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ULTRASONICATION AND RSM-BASED OPTIMIZATION OF ANTIOXIDANT ACTIVITY, SACCHARIDE COMPOSITION AND FATTY ACIDS FROM *Phoenix dactylifera* L. MEDJOOL DATE SEEDS INFLUENCED BY ETHANOL

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ABSTRACT. In response with the demand in date industry finding on sustainable solution for date seeds management and its bioactive rich constituent, current study envisaged the optimum condition for the ultrasound extraction of *Phoenix dactylifera* L. Medjool date seeds and its antioxidative activity by employing a three-level three-factor Box–Behnken design via response surface methodology (RSM). Ethanol (EtOH) concentration (50-80%), time (30-90 min) and temperature (40-70 °C) were the independent variables investigated for $ABTS^{\bullet+}$ scavenging antioxidant activity and subjected to analysis of variance (ANOVA). The optimum conditions for maximum antioxidant activity (60.93% ± 0.021) were achieved at 80% EtOH, 44 min and at 57 °C, where the effect of EtOH concentration were notably significant. The observed agreement between the experimental (60.93% ± 0.021) and predicted (60.35%) values indicated the employed model suitability while substantiates the successful implementation of RSM for optimizing extraction parameters. The optimized extract characterized through UPLC-QTOF/MS and GC-MS/MS, detailed the presence of saccharides (isomaltose, mannotriose and stachyose) and volatile compounds, namely 5 saturated fatty acids that encompassed within the 8.42% (w/w) of total fat obtained. This verifies the ability of the solvent mixture extracting fatty acids and saccharides even under high EtOH concentration.

KEY WORDS: Date seeds, Response surface methodology (RSM), Antioxidant, Saccharide, GC-MS/MS, UPLC-QTOF/MS

INTRODUCTION

Date seeds (*Phoenix dactylifera* L.) which comprise 10 - 15% w/w of total fruit [1], often overlooked as waste products in date processing industries. Abundantly available in the Middle Eastern and North African countries, date palm seeds represent a substantial agricultural waste stream [2]. Based on the information released by the Statista [3] platform, global date production in 2021 was estimated at 9.66 million metric tons, covering a surface area of 1,301,979 ha, resulting in approximately 1 million tons of date seeds annually, leading to potential environmental challenges if not properly managed [4].

Scientific investigations have demonstrated that date seeds possess substantial quantities of dietary fiber, noteworthy proportions of proteins, carbohydrates, vitamins and lipids [2, 5]. Moreover, these seeds exhibit a remarkable abundance of bioactive compounds, including

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flavonoids (epicatechin, catechin and rutin) and polyphenolic compounds (*m*-coumaric, caffeic acid, ferulic acid, β-carotene and α-tocopherol) [6, 7]. These bioactive constituents are attributed with a range of health-promoting effects, encompassing anti-inflammatory properties, potent antioxidant action, antigenotoxic activity and anti-carcinogenic potential [6], as well holding promising potential as functional food ingredients or functional food. Therefore, date seeds possess significantly higher economic worth beyond their current utilization as inexpensive animal feed or mere waste disposal [2].

Despite extensive research on the topic, establishing universal guidelines on the optimum extraction conditions for the retrieval of bioactive phytocompounds remains challenging. The quantity and quality of phytoextracts depend on various factors, including solvent-to-solid ratio, solvent composition, extraction pressure [8], number of extraction steps and particle size of solutes. Recent studies have particularly emphasized the critical influence of solvent concentration, incubation temperature and time [9] on natural product extraction from plants. The selection of an appropriate extraction solvent is also essential in extracting phenolic antioxidants from plants as their chemical composition and stability can be preserved [10]. Therefore, ethanol is often favored over methanol due to its lower toxicity and comparable yields [11], despite the fact that both solvents are highly effective for extracting polar phytochemicals [12]. Among modern extraction methods, ultrasonic assisted extraction (UAE) is preferred over microwave-assisted extraction, supercritical fluid extraction and other conventional extraction, for its environmentally friendly approach, cost-effective, simpler operation, reduced solvent usage, shorter extraction time, increased yield and improved extract quality [13, 14]. UAE achieves this by enhancing mass transfer through acoustical cavitation in the solvent via its wave frequency and distribution, which disrupts the sample cell walls and elevating the contact surface area between solid and liquid phases [15].

