite design (CCD) was employed to optimize the integration of independent variables - A: time (30–60 min), B: mass of seed powder to volume of solvent (50–100 %), and C: amplitude (30–60 %) - with dependent variables, including DPPH activity (%), total phenolic content as *w*(GAE)/(mg/g), and extraction yield (%). The experimental design is detailed in Table 1. The results present the optimized and analysed factors (time, mass of seed powder to volume of solvent, and amplitude) in relation to the response variables (DPPH activity, total phenolic content, and yield). A total of 20 experimental trials, including 6 replications, were conducted using a central composite design to generate various combinations of

these factors. Table 2 presents the results of the analysis of variance (ANOVA), which includes an evaluation of the model's fit, statistical significance, and regression coefficients. In addition to the ANOVA results, several statistical parameters were used to assess the accuracy and reliability of the model. The analysis encompassed the coefficient of determination (R^2), modified R^2, and coefficient of variation (CV), all of which are crucial for assessing the model's fit to the experimental data and the variability of the responses. The correlation coefficients (R^2) for the three - were determined to be 0.980, 0.948, and 0.903, respectively. The elevated R^2 values indicate that the models effectively describe the data and are dependable for forecasting the system's behaviour. The R^2 value is crucial for evaluating the quality of fit, with values approaching 1 signifying a superior alignment between the model and the observed data.

The F-values obtained for the models corresponding to DPPH, TPC as GAE, and yield were 37.09 %, 13.31 mg/g and 16.78 %, respectively, all of which were statistically significant. The F-value is a critical parameter in ANOVA as it indicates the ratio of variance explained by the model compared to the unexplained variance. Higher F-values suggest a stronger and more reliable model. In this particular instance, the significant F-values for each response provide evidence that the models are successful in capturing the fundamental correlations that exist between the extraction parameters and the recovery of the bioactive compound recovery (*35,36*). The reliability of optimizing the extraction process using the fitted RSM is contingent on the strength of the model’s fit. This is determined by both the adequacy of the model and the statistical significance of the F-values. The high R^2 values and significant F-values indicate that the models are robust and can be confidently used for further optimization of the ultrasonic-assisted extraction process. These results highlight the importance of using appropriate statistical tools, such as ANOVA and response surface methodology (RSM), to optimize extraction conditions for maximizing the recovery of bioactive compounds from black cumin seeds. The 3D surface plots presented in Fig. 4, Fig. 5 and Fig. 6 were used to evaluate the adequacy of the model and to examine the relationships between the experimental variables under investigation. These plots visually represent the correlation between the independent factors and the response variables, providing valuable insight into how the extraction parameters influence the outcomes. These findings unequivocally revealed that the variables involved in the extraction process - such as extraction time, solvent volume, and amplitude - significantly affected the response variables, including antioxidant activity, total phenolic content (TPC), and yield percentage (*37*). The study revealed that interactions between the extraction time and mass of seed powder to volume of solvent ratio (A, B), amplitude and time (C, A), as well as solvent volume and amplitude (B, C), had a marked impact on the extraction outcomes. Specifically, the antioxidant activity was notably enhanced under

three conditions: a methanol volume of approximately 75 %, an extraction time of 30–45 min, and an amplitude setting of 30–45 %. These findings suggest that optimizing these parameters can significantly improve the yield of bioactive compounds, particularly those with antioxidant properties. Supporting the results of previous studies, such as by Chakraborty *et al.* (*38*), the ultrasonic-assisted extraction (UAE) process promotes the dissolution of phenolic compounds, with higher ethanol concentrations contributing to a more efficient extraction. As ethanol levels increase, the solubility of phenolic compounds improves, which enhances the overall antioxidant activity of the extracts. Thus, the interaction of key factors in UAE highlights the importance of optimizing these parameters for achieving the best possible outcomes in terms of both yield and antioxidant potential.

