

Article

Optimization of Thymoquinone Extraction from Black Seed Using Natural Deep Eutectic Solvent and Ultrasound-Assisted Extraction

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Abstract

Black seed (*Nigella sativa* L., Ranunculaceae) possesses antimicrobial activity due to its bioactive compound, thymoquinone. This study aimed to extract thymoquinone from black seed using an alternative natural deep eutectic solvent (NADES) through an optimization approach. A NADES based on malic acid and choline chloride was employed in this work. The Box–Behnken design was used to investigate the effects of the molar ratio of malic acid to choline chloride, water addition, and ultrasonication time. These variables were varied as follows: the malic acid to choline chloride molar ratio ranged from 2:1 to 4:1, the mass ratio of water to NADES from 0.5:2 to 1.5:2, and the ultrasonication time from 60 to 120 min. The results showed that the malic acid to choline chloride molar ratio, the quadratic term of the malic acid to choline chloride molar ratio, and the quadratic term of ultrasonication time significantly affected thymoquinone extraction. The optimal condition, which yielded the highest thymoquinone concentration, was determined to be a malic acid to choline chloride molar ratio of 4:1, a mass ratio of water to NADES of 1:2, and an ultrasonication time of 60 min. Under these conditions, the thymoquinone concentration was 70.57 ± 5.78 µg/mL, closely aligning with the predicted value. The thymoquinone-rich extract obtained using NADES exhibited antimicrobial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Candida albicans*. In conclusion, NADES serves as an effective alternative solvent for extracting thymoquinone from black seed.

Keywords: black seed; Box–Behnken design; response surface methodology; thymoquinone; ultrasound-assisted extraction



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1. Introduction

Black seed (*Nigella sativa* L.), a member of the Ranunculaceae family, exhibits notable antimicrobial properties. The extract of black seed demonstrates antibacterial activity against several pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans* [1]. Additionally, black seed oil is effective against clinical isolates of multi-drug-resistant *S. aureus* [2,3] and other pathogens, including methicillin-resistant *S. aureus*, *Bacillus subtilis*, and *B. cereus* [3]. It also inhibits the growth of *Listeria monocytogenes* [4]. The primary antimicrobial compound in black seed is thymoquinone [1], with carvacrol and *p*-cymene also reported as active antimicrobial agents [4].

Green chemistry principles have been applied in various industries to create safer, eco-friendly chemicals and processes by replacing hazardous solvents with safer alternatives [5]. Deep eutectic solvents (DES), formed by combining a hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA) in a specific molar ratio, become liquid at room temperature due to strong hydrogen bonding [5,6]. Natural deep eutectic solvents (NADES) are a type of DES using plant metabolites like sugars and amino acids, offering low toxicity, biodegradability, and high solubility [7]. However, these advantages depend on the specific formulation and should not be generalized across all NADES systems [8]. NADES are particularly effective for extracting plant metabolites, with factors like viscosity, polarity, and pH influencing extraction efficiency [9]. Common components include sugars, organic acids, and amino acids, which are mixed in specific molar ratios to achieve the desired eutectic properties. The preparation process generally involves dissolving the HBD in an HBA at elevated temperatures until a homogeneous liquid is formed [10,11]. The cost associated with the preparation of NADES is significantly lower compared to that of ionic liquids, as the raw materials used for NADES preparation—primarily derived from biologically sourced components—are generally more affordable and easily accessible [12]. Furthermore, the utilization of these materials enhances the sustainability of extraction processes. The favorable environmental profile of NADES contributes to their growing acceptance in various sectors, including pharmaceuticals, food science, and biomass valorization [13]. Due to the low-cost preparation process and the low toxicity of the components involved, NADES are increasingly being explored for the efficient extraction of valuable compounds from plant materials [14–18].

Ultrasound-assisted extraction (UAE) is widely used for extracting plant bioactive compounds because of its high extraction efficiency, reduced solvent consumption, and ability to minimize heat-induced degradation of thermolabile compounds [19,20]. Therefore, UAE was employed in the present study to facilitate thymoquinone extraction from black seed using NADES.