The classical optimization method involves varying only one factor at a time, which is a time-consuming and costly approach that overlooks interactions between variables. Response Surface Methodology (RSM), developed by Box and Wilson [16], serves as a valuable statistical experimental protocol used in mathematical modelling for optimizing the process while incorporating experimental designs such as the Box-Behnken design (BBD). BBD employs a spherical, rotating design with 3 interlocking 3^3 factorial designs with a set of points arranged on the surface of a sphere surrounding the central point of the design. RSM offers advantages such as accurate results with fewer experiments, statistical data analysis, identification of variable interactions and cost-effectiveness [17].

The objectives of this research were to optimize the ultrasonic-assisted extraction process of phytocompound enriched *P. dactylifera* seed extracts by utilizing a 3-factor and 3-level BBD and to investigate the influence of operational parameters (EtOH concentration, time, and extraction temperature) on the antioxidative $ABTS^{\bullet+}$ scavenging potential. The composition of the extract acquired under the ideal conditions was further analyzed through UPLC-QTOF/MS and GC-MS/MS to gain a deeper understanding of its chemical makeup. The findings could prompt the use of overlooked date parts for new product development, benefiting the food and pharmaceutical industries with insights into optimal extraction methods and functional phytocompounds.

EXPERIMENTAL

Chemicals and reagents

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate were purchased from Sigma-Aldrich, St. Louis, MO, USA, while 99% ethanol (EtOH) and 95% EtOH were obtained from Systerm Chem AR (Malaysia). All chemicals used throughout this experiment were of analytical grade.

Preparation of Medjool date seed (MDS) powder

Medjool date seed (MDS) powder was prepared following a modified protocol [18] where a total of 5 kg of imported Medjool date fruits from Palestine were procured locally and pitted to obtain the seeds. The seeds were thoroughly washed to remove any remnants of date flesh. Approximately 500 g seeds were dried for 24 h at 45 °C in an oven. The dried seeds were then homogenized and ground using a heavy-duty grinder to obtain a fine powder, which was further screened through a 1-2 mm mesh. The resulting powder was stored at 4 °C until further analysis was conducted.

Ultrasound-assisted extraction (UAE)

The experiment involved the mixing of 2 g of dried Medjool date seed powder with 100 mL solvent, followed by ultrasound-assisted extraction (UAE) using a sonication water bath machine (Elmasonic S60 H, Elma Hans Schmidbauer GmbH, Singen, Germany). Various extraction conditions were implemented based on the outlined experimental plan. After extraction, the mixture underwent centrifugation at 3000 rpm for 5 min to separate insoluble materials. The supernatant was then filtered using vacuum filtration with Whatman No.1 filter papers. The solvent from the extract filtrate was eliminated by rotary evaporation at 45 °C to preserve the extract's phytocompound constituents. To prevent the degradation of components, elevated temperatures were avoided. The resulting crude extract was designated as Medjool date seed extract (MDSE), saved in storage glass vials at -20 °C and utilized for future analytical assays.

Total fat, ash and moisture content

The ash and moisture contents of MDSE were assessed following the AOAC methods [19]. Moisture content was determined by subjecting the date seed powder to oven drying at 105 °C, while ash content was measured through incineration in a furnace at 550 °C for 24 h. For the fat content of MDSE, a standard Soxhlet extraction procedure was employed using an automated Soxtec extraction system (FOSS Soxtec 8000, Hilleroed, Denmark). A 2 g sample of MDSE was combined with petroleum ether, and the subsequent boiling, rinsing and recovery durations were set at 20, 40 and 8 min, respectively. The total fat content was quantified as a percentage by calculating the ratio of the weight of extracted fat to the weight of the MDSE sample (g/g) based on its dry weight.