As water and ethanol are combined, water acts as a swelling agent, while ethanol plays a key role in dissolving the bonds that hold solutes within the cellular matrix facilitates the extraction of bioactive compounds (*39,40*), particularly thymoquinone, known for its strong antioxidant activity (*41*). Increased solvent enhances the extraction process by improving the dissolution of these compounds. However, the optimal mass of seed powder to volume of solvent for extracting thymoquinone was found to be 75 %, especially when coupled with an extraction time of 45 min. Under these conditions, the total phenolic content reached a moderate level, while the yield percentage peaked at 4.5 %, suggesting that these parameters provide the most efficient extraction. Similar findings have been reported in other studies (*42-44*), confirming that the recovery of bioactive compounds. In the current study, the thymoquinone content was found to be 28.67 % in the methanol extract, a result consistent with previous research (*45,46*), which reported thymoquinone content of 22 %. These findings underscore the effectiveness of ultrasonic-assisted extraction and highlight the use of methanol as an optimal solvent for maximizing thymoquinone yield from black cumin seeds.

CONCLUSIONS

The current work optimized the extraction conditions for bioactive compounds using response surface approach. In this study, the antioxidant activity, total phenolic content, and yield percentage of black cumin extract were analyzed. An enhanced extraction efficiency for thymoquinone (28.62 %) was also achieved using an ultrasonic-assisted extraction method. Ultrasonic extraction was optimized using a three-factor, three-response design, with quadratic polynomial statistical models developed and validated to enhance thymoquinone extraction. The study findings indicated that thymoquinone extraction efficiency reached 28.62 % when using ultrasonic extraction with methanol as the solvent. Total phenolic content (TPC) as GAE, yield and DPPH radical scavenging activity were improved to 271.03 mg/g, 4.5 % and 83.06 %, respectively. Thymoquinone content was significantly

influenced by both amplitude and mass of seed powder to volume of solvent. Additionally, the enhanced extraction efficiency of ultrasonic extraction was confirmed through SEM image analysis, which revealed notable disruption and disintegration of the matrix cell wall. GC-MS and FT-IR analyses confirmed the predominant presence of thymoquinone and thymohydroquinone. Consequently, black cumin seeds are considered a rich source of phenolic compounds and thymoquinone, which are key contributors to antioxidant activity. The novelty of this work lies in the detailed statistical optimization approach and validation of ultrasonic-assisted extraction, demonstrating its superiority in disrupting and disintegrating the matrix cell wall, as confirmed through SEM analysis. Additionally, the application of GC-MS and FT-IR analyses highlighted the dominance of thymoquinone and thymohydroquinone in the extracts, further emphasizing *Nigella sativa* as a rich source of phenolic compounds with potent antioxidant activity. This study contributes to advancing green extraction technologies and promotes the use of *Nigella sativa* as a sustainable source for developing nutraceuticals and functional foods. The findings offer a promising foundation for the large-scale production of bioactive-rich extracts and their potential application in health and pharmaceutical industries. To build upon the findings of this research, future studies could explore the encapsulation and formulation of thymoquinone-enriched extracts for improved bioavailability and stability. Additionally, investigating the therapeutic efficacy of these extracts in clinical settings for managing oxidative stress, inflammation, and immune-related disorders will further validate their potential. Expanding this work to include other bioactive compounds from *Nigella sativa* and exploring synergistic effects with other natural antioxidants could lead to novel functional food and pharmaceutical innovations.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

AUTHOR'S CONTRIBUTION

Nita Kaushik involved in writing the manuscript, designing and conducting the experiments as well as in the processing and interpretation of the data. Aradhita Barmanray read and approved the manuscript. All authors contributed to the final approval of the version to be published.

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Table 1. Central composite design with coded variables and measured values

	Factor			Response		
Run	A: *t*/min	B: (*m*(seed powder)/*V*(solvent))/%	C: Amplitude/%	DPPH scavenging activity/%	TPC as *w*(GAE)/(mg/g)	*Y*/%
1	45	32.9552	45	33.67	235.65	4.3
2	45	75	70.2269	21.83	238.98	3.2
3	45	75	45	83.06	271.03	4.5
4	45	75	45	83.06	271.03	4.5
5	30	100	30	51.73	245.07	2.5
6	60	100	30	61.73	333.91	1.3
7	30	50	60	7.14	192.02	4.5
8	60	50	30	13.57	218.26	4.2
9	70.2269	75	45	28.67	300.2	2.8
10	30	50	30	3.87	339.85	3.7
11	45	75	45	83.06	271.03	4.5
12	45	75	45	83.06	271.03	4.5
13	19.7731	75	45	37.95	308.98	3.0
14	45	117.045	45	31.12	356.52	2.3
15	60	100	60	65.00	299.13	1.0
16	45	75	19.7731	41.02	301.44	3.3
17	45	75	45	83.06	271.03	4.5
18	45	75	45	83.06	271.03	4.5
19	30	100	60	46.22	312.46	1.3
20	60	50	60	18.57	278.26	3.0