In the extraction of thymoquinone from black seed, conventional solvents such as ethanol, methanol, benzene, and hexane have commonly been used because of their high extraction efficiency for lipophilic compounds [21–24]. However, these organic solvents require removal before use and may pose toxicity concerns. In contrast, NADES-based extracts can be used directly without solvent removal, making them suitable as ready-to-use ingredients for formulation development. Owing to their tunable polarity and hydrogen-bonding network, NADES can effectively solubilize thymoquinone while providing a greener alternative to conventional organic solvents [25,26]. Moreover, NADES have been reported to enhance the stability of bioactive compounds during storage, further supporting their application in the extraction of thymoquinone from black seed [27].

Despite the well-documented antimicrobial properties of black seed and its primary bioactive compound thymoquinone, significant research gaps remain in optimizing its extraction using eco-friendly and sustainable methods. Conventional extraction processes rely heavily on hazardous organic solvents, raising environmental and safety concerns [28]. While NADES have demonstrated potential as green solvents for plant metabolite extraction [5], their application to thymoquinone extraction from black seed is largely unexplored. Furthermore, the influence of NADES composition and UAE parameters on the efficiency and bioactivity of the extracted compounds remains inadequately studied. These gaps highlight the need for a systematic investigation into the optimization of NADES-UAE methods to improve the yield, bioactivity, and sustainability of thymoquinone extraction from black seed.

Given the notable antimicrobial properties of black seed and its key active compound thymoquinone, this study aims to enhance the extraction process of thymoquinone using

UAE. By employing NADES as green alternative solvents, the objective is to optimize extraction efficiency while adhering to sustainable and eco-friendly practices. In addition, the antimicrobial activity of the optimized NADES extract was evaluated to determine whether the extract retained its biological activity after extraction. This approach seeks to improve the recovery of thymoquinone from black seed, addressing the need for safer and more effective extraction methods in antimicrobial applications.

2. Materials and Methods

2.1. Materials

Standard thymoquinone was purchased from Sigma-Aldrich, Inc., St. Louis, MO, USA. Malic acid was purchased from Krungthepchemi, Bangkok, Thailand. Choline chloride was purchased from Chanjao Longevity Co., Ltd., Bangkok, Thailand. Ultrapure water was produced by a Direct-Q 3UV water purifier (Merck KGaA, Darmstadt, Germany). Methanol was purchased from Fisher Chemical, Loughborough, UK. Mueller-Hinton Broth (MHB), Sabouraud Dextrose Broth (SDB), and agar were purchased from HiMedia Laboratories LLC, Kennett Square, PA, USA. Black seeds were obtained from Charoensuk Osod, Nakhon Pathom, Thailand. They were pulverized, passed through a 60-mesh sieve, and stored in a dry place until use.

2.2. Experimental Design Using Box–Behnken Design

Three factors, including the molar ratio of malic acid to choline chloride, the mass ratio of water to NADES, and the ultrasonication time, were varied based on the Box–Behnken design (Table 1). The selected ranges were established based on preliminary studies.

Table 1. Factors and their levels based on the Box–Behnken design.

Factor	Level		
	Low (−1)	Medium (0)	High (+1)
Malic acid: choline chloride (molar ratio)	2:1	3:1	4:1
Water:NADES (mass ratio)	0.5:2	1:2	1.5:2
Ultrasonication time (min)	60	90	120

2.3. Preparation of NADES and Extraction Procedure

NADES was prepared by mixing malic acid and choline chloride in various molar ratios. Then, ultrapure water was added to the NADES in a specific mass ratio and heated to no more than 80 °C to accelerate solubilization, resulting in the NADES used for the extraction of black seed powder.

Black seed powder (50 mg) was added to a 2 mL microfuge tube ($n = 3$). The pre-mixed NADES was then added and mixed using a vortex mixer for 10 s. Subsequently, the mixture was subjected to UAE using an ultrasonic bath (Elmasonic Easy 300 H, Elma Schmidbauer GmbH, Singen, Germany; Internal tank dimensions, width $\times$ depth $\times$ height, 475 mm $\times$ 270 mm $\times$ 195 mm; total volume 27.5 L; Effective ultrasonic power of 300 W; Ultrasonic frequency of 37 kHz) with no temperature control, for a specified time. However, the extraction temperature remained below 40 °C throughout the process. The initial temperature was typically 25–30 °C and increased to approximately 33–35, 35–37, and 37–38 °C after 60, 90, and 120 min of ultrasonication, respectively. At predetermined intervals, the samples were centrifuged at 6000 rpm for 10 min at ambient temperature. The supernatant was diluted with methanol to achieve a 250-fold dilution, vortexed, and filtered before being analyzed for thymoquinone concentration using a high-performance liquid chromatography (HPLC) method as described in previous work [29]. The linear equation for the concentration range