$ABTS^{\bullet+}$ scavenging assay

The inhibition activity of MDSE on $ABTS^{\bullet+}$ (2,2′-azino-bis(3-ethylbenothiazoline-6-sulphonic acid) radical cations was determined following a modified method [20]. The $ABTS^{\bullet+}$ reagent was prepared by mixing 7 mM $ABTS^{\bullet+}$ solution with 2.45 mM potassium persulfate ($K_2S_2O_8$) in 95% EtOH. The mixture was left to stand in an amber bottle in darkness for 16 h at room temperature, permitting complete radical generation. Working solution of $ABTS^{\bullet+}$ was prepared by adjusting and diluting the incubated $ABTS^{\bullet+}$ stock solution with 95% EtOH until an absorbance of 0.7 ± 0.05 at 734 nm was obtained. The antioxidant activity was evaluated by adding approximately 10 µL of 500 µg/mL MDSE to 190 µL of $ABTS^{\bullet+}$ working reagent. Changes in the absorbance and discoloration were measured at 734 nm via UV-Vis microplate reader (SpectraMax iD3 Multi-Mode, Molecular Devices, San Jose, CA, USA) after 10 min of incubation. The radical scavenging effect of MDSE tested was expressed as a percentage of scavenging $ABTS^{\bullet+}$ radical using the following equation:

$$ABTS^{\bullet+} \text{ Scavenging effect (inhibitory activity) \%: } ((A_0 - A_1)/A_0) \times 100$$

where, A_0 is the absorbance of control $ABTS^{\bullet+}$ radical with EtOH and A_1 is the absorbance of samples ($ABTS^{\bullet+}$ radical + sample).

Experimental design and statistical analysis

Box-Behnken Design (BBD) with three independent variables (X_i, X_{ii} and X_{iii}) was employed to optimize the extraction conditions of MDSE using UAE. The design consisted of a three-factorial level (3^3) experimental plan with 3 independent variables, namely X_i (EtOH concentration, 50 - 80%), X_{ii} (extraction time, 30 - 90 min) and X_{iii} (extraction temperature, 40 - 70 °C). Design-Expert software version 12 was used to design and analyze the experimental conditions. The variables were coded at three levels (-1, 0, 1) (Table 1) and a total of 17 experimental runs were performed (Table 2). The effects of X_i, X_{ii} and X_{iii} on the responses were determined using a second-order-polynomial equation retrieved from RSM (Equation 1), as detailed below:

$$Yi = \alpha_0 + \alpha_1 X_i + \alpha_2 X_{ii} + \alpha_3 X_{iii} + \alpha_{11} X_i^2 + \alpha_{22} X_{ii}^2 + \alpha_{33} X_{iii}^2 + \alpha_{12} X_i X_{ii} + \alpha_{13} X_i X_{iii} + \alpha_{23} X_{ii} X_{iii} \quad (1)$$

where Y is the response variable; input variables consisting X_i (EtOH concentration), X_{ii} (time) and X_{iii} (extraction temperature); X_i^2, X_{ii}^2 and X_{iii}^2 representing the square effects; interaction terms were indicated by $X_i X_{ii}$, $X_i X_{iii}$ and $X_{ii} X_{iii}$; α_0 being the constant regression coefficient; while (α_1, α_2, α_3) , (α_{11}, α_{22}, α_{33}) and (α_{12}, α_{13}, α_{23}) are the regression coefficient for linear, quadratic and interactions, respectively.

The experimental data was analyzed using RSM to obtain the regression coefficients and statistical significance of the model terms while fitting it to the mathematical models. Model adequacies were checked through the regression analysis in terms of the values by R^2 (actual-R^2 and adjusted-R^2). One-way analysis of variance (ANOVA) at $p < 0.05$ was utilized to identify the significant terms in the model by F-value and lack of fit for each response. According to the software's recommendation, a quadratic polynomial model was selected and found to be a well-fitted for both the independent variables and their corresponding responses.

Table 1. Independent extraction variables selected for BBD optimization.

Independent variables	Levels			Dependent variables	Goal
	-1	0	1		
EtOH concentration (%) (X_i)	50	65	80	$ABTS^{\bullet+}$ scavenging activity (%) (Y_i)	Y_i - Maximised
Time (min) (X_{ii})	30	60	90		
Extraction temperature (°C) (X_{iii})	40	55	70		

Verification of the model

The extraction condition for MDSE were optimized for a maximum yield of antioxidant activity by $ABTS^{\bullet+}$, as determined by regression analysis, contour and 3D surface plots depicting the independent variables. The responses were measured based on the suggested optimal extraction parameters for EtOH concentration, time and extraction temperature. The proposed optimal parameters were then tested to validate the models, which is a crucial step in confirming the adequacy of the final reduced models. Finally, the predicted values were compared against the actual experimental values.