DPPH=2,2-diphenyl-1-picrylhydrazyl radical, TPC=total phenolic content, GAE=galic acid equivalent, *Y*=yield

Table 2. ANOVA result of ultrasonic assisted extraction for methanol solvent extracts

Methanol			
	DPPH scavenging activity/%	TPC as *w*(GAE)/(mg/g)	*Y*/%
	F-value	F-value	F-value
Model	37.09***	13.31***	16.78***
A: *t*/min	19.17***	21.15***	19.43***
B: (*m*(seed powder)/*V*(solvent)/)%	14.84***	14.7***	11.41**
C: Amplitude/%	23.0***	12.30**	12.50**
AB	33.0***	49.20***	10.04 **
AC	63.50***	70.58***	11.51**
BC	61.75***	80.42***	21.21***
Â²	75.01***	11.67**	43.38***
B²	79.62***	4.48 ns	63.73***
C²	84.67***	8.62*	49.0***
Residual	21.90	17.97	10.21
Lack of fit	0.584 ns	0.813 ns	0.565 ns
R^2	0.980	0.948	0.903
R^2 (adjusted)	0.954	0.922	0.890

DPPH=2,2-diphenyl-1-picrylhydrazyl radical, TPC=total phenolic content, *Y*=yield. All the linear and quadratic values are significant and lack of fit was non-significant; ns=not significant ($p>0.05$), * significant at ($p<0.05$), **significant at ($p<0.01$), ***significant at ($p <0.0001$)

a)

b)