of 1.5625–100 µg/mL was $y = 89,635.9x - 29,926.2$, with a coefficient of determination (R^2) of 1.0000 [29]. The thymoquinone concentration was calculated from the calibration curve of standard thymoquinone.

2.4. Optimization Procedure

The response surface for thymoquinone concentration was created using Design-Expert® 11 (Stat-Ease Inc., Minneapolis, MN, USA). Diagnostic plots showing predicted versus actual values and externally studentized residuals versus run numbers were presented. Both coded and actual equations were provided. Analysis of Variance (ANOVA) was conducted to assess thymoquinone concentration. The optimal conditions for achieving the highest thymoquinone concentration were identified using the desirability function. Model verification was performed by replicating the extraction under this optimized condition, and percent error calculations were used to evaluate the model's accuracy.

2.5. Determination of Clear Zone Against *S. aureus*, *S. epidermidis*, and *C. albicans*

Antimicrobial activities against *S. aureus*, *S. epidermidis*, and *C. albicans* were assessed following previously described methods [29,30]. The antibacterial activity against *S. aureus* (Thailand Institute of Scientific and Technological Research [TISTR]: 1466) and *S. epidermidis* (TISTR: 518), as well as the antifungal activity against *C. albicans* (American Type Culture Collection [ATCC]: 10231), were evaluated using the agar well diffusion method [29,30]. Bacterial and fungal cultures were prepared and incubated on Mueller-Hinton Agar (MHA) for *S. aureus* and *S. epidermidis*, and Sabouraud Dextrose Agar (SDA) for *C. albicans* at 37 °C for 18–24 h. The bacterial or fungal density was adjusted to a 0.5 McFarland standard using a 0.85% sodium chloride solution. The standardized inoculum was evenly spread on the MHA or SDA surface using a sterile swab. Wells with a diameter of 0.7 mm were created in the agar, and 70 µL of NADES containing black seed extract ($n = 3$) was added to the wells. The plates were incubated at 37 °C for 24 h, after which the diameters of the clear zones were measured using a Vernier caliper. The results were reported as the mean diameter and standard deviation (SD) of the clear zones. Blank NADES was also tested. Gentamicin (10 µg/well) served as a positive control for *S. aureus* and *S. epidermidis*, while nystatin (7000 units/well) served as a positive control for *C. albicans*. Sterile water was used as the negative control.

2.6. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) or Minimum Fungicidal Concentration (MFC)

The MIC, MBC, and MFC of NADES containing black seed extract were evaluated. The MIC was determined using the broth microdilution method. Initially, the test sample (100% *v/v*, corresponding to 70.57 µg/mL thymoquinone) was subjected to two-fold serial dilutions to achieve 12 concentrations ranging from 50% to approximately 0.02% *v/v* in the appropriate medium. For antifungal testing, the dilutions were prepared in SDB, and for antibacterial testing, in MHB. From each dilution, 50 µL was transferred into 96-well plates ($n = 3$). For antifungal activity, a 50 µL suspension of *C. albicans*, adjusted to a 0.5 McFarland standard, was added to each well containing the test sample in SDB. For antibacterial activity, an equal volume of bacterial suspension (*S. aureus* or *S. epidermidis*), standardized to a 0.5 McFarland standard, was added to the wells containing the test sample in MHB. The plates were incubated at 37 °C for 24 h. After incubation, 10 µL of 0.1% resazurin sodium salt solution was added to each well and incubated for an additional 4 h at 37 °C. The MIC was recorded as the lowest concentration at which the resazurin dye remained blue, indicating no microbial growth.