Table 2. The BBD matrix and the RSM's experimental response data observed using UAE.

Run	Independent variables			Response
	X_i	X_{ii}	X_{iii}	Y_i
	EtOH concentration (%)	Time (min)	Extraction temperature (°C)	$ABTS^{\bullet+}$ scavenging activity (%)
1	80	60	40	57.66
2	65	90	40	34.45
3	50	60	70	38.74
4	80	60	70	61.52
5	50	30	55	38.37
6	65	60	55	43.32
7	65	60	55	48.49
8	65	60	55	46.24
9	80	90	55	56.34
10	50	60	40	36.68
11	65	60	55	45.31
12	80	30	55	56.16
13	65	30	70	35.83
14	65	30	40	33.58
15	65	90	70	38.58
16	50	90	55	32.33
17	65	60	55	41.62

Identification of extracted saccharides in the optimized MDSE via UPLC-QTOF/MS

Saccharides within MDSE were characterised using ultra-high-performance liquid chromatography (UPLC-MS) and analyzed using Waters Acquity ultra-performance LC system (Waters, Milford, MA, USA). Separation through chromatography was achieved using a column (ACQUITY UPLC HSS T3, dimensions: 100 mm × 2.1 mm × 1.8 µm) sourced from Waters in Manchester, UK while Vion IMS QTof detector (Waters, Milford, MA, USA) was connected to the UPLC system. The mobile phase employed consisted of 0.1% formic acid (A) and acetonitrile (B), with a multi-step linear gradient comprising the subsequent stages: at 0 min, 1% B and 99% A; at 0.5 min, 1% B and 99% A; at 16.00 min, 35% B and 65% A; at 18.00 min, 100% B and 0% A; and finally at 20.00 min, 1% B and 99% A. Samples were injected at a volume of 1 µL, while the flow rate was set at 0.6 mL/min. Data acquisition was conducted within the m/z range of 50 to 1500, using 0.1 s/scan in high-definition mass spectrometry elevated energy (HDMSE). Collision energies (CE) were maintained at a constant 4 eV with a ramped increase from 10 (low energy) to 40 eV for high-energy scans.

Identification of volatile compounds and fatty acids (GC-MS/MS)

The analysis of optimized extracted phytochemicals from MDSE was performed using GC-MS/MS Agilent Technologies-7890A GC system equipped with an Agilent 7693B autosampler and an Agilent 7000B triple quadrupole mass spectrometry system (Agilent Technologies, Palo Alto, USA). A 30 m long × 0.25 mm internal diameter × 0.25 µm film thickness HP-5 column was utilized for the separation of phytochemical compounds. Injection was carried out using 7890A GC multimode inlet system in cold-splitless mode, with a volume of 1 µL. The carrier gas and quenching gas employed were high-purity helium (99.999%) at a constant flow rate of 1.2 mL/min and 2.25 mL/min, respectively. During the experiment, nitrogen gas with a purity of 99.999% served as the collision gas, flowing at a rate of 1.5 mL/min.

To initiate the analysis, the oven temperature was set at 70 °C for a duration of 2 min. Subsequently, the temperature was elevated to 150 °C at a rate of 25 °C/min, followed by a further increase to 200 °C at a rate of 10 °C/min. Finally, a final temperature ramp of 5 °C/min was applied, leading to a final temperature of 280 °C. This temperature was maintained for 9 min, resulting in a total run time of 36 min. For ionization, the mass spectrometer was operated in electron impact (EI) mode. In this mode, the transfer line, ion source and both quadrupoles were set at temperatures of 280, 300, 180 and 180 °C, respectively. Agilent MassHunter B.05.00 software was used for instrument control and data analysis.

RESULTS AND DISCUSSION

Total fat, ash and moisture content

As shown in Table 3, the total crude fat, ash and moisture content of Medjool seed powder were 8.42%, 1.03% and 8.25%, respectively. In regard to fat content, different date seed varieties exhibit a range of 5.0% to 13.2% [21], with seeds generally containing higher fat content compared to the date fruit itself [22]. The variability in fat content may be attributed to variations in variety, origin, harvesting time and fertilizer application [23]. The fat content in the current study represents a higher lipid content in Medjool seeds compared to Boufgous (6.76%), Bousthammi (6.97%), Majhoul (5.66%), Khalt a (6.05%) and Khalt z (7.75%) date seeds [18, 24]. Literature studies have reported a wide range of ash and moisture content in date seeds, with ash levels ranging from 1% to 17.5% and moisture content ranging from 1.2% to 10.8% [25-27]. Research suggests that biomass with a moisture content < 10 wt% is considered suitable as a feedstock, aligning with the conventional use of Medjool seeds in animal feed [28].