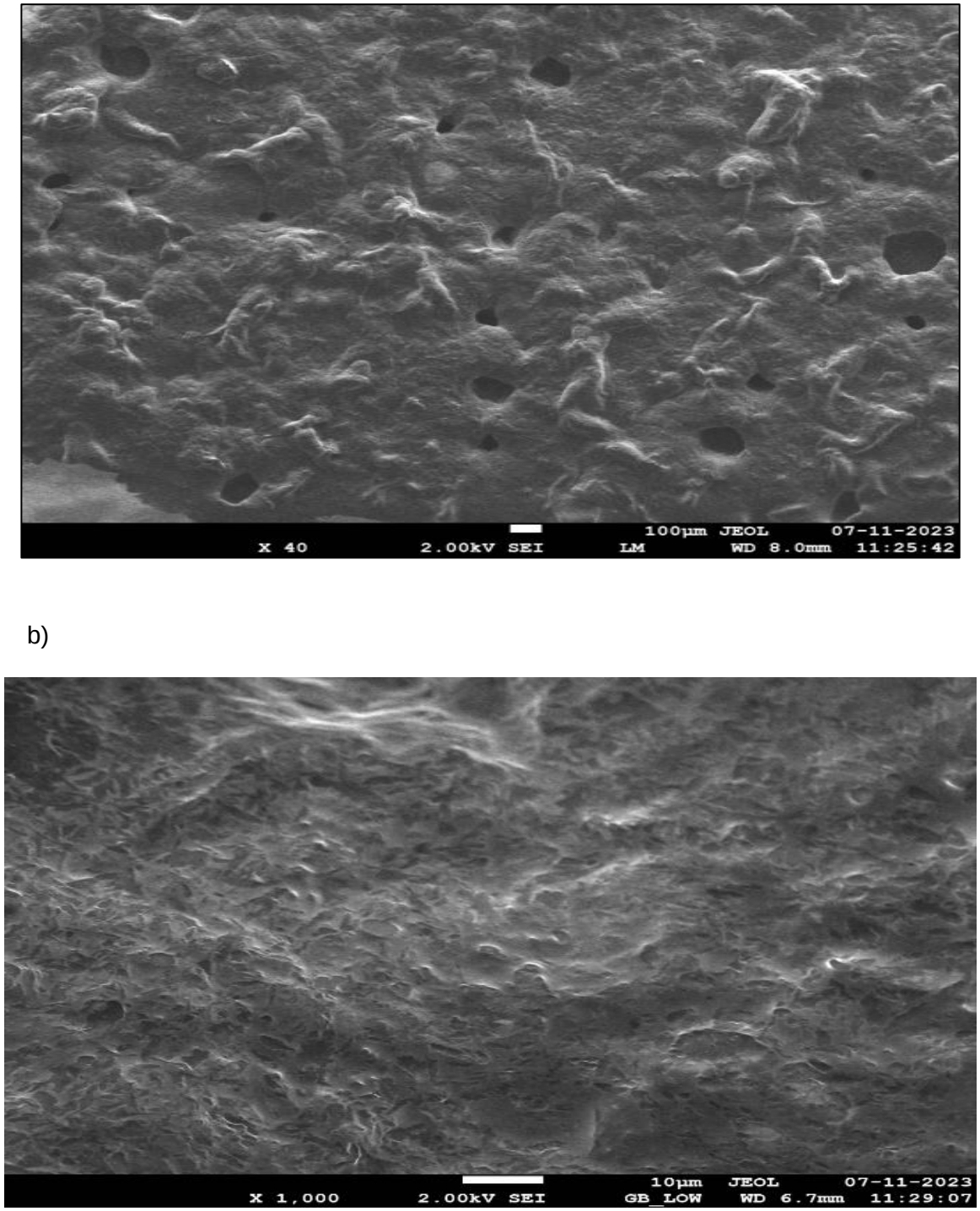


Fig. 1. Image analysis of *Nigella sativa* extracts by SEM using ultrasonic extraction: a) 40× and b) 1000×

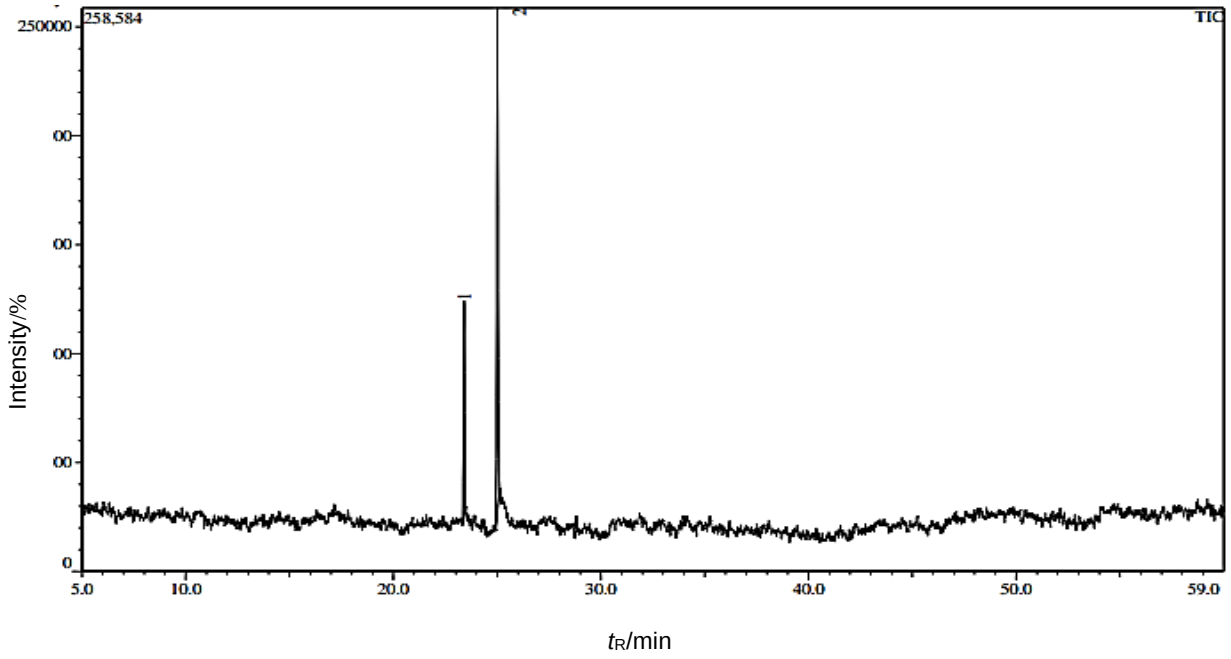


Fig. 2. Gas chromatogram of black cumin seed extract revealing presence of thymoquinone