To determine the MFC for *C. albicans*, the mixtures from wells showing no color change in the MIC test were streaked onto SDA plates and incubated at 37 °C for 24 h. The MFC

was defined as the lowest concentration at which no fungal growth was observed. Similarly, the MBC for *S. aureus* and *S. epidermidis* was determined by streaking the mixtures from the MIC wells onto MHA plates, followed by incubation at 37 °C for 24 h. The MBC was recorded as the lowest concentration at which no bacterial growth was observed.

2.7. Statistical Analysis

Statistical analysis was conducted using ANOVA, with post hoc comparisons performed using the least significant difference (LSD) test to evaluate differences between multiple groups. The analysis was carried out using IBM SPSS Statistics version 22 (IBM Corp., Armonk, NY, USA). A *p*-value of less than 0.05 was considered statistically significant at a 95% confidence level.

3. Results and Discussion

3.1. Optimization to Obtain the Optimal Condition

Factors and responses based on the Box–Behnken design are shown in Table 2.

Table 2. Factors and response based on the Box–Behnken design.

Standard Order	Run Order	Malic Acid:Choline Chloride (Molar Ratio)	Water:NADES (Mass Ratio)	Ultrasonication Time (min)	Thymoquinone Concentration (µg/mL) *
14	1	3	1:2	90	37.40
2	2	4	0.5:2	90	67.16
1	3	2	0.5:2	90	51.30
5	4	2	1:2	60	58.87
12	5	3	1.5:2	120	47.33
3	6	2	1.5:2	90	56.02
10	7	3	1.5:2	60	52.74
15	8	3	1:2	90	37.88
9	9	3	0.5:2	60	59.01
13	10	3	1:2	90	52.06
7	11	2	1:2	120	52.41
11	12	3	0.5:2	120	50.17
17	13	3	1:2	90	41.48
4	14	4	1.5:2	90	53.27
6	15	4	1:2	60	70.44
16	16	3	1:2	90	45.04
8	17	4	1:2	120	70.10

* Average value of triplicates.

The coded and actual equations are shown in Equations (1) and (2). The coded equation expresses the response in terms of normalized (coded) values of the independent variables. These coded values are dimensionless and are scaled to a standardized range (typically −1 to +1) to simplify the analysis and comparison of the effects of different variables. The coded equation helps in identifying the relative significance and direction of influence (positive or negative) of each factor on the response. In contrast, the actual equation presents the response as a function of the original (uncoded or real-world) units of the independent variables, such as molar ratio, mass ratio, or time. This equation is useful for practical implementation, as it directly relates to the experimental conditions and provides a means for predicting the response based on actual process parameters. Including both equations allows for statistical interpretation through coded values and practical application using actual values. These equations were used to predict the thymoquinone concentration. Additionally, the coefficients in the coded equation demonstrated that X_1^2 had the greatest effect on thymoquinone concentration, followed by X_3^2 and X_1 , respectively.

Coded equation:

$$Y_1 = 43.51 + 5.30X_1 - 2.29X_2 - 2.63X_3 - 4.65X_1X_2 + 12.50X_1^2 + 7.87X_3^2 \quad (1)$$

where Y_1 is thymoquinone concentration, and X_1 , X_2 , and X_3 are the molar ratio of malic acid: choline chloride, the mass ratio of water:NADES, and ultrasonication time as coded values, respectively.

Actual equation:

$$Y_1 = 195.49 - 60.38X_1 + 23.35X_2 - 1.66X_3 - 9.31X_1X_2 + 12.50X_1^2 + 0.01X_3^2 \quad (2)$$

where Y_1 is thymoquinone concentration, and X_1 , X_2 , and X_3 are the molar ratio of malic acid: choline chloride, the mass ratio of water:NADES, and ultrasonication time as actual values, respectively.

The model was considered adequate as the coefficient of determination ($R^2 > 0.8$) indicated a strong correlation between predicted and actual values [31]. The difference between adjusted R^2 and predicted R^2 was less than 0.2, and the adequate precision exceeded 4 (Figure 1a). Additionally, the plot of externally studentized residuals versus run numbers showed a random distribution within the 95% confidence interval, with no apparent pattern (Figure 1b), supporting the model's validity.