Table 3. Total fat, ash and moisture content (dry weight) of Medjool seed powder (*P. dactylifera*).

Component	Values*, %, (w/w) dry matter basis
Total fat	8.42 ± 0.06
Ash	1.03 ± 0.02
Moisture	8.25 ± 0.03

*The results are shown as the mean ± standard deviation of triplicates.

Fitting model

Response surface methodology (RSM) is a mathematical approach employed to optimize and analyze multivariable systems by identifying the relationship between independent variables and the response. The aim of this study was to use RSM to optimize 3 operational variables, namely X_i, EtOH concentration, X_{ii}, extraction time and X_{iii}, extraction temperature, to obtain the highest possible antioxidant activity ($ABTS^{\bullet+}$ scavenging activity) (Y_i). Table 2 displays the design template for the BBD employed in the evaluation. The independent variables were represented by a quadratic equation with second-order terms and interaction coefficients. The resultant equations, expressed in terms of coded factors, are presented below,

$$Y_i\ (ABTS^{\bullet+}\ \text{scavenging activity}) = +\ 79.23519\ -\ 3.60406X_i\ +\ 0.524730X_{ii}\ +\ 1.50693X_{iii}\ +$$
$$0.003455X_iX_{ii} - 0.002004X_iX_{iii} + 0.001038X_{ii}X_{iii} + 0.030766X_i^2 - 0.006798X_{ii}^2\ -\ 0.014518X_{iii}^2$$

The statistical models employed have demonstrated the absence of a significant lack of fit ($p > 0.05$) for the response surface model at 95% confidence level which indicates that the regression equation adequately explains the observed responses based on the ANOVA table generated (Table 4). Therefore, this suggests that the model could efficiently predict the $ABTS^{\bullet+}$ scavenging activity

of *P. dactylifera* MDSE given that the independent variables fall within the ranges specified. The use of an adjusted R^2 is recommended for assessing the appropriateness of the model and a threshold of at least 90% is desirable to indicate its adequacy [29], which alligns with all the models generated for X_i, X_{ii} and X_{iii}. The adjusted R^2 serves as a refined value for R^2 by accounting for the exclusion of unnecessary model terms [30]. Based on the ANOVA produced, a high F-value and a low p-value for each term in the model indicated a more significant influence on the corresponding response variables. Besides, according to the statistical analysis, a remarkably high level of significance will be shown by the regression model if evidenced by a p-value that is less than 0.0001, while a value of less than 0.5 satisfactorily describes a model is significant. The model term X_i (EtOH concentration) demonstrated an exceptionally high level of statistical significance or effect on the Y_i, $ABTS^{\bullet+}$ scavenging activity recorded, as indicated by their corresponding p-values of less than 0.0001. Another significant effect was observed on $ABTS^{\bullet+}$ scavenging activity caused by the interaction of X_i^2 (EtOH concentration^2), X_{ii}^2 (time^2) and X_{iii}^2 (extraction temperature^2) where its probability value was less than 0.05. It was determined that the remaining terms did not have a statistically significant impact on the responses. The coefficient of variation (CV) is a measure that reflects the level of dispersion in the experimental data points from the predictions of the models. Typically, CV should not exceed 10% and a low CV value signifies a small variation in the mean value, which is indicative of a satisfactory development of an appropriate response model [31]. In the present case, a low CV of 5.44% for Y_i denotes that the experiments performed are highly reliable and precise. Thus, the quadratic model generated can be employed for navigating the design space.

Table 4. Analysis of variance (ANOVA) of the regression equation for the optimization of $ABTS^{\bullet+}$ radical scavenging activity of MDSE.