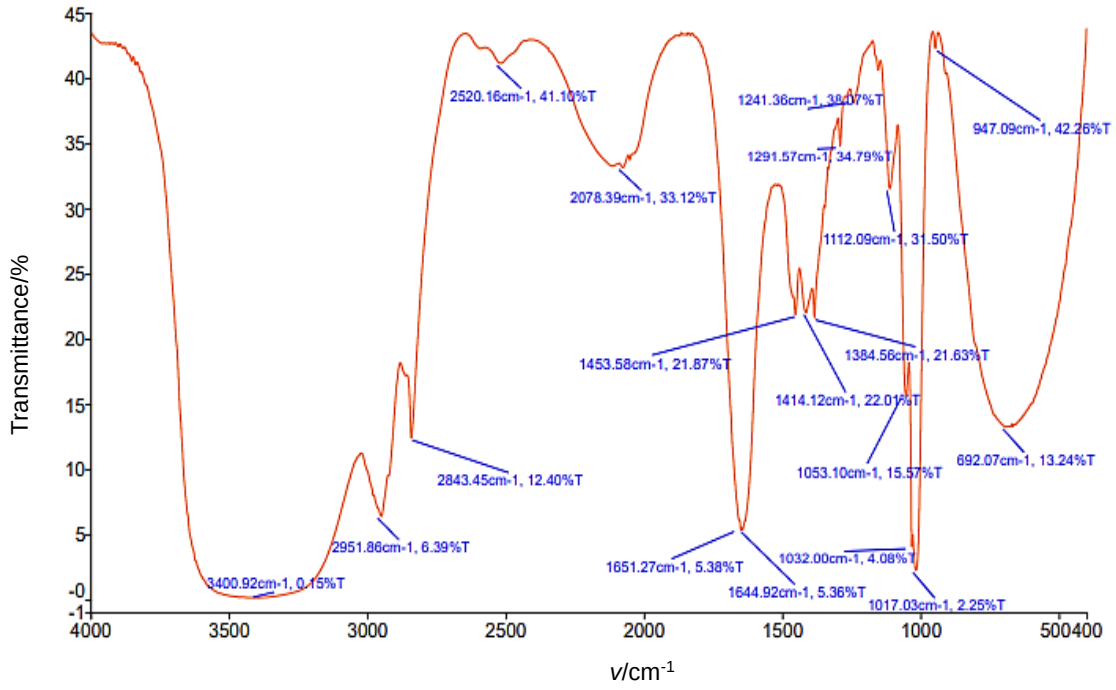
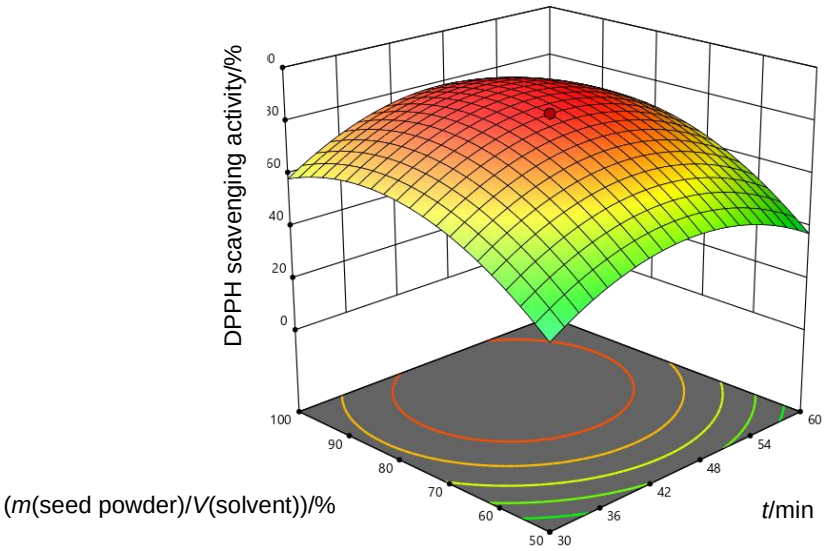
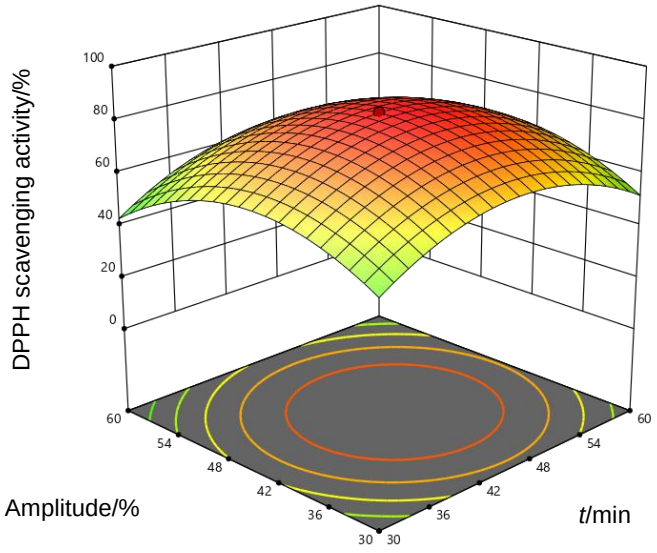