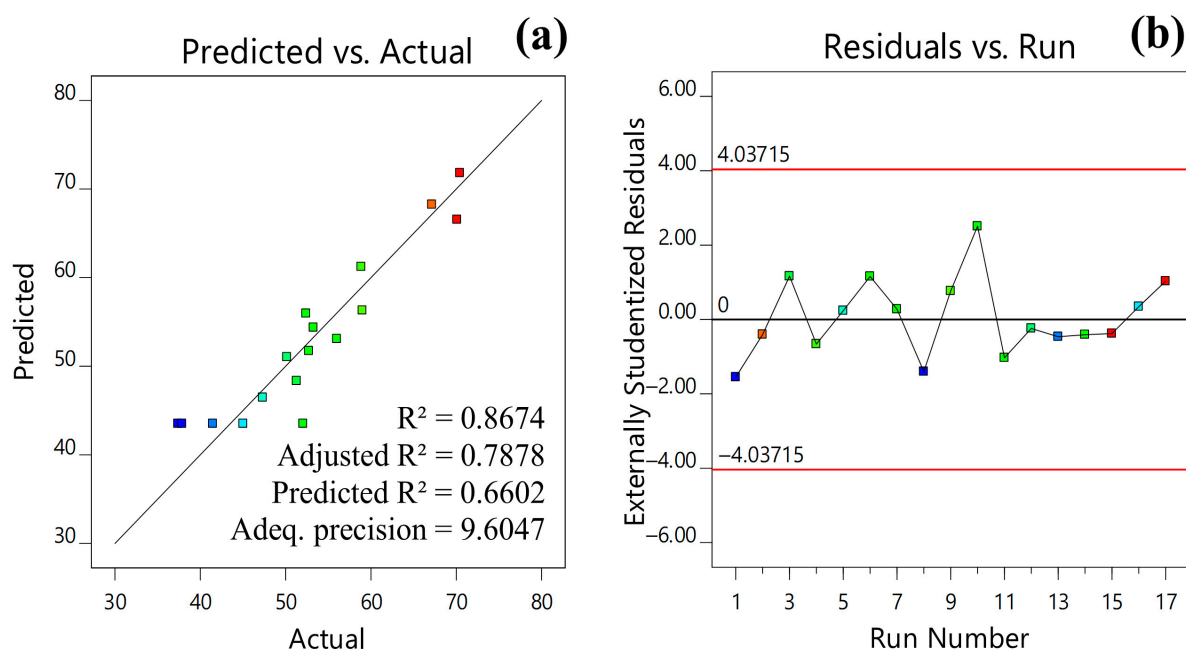


Figure 1. Diagnostic plots showing (a) predicted versus actual values and (b) externally studentized residuals versus run numbers.

Response surfaces of thymoquinone concentration under different ultrasonication times are shown in Figure 2. The results indicate that increasing the molar ratio of malic acid to choline chloride from low to medium decreased thymoquinone concentration, while further increasing the molar ratio resulted in higher thymoquinone concentration. An increase in the mass ratio of water to NADES led to a decrease in thymoquinone concentration. Additionally, increasing the ultrasonication time from low to medium reduced thymoquinone concentration, but further increasing the ultrasonication time caused an increase in thymoquinone concentration.

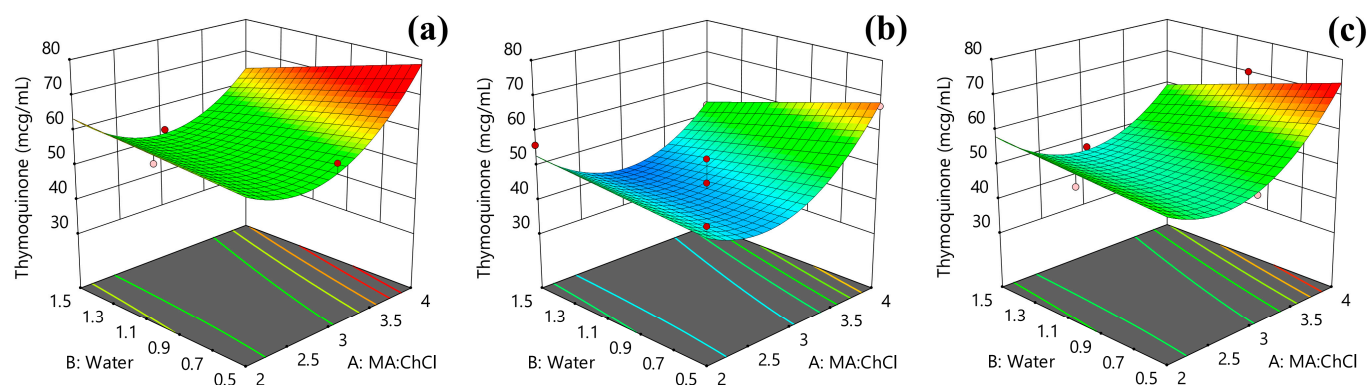


Figure 2. Response surfaces of thymoquinone concentration with different ultrasonication times: (a) 60 min, (b) 90 min, and (c) 120 min.