Source	$ABTS^{\bullet+}$ scavenging activity (Y_i)			
	dF[a]	SS[b]	F-value	p-value
Model	9	1331.43	26.01	0.0001
X_i	1	915.06	160.87	< 0.0001
X_{ii}	1	0.6279	0.1104	0.7494
X_{iii}	1	18.89	3.32	0.1112
X_iX_{ii}	1	9.67	1.7	0.2335
X_iX_{iii}	1	0.8129	0.1429	0.7166
$X_{ii}X_{iii}$	1	0.872	0.1533	0.7071
X_i^2	1	201.76	35.47	0.0006
X_{ii}^2	1	157.59	27.71	0.0012
X_{iii}^2	1	44.93	7.9	0.0261
Residual	7	39.82		
Lack of fit	3	11.72	0.5564	0.6711
R^2	0.8312			
R^2 adjusted	0.9336			
CV (%)	5.44			

Response surface analysis

This section elucidates the impact of operational parameters and their interactive effects on the extraction of natural antioxidants ($ABTS^{\bullet+}$ scavenging activity). To visualize this impact, three-dimensional and contour profiles were depicted by varying two variables within the experimental range, while keeping the other variables at their central level (0 levels).

Antioxidant response – $ABTS^{\bullet+}$ radical scavenging activity

The contour and 3D plot graphs in Figure 1a(i and ii) and 1b(i and ii) highlighted the significance of X_i (EtOH concentration) as the single determining factor that highly influences the $ABTS^{\bullet+}$ radical scavenging activity ($p < 0.05$) of MDSE. All quadratic effects, namely X_i^2, X_{ii}^2 and X_{iii}^2, were shown to be significant ($p < 0.05$), while all 2-way interactions on $ABTS^{\bullet+}$ activity were non-significant with p-values > 0.05. Based on the fitted model results demonstrated by ANOVA (Table 4), R^2 values between the RSM model-adjusted (0.9336) and actual values (0.8312) of target responses satisfactorily supports the goodness of fit of the model with $p < 0.05$. Overall, *P. dactylifera* MDSE showed an antioxidant activity ranging from 32.33% to 61.52% with the highest activity recorded in run 4 and relatively weaker free radical scavenging in run 16.

The ABTS (2,2-azino-*bis*-3-ethylbenzothiazoline-6-sulphonic acid) assay works on the basis of creating $ABTS^{\bullet+}$ radical cation by reacting ABTS salt with a potent oxidizing agent, such as potassium persulfate or potassium permanganate. The reduction or termination of the blue-green $ABTS^{\bullet+}$ radical caused by the presence of hydrogen-donating antioxidants in the extract solution is measured by the suppression in its absorbance [32]. The free radical $ABTS^{\bullet+}$ scavenging capacity of MDSE increased dependently to the increased EtOH: water (v/v) factor (50 - 80% EtOH) and the EtOH concentration at 80% marks the highest scavenging capacity from 56.16% to 61.52%. The proportion of organic solvent present in water not only impacts the overall yield but also influences the yield of individual compounds that are prevalent in the plant. The positive effect created from increasing EtOH concentration may have been attributed to the enhanced polyphenol solubility with high antioxidant potential and its extraction rate. This is due to variations in affinity attributed to the differing polarities of a target solvent and compounds. Studies have demonstrated that higher ethanol concentration (beyond 70%) is more effective in extracting free radical scavenging flavonoids and flavonoid glucosides than water owing to its ability to interact with the compounds through non-covalent interactions and facilitate their diffusion into the solution [33]. While it has been observed that tannin constituents can solely be extracted using solvents possessing a dielectric constant greater than 20, such as ethanol. A plethora of research studies also have consistently demonstrated a correlation between $ABTS^{\bullet+}$ activity and the levels of total phenolic and flavonoid content (TPC and TFC) found in various plants, including *P. dactylifera* fruits [34] and *Mimosa pudica* [35]. Furthermore, previous studies have contributed to the present findings, indicating that an optimal ethanol concentration of approximately 80–85 v/v% is necessary to achieve the highest total phenolic content in *Curcuma zedoaria* leaves and distillery stillage from cereals [36, 37]. Hence, this justified the elevated antioxidant activity with increasing EtOH can be explained as the recovery of abundant flavonoids (epicatechin and catechin), tannins and condensed tannins (procyanidins B1, B2 and B2–3-*O*-gallate) were achieved from date seeds in previous studies [38, 39].