Fig. 3. Results of Fourier transform infrared spectroscopy (FTIR) analysis

a)



b)



c)

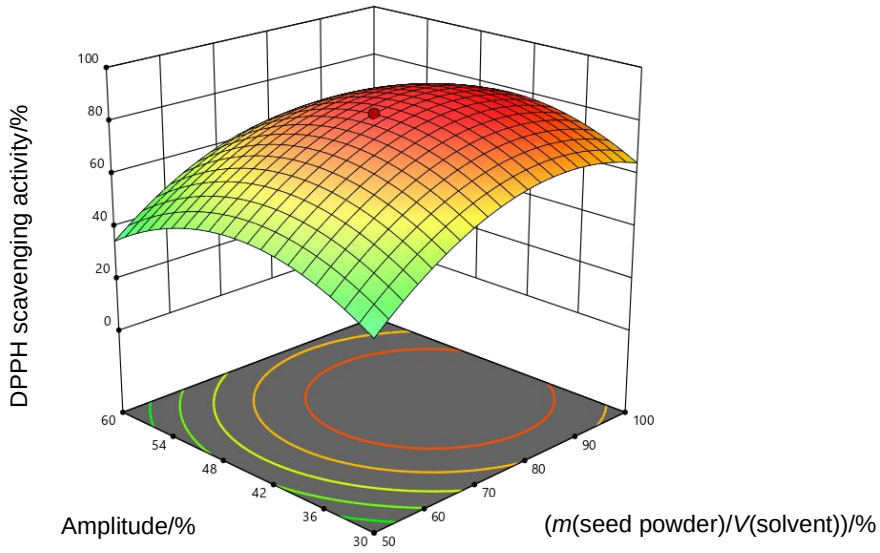
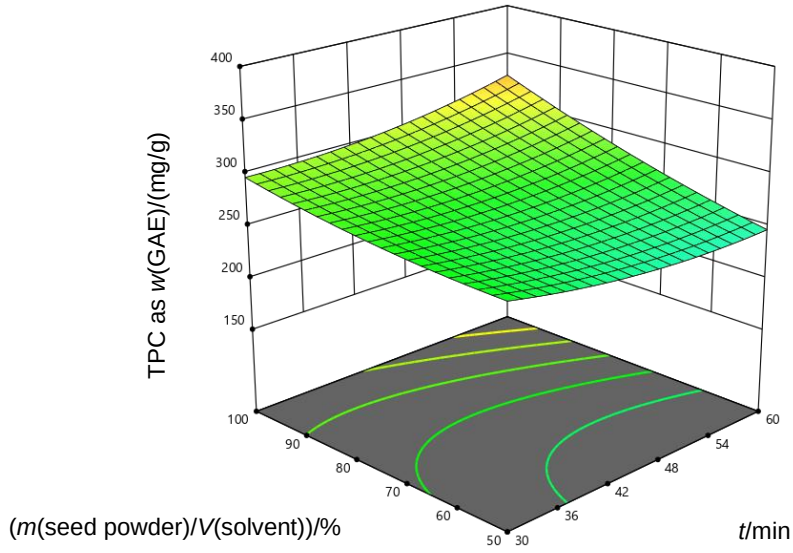
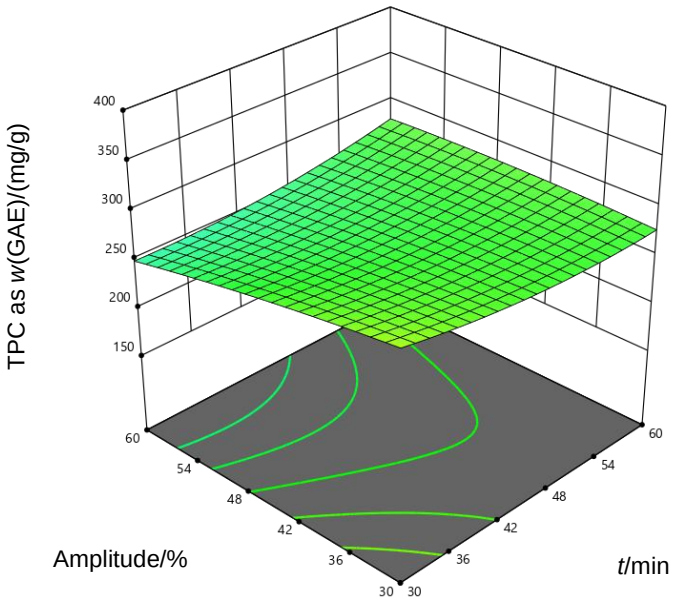