The ANOVA data demonstrated that the significant factors influencing thymoquinone concentration were the molar ratio of malic acid: choline chloride, the quadratic term of the malic acid: choline chloride molar ratio, and the quadratic term of ultrasonication time (Table 3).

Table 3. ANOVA for the reduced quadratic model of thymoquinone concentration.

Source	Sum of Squares	df	Mean Square	F-Value	p-Value
Model	1378.29	6	229.71	10.90	0.0007 *
X_1 -Malic acid: choline chloride	224.40	1	224.40	10.65	0.0085 *
X_2 -Water:NADES	41.77	1	41.77	1.98	0.1895
X_3 -Ultrasonication time	55.39	1	55.39	2.63	0.1361
X_1X_2	86.58	1	86.58	4.11	0.0702
X_1^2	659.38	1	659.38	31.28	0.0002 *
X_3^2	261.63	1	261.63	12.41	0.0055 *
Residual	210.78	10	21.08		
Lack of Fit	64.91	6	10.82	0.2967	0.9102
Pure Error	145.87	4	36.47		
Cor Total	1589.07	16			

An asterisk (*) denotes significant value.

The varying molar ratios of HBAs and HBDs can influence the extraction efficiency of plant bioactive compounds by altering the physicochemical properties of NADES, including viscosity, polarity, conductivity, solubility, density, and pH [32]. Changes in the HBA:HBD molar ratio modify the intermolecular hydrogen-bonding network and solvent polarity [33], thereby affecting the solubilization of thymoquinone. Generally, water decreases the viscosity of NADES, which improves the mass transfer of plant bioactive compounds [32,34]. However, excessive water can weaken the hydrogen-bonding network and dilute the solvent system, thereby reducing the solubilization capacity of NADES for thymoquinone, as observed in the present study. Additionally, NADES can maintain its structure only when the water content is below 50% (v/v). Excess water can weaken the hydrogen bonds, leading to dissociation [34]. Ultrasonication time was another key parameter examined in this study. The results showed that prolonging ultrasonication time beyond a certain limit did not significantly improve thymoquinone extraction. As a result, a shorter ultrasonication duration was determined to be optimal, achieving high thymoquinone yields while minimizing energy consumption and processing time [32]. However, according to the ANOVA results, the quadratic term significantly affected thymoquinone yield. The

authors note that a wide range of ultrasonication times might have contributed to the observed significant differences.

The optimal conditions that yielded the highest thymoquinone concentration with a desirability of 1.000 are shown in Figure 3. These conditions were a malic acid: choline chloride molar ratio of 4:1, a water-to-NADES mass ratio of 1:2, and an ultrasonication time of 60 min. To verify the accuracy of the predictions made by the computer software, the extraction was replicated under this optimized condition. The results showed that the experimental value closely matched the predicted value, with a low percent error (Table 4). Conventional organic solvents, such as ethanol, methanol, benzene, and hexane, have been widely used for thymoquinone extraction because of their high extraction efficiency [21–24]. However, these solvents generally require removal before further application and may pose environmental and safety concerns. In contrast, the optimized NADES developed in the present study provided a thymoquinone-rich extract that can be directly used without solvent removal, offering a greener alternative for thymoquinone extraction.