The lower antioxidant activity observed at lower EtOH concentrations could be attributed to the fact that water tends to extract more carbohydrates and other non-bioactive compounds, leading to a decrease in the purity of the bioactive extracts. The aforementioned suggests that the choice of solvent is pivotal in accessing the purity of the extract, specifically with regards to the concentration of antioxidative polyphenol compounds. In light of this, the present study has identified EtOH solvent at 80% as the optimal selective solvent for extracting polyphenols from Medjool date seeds. Based on Figure 1a (i and ii), 1b(i and ii), 1c(i and ii) and Table 4, temperature and time could be observed to have no significant influence on $ABTS^{\bullet+}$ radical scavenging activity of MDSE.

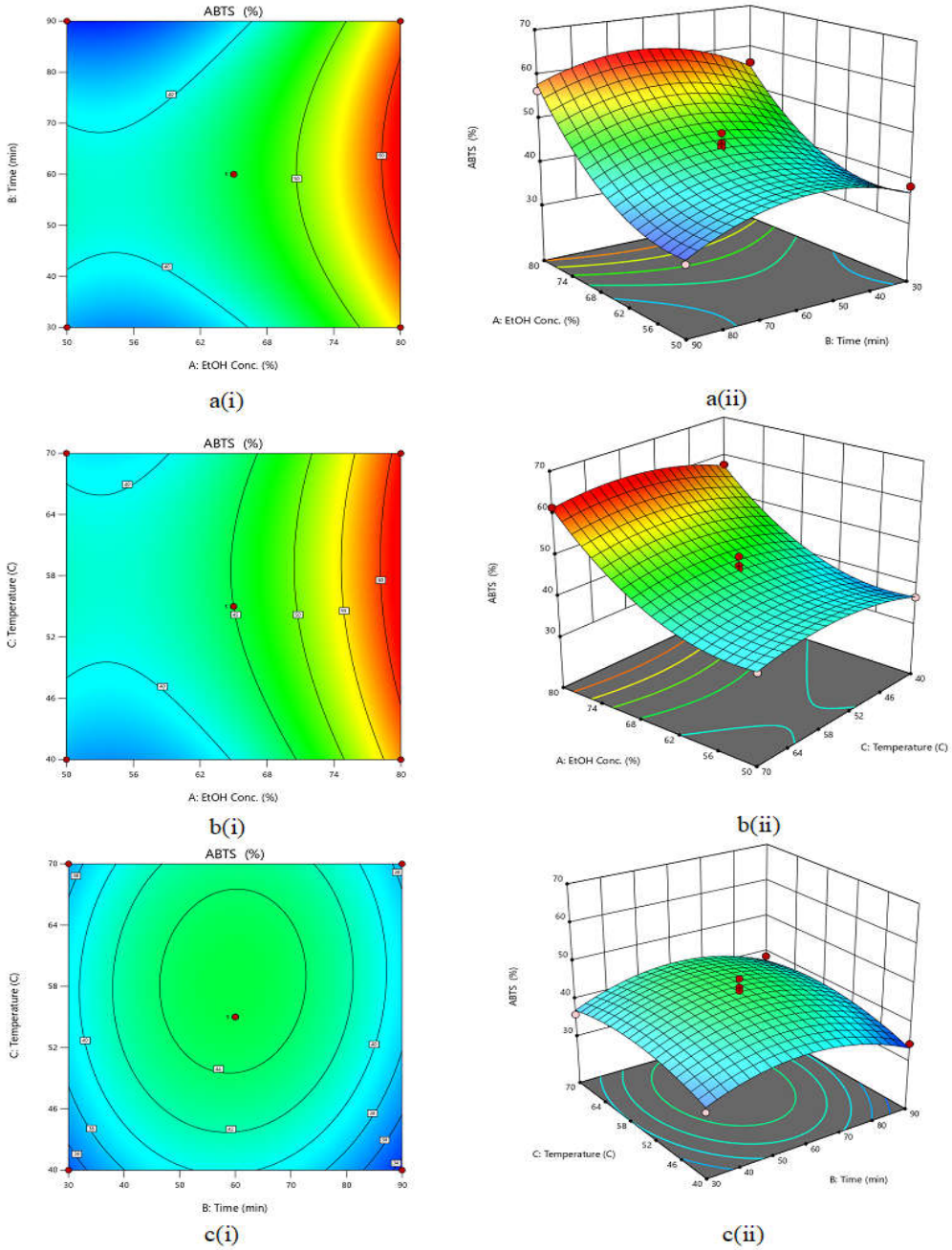

Figure 1. RSM generated (i) contour plot and (ii) 3D surface plots for $ABTS^{\bullet+}$ scavenging antioxidant activity (Y_i), (a) effect of EtOH concentration and time; (b) effect of EtOH concentration and temperature; (c) effect of temperature and time.