Fig. 4. 3D surface plots illustrating the synergistic impact of process factors for DPPH scavenging activity: a) ratio of mass of seed powder to volume of solvent *vs.* time, b) amplitude *vs.* time and c) amplitude vs. ratio of mass of seed powder to volume of solvent

a)



b)



c)

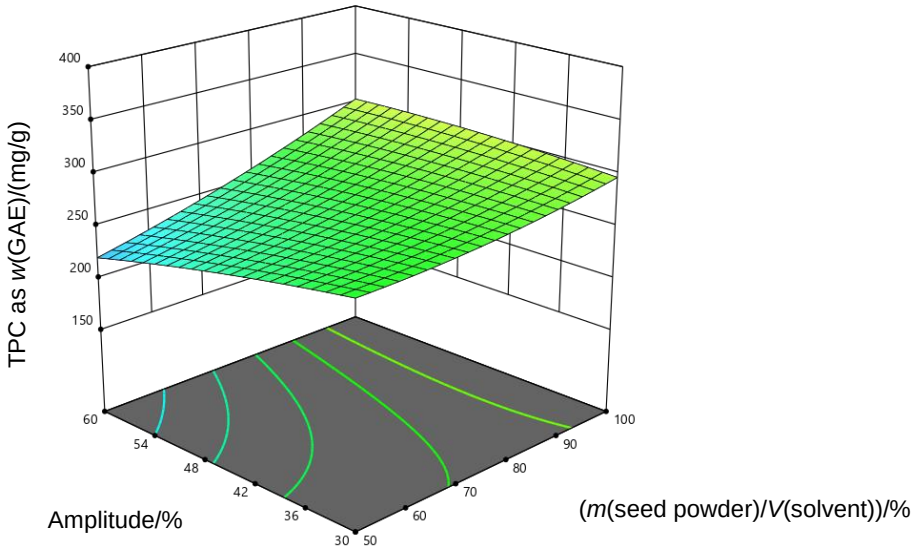
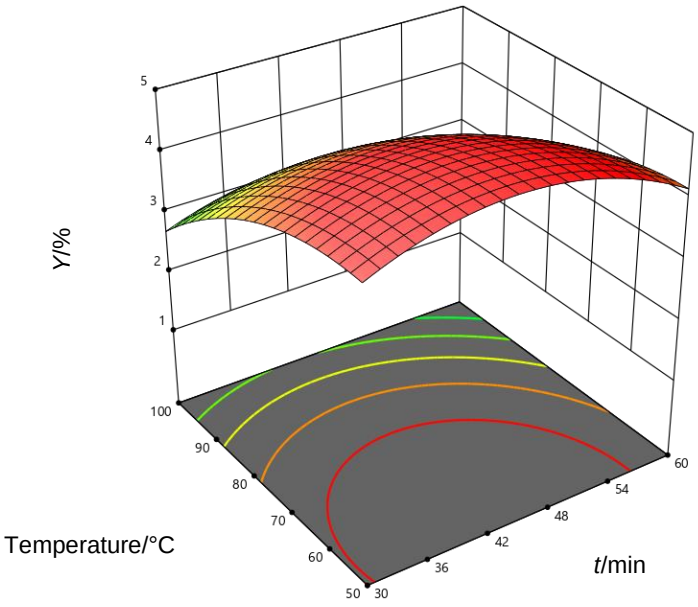
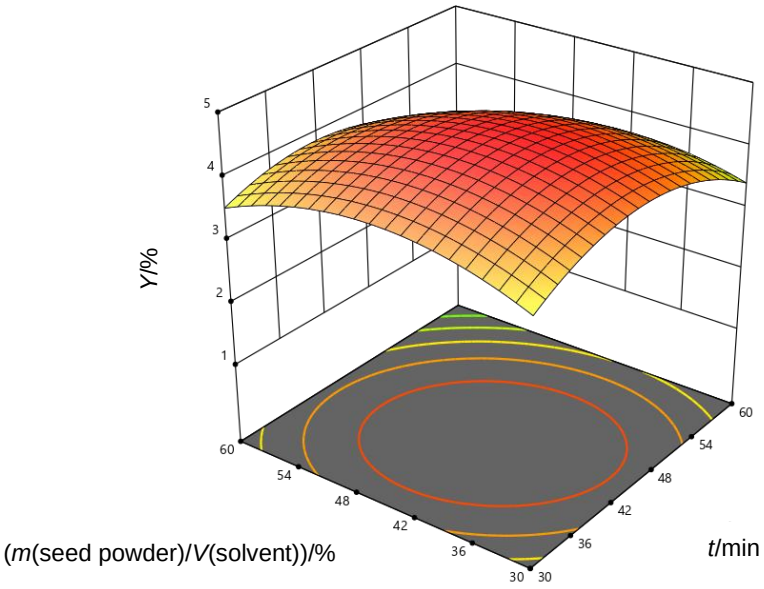