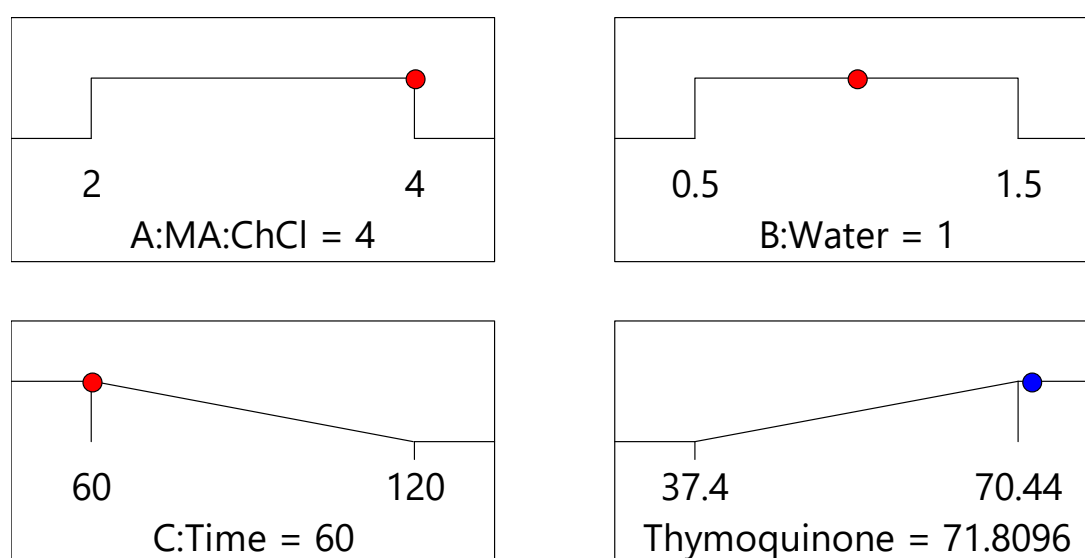


Figure 3. The optimal conditions for achieving the highest thymoquinone concentration and the predicted thymoquinone concentration derived from this condition.

Table 4. Verification data of thymoquinone concentration.

Response	Predicted Value	Experimental Value (<i>n</i> = 3)	Error (%) *
Thymoquinone concentration (µg/mL)	71.81	70.57 ± 5.78	−1.76

* Error = (Experimental value—Predicted value) × 100/Experimental value.

Previous studies have demonstrated that both the extraction method and extraction conditions markedly influence thymoquinone recovery from black seed. A comparative study of maceration, percolation, and UAE using different solvents showed that hexane extraction by maceration yielded the highest thymoquinone content, highlighting the importance of extraction method selection [24]. In addition, the extraction efficiency of conventional solvents varied considerably, with benzene producing the highest thymoquinone content (0.009%), followed by hexane (0.001%) and methanol (0.0009%), whereas thymoquinone was negligible in water and ethanol extracts [22]. Consistent with these findings, another study reported that the thymoquinone content ranged from 8.34 to 63.13 mg/g extract (0.83–6.31%, *w/w*) depending on both the extraction method and sol-

vent used, with hexane extraction producing the highest thymoquinone content among the evaluated methods [35]. These findings collectively demonstrate that optimization of the extraction process is essential for maximizing thymoquinone recovery and support the application of response surface methodology for optimizing NADES-based extraction in the present study. However, direct comparison with the present study is challenging because previous studies generally reported thymoquinone content relative to the dry extract, whereas the present study reports thymoquinone concentration in the NADES extract solution. In addition, the initial thymoquinone content of the black seed material may vary among studies and should be considered when comparing extraction efficiencies.

3.2. Antimicrobial Activity of the Optimal NADES-Containing Black Seed Extract

The NADES-containing black seed extract exhibited antimicrobial activity against three microorganisms, including *S. aureus*, *S. epidermidis*, and *C. albicans*. The authors mentioned that blank NADES without black seed extract also exhibited antimicrobial activity, as in a previous report [36]. NADES exhibit inherent high osmotic pressure properties, which result in adverse dehydration effects by rapidly extracting water from microbial cells, leading to cell dehydration and subsequent lysis [37]. It was observed that black seed extract enhanced anti-*S. aureus* and *C. albicans*, while it did not enhance antimicrobial activity against *S. epidermidis* (Table 5). The antimicrobial activity of the optimized NADES containing black seed extract was further evaluated by determining the MIC and MBC or MFC (Table 6). Compared with blank NADES, the extract-containing NADES exhibited lower MIC values against all tested microorganisms, indicating enhanced growth inhibition. The greatest improvement was observed against *S. aureus*, where the MIC decreased from 0.63% to 0.16%. Similarly, the MIC values against *S. epidermidis* and *C. albicans* were reduced by approximately two-fold. In contrast, the MBC and MFC values showed less pronounced improvements than the MIC values. The MBC against *S. aureus* remained unchanged, whereas the MFC against *C. albicans* decreased from 25.00% to 10.00%. These findings suggest that the incorporation of black seed extract primarily enhanced microbial growth inhibition, while its effect on bactericidal or fungicidal activity varied among the tested microorganisms. This antimicrobial activity is likely attributable to thymoquinone, the principal antimicrobial constituent of black seed, together with other bioactive constituents that may contribute through additive or synergistic effects. It can be concluded that NADES containing black seed extract has the potential for incorporation into topical preparations.