Fig. 5. 3D surface plots illustrating the synergistic impact of process factors for total phenolic content (TPC) as *w*(GAE)/(mg/g) as a function of: a) ratio of mass of seed powder to volume of solvent *vs.* time, b) amplitude *vs.* time and c) amplitude *vs*. ratio of mass of seed powder to volume of solvent

a)



b)



c)

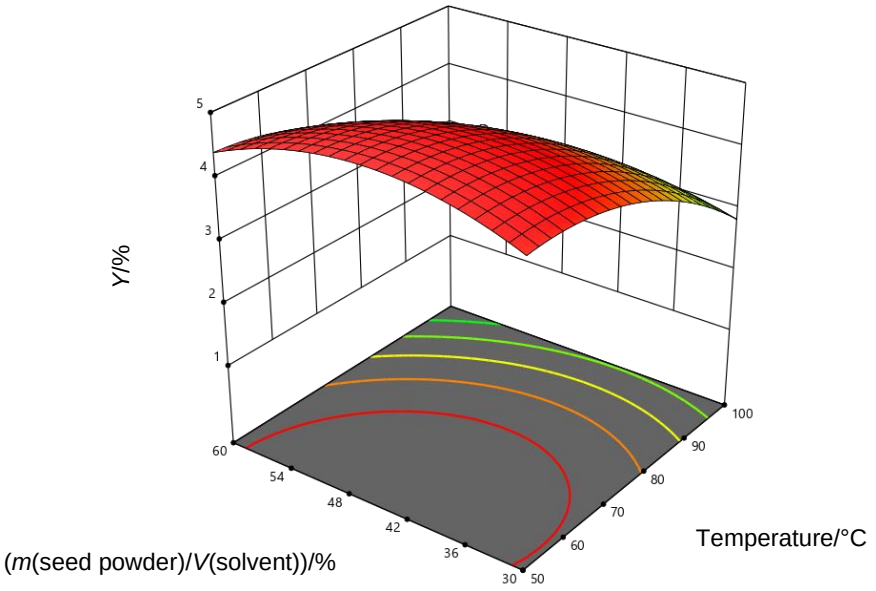


Fig. 6. 3D surface plots illustrating the synergistic impact of process factors of yield (*Y*) as a function of: temperature vs. time, b) ratio of mass of seed powder to volume of solvent *vs.* time and c) ratio of mass of seed powder to volume of solvent *vs*. temperature