Table 5. Clear zone against *S. aureus*, *S. epidermidis*, and *C. albicans* by agar well diffusion method.

Sample	Diameter of Clear Zone (mm) ***		
	<i>S. aureus</i>	<i>S. epidermidis</i>	<i>C. albicans</i>
NADES containing black seed extract	34.05 ± 0.00 ^a	35.97 ± 0.23 ^b	19.12 ± 0.90 ^a
Blank NADES	33.13 ± 0.32 ^b	39.12 ± 0.42 ^a	16.75 ± 0.95 ^b
Positive control *	22.15 ± 0.00 ^c	28.02 ± 0.31 ^c	13.45 ± 0.00 ^c
Negative control **	Not detected	Not detected	Not detected

* Positive control for *S. aureus* and *S. epidermidis* was gentamicin at 10 µg/well, and for *C. albicans* was nystatin at 7000 units/well. ** Negative control was sterile water. *** Different letters on the same column indicate statistically significant differences among the tested groups ($p < 0.05$).

Future research should focus on expanding the optimization parameters to include additional factors influencing thymoquinone extraction using NADES. Variables such as ultrasonic power, extraction temperature, solvent-to-solid ratio, particle size, and the types of HBAs and HBDs should be systematically investigated to further improve extraction efficiency and reproducibility. Additionally, assessing the stability of thymoquinone in

NADES is crucial for evaluating its practicality for storage and applications. Addressing scalability challenges, including operational feasibility, is essential for translating the process to an industrial scale. A detailed economic analysis, encompassing solvent recovery and cost-effectiveness, will provide critical insights into the commercial and environmental viability of this green extraction method.

Table 6. MIC, MBC, and MFC of NADES containing black seed extract and blank NADES against *S. aureus*, *S. epidermidis*, and *C. albicans*.

Microorganism	Sample	MIC	MBC	MFC
<i>S. aureus</i>	NADES containing black seed extract	0.16%	1.25%	-
	Blank NADES	0.63%	1.25%	-
<i>S. epidermidis</i>	NADES containing black seed extract	0.31%	1.25%	-
	Blank NADES	0.63%	0.63%	-
<i>C. albicans</i>	NADES containing black seed extract	2.50%	-	10.00%
	Blank NADES	5.00%	-	25.00%

4. Conclusions

This study successfully demonstrated the use of the NADES based on malic acid and choline chloride for the extraction of thymoquinone from black seed. The Box–Behnken design allowed for the optimization of key variables, including the malic acid to choline chloride molar ratio, water addition, and ultrasonication time. The results highlighted that the malic acid-to-choline chloride molar ratio had the most significant influence on the extraction efficiency of thymoquinone, with the optimal conditions being a malic acid-to-choline chloride molar ratio of 4:1, a water-to-NADES mass ratio of 1:2, and an ultrasonication time of 60 min. This optimized condition yielded a thymoquinone concentration closely matched the predicted value. Additionally, the NADES containing the black seed extract exhibited antimicrobial activity against *S. aureus*, *S. epidermidis*, and *C. albicans*, indicating its potential for use in topical formulations to treat infections caused by these pathogens. These findings indicate that NADES holds promise as an alternative solvent for thymoquinone extraction from black seed. While the study demonstrated antimicrobial activity of the extract, further work comparing NADES with conventional solvents and assessing the activity of isolated thymoquinone is warranted to strengthen these conclusions.

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grammatical errors during the manuscript writing process. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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