Optimization and validation of predictive model

The optimization of extraction parameters was aimed to maximize the desirability of responses using numerical optimization in Design-Expert statistical software version 12.0 (Stat-Ease, Inc.) based on the initial experimental outcomes. The objective was to achieve maximum $ABTS^{\bullet+}$ scavenging antioxidant activity from MDSE. The outcome of the simultaneous optimization, employing a high desirability function approach at 0.96, indicated that the optimal ethanolic extraction conditions for MDSE were as follows: 80% EtOH concentration, 44 min and 57 °C. To assess the model's adequacy in predicting these optimal conditions and response, a validation of the optimal conditions was conducted by comparing the observed and predicted values of the response using the same extraction conditions (80% EtOH, 44 min and 57 °C). The validation experiment was performed in triplicates, and the predicted and mean experimental values under the optimized condition are presented in Table 5. The percentage deviation between the experimental and theoretical results of $ABTS^{\bullet+}$ scavenging potential was calculated as 1.79% respectively, indicating a close agreement. The verification experiments conducted at the predicted optimum conditions confirmed the validity and adequacy of the predictive model obtained through the BBD design of experiment. The experimental values closely matched the predicted values, further supporting the accuracy of the predictive model in suggesting the specified optimal UAE conditions for antioxidant activity of MDSE to be valid. Additionally, the validation experiments also confirm that the predicted values of $ABTS^{\bullet+}$ scavenging activity as determined by the model were successfully achieved within 96.6% confidence interval of experimental values.

Table 5. Comparison of predicted and experimental values for the response at optimal extraction condition.

Response	Predicted value[a]	Experimental value[b]	% Deviation
Yi, $ABTS^{\bullet+}$ scavenging activity (%)	60.35	60.93 ± 0.021	1.79

[a]Optimized predicted value using response surface quadratic model. [b]Mean ± standard deviation of triplicate determinations from experiments.

UPLC-QTOF/MS Identification of Saccharides in the optimized MDSE

The UPLC-QTOF/MS analysis (Table 6) unveiled that the optimized extract of MDSE is composed of isomaltose, a disaccharide with α-(1, 6)-linkage (Figure 2a(i, ii and iii)), along with mannotriose, a trisaccharide manno-oligosaccharide (MOS) (Figure 2b(i, ii and iii)), and a trisaccharide from the raffinose family oligosaccharides (RFOs) known as stachyose (Figure 2c(i, ii and iii)). Isomaltose, known for its lower glycemic index (GI) than sucrose, is a beneficial pharmaceutical candidate and a promising option as a low-calorie sweetener in the food industry (when ingested 8-10 g/day), in addition to its role in boosting intestinal microflora [40]. Recent findings indicate that among the probiotic *Lactobacillus* sp., mannotriose is the preferred oligosaccharide [41]. Research has shown that date seed powder promotes the growth of the probiotic *Lactobacillus paracasei* [42]. RFOs such as stachyose, with one glucose, one fructose and two galactose units, are usually known for their significant roles in plant development and stress responses [43], which tend to be more advantageous for the survival of plant compared to humans. Despite being linked to causing flatulence in humans and monogastric animals due to the lack of necessary enzymes for breakdown, these compounds, when maintained at controlled levels, offer positive impacts in the colon. They are also known to exhibit prebiotic potential by fostering the growth of beneficial bacteria while reducing the presence of pathogens and putrefactive bacteria in the colon [44]. Following its benefits to the gastrointestinal tracts, stachyose is also recognized for its ability to prevent ulcerative colitis in mice [45]. This clearly shows the saccharides-rich matrix in date seed extracts would serve as the reason behind the role of date seeds in modulating gut microbiota and preventing gastrointestinal ailments.

Table 6. Tentatively identified saccharides in the optimized extract from MDSE.

No	Compound name	Formula	Natural mass (Da)	Observed *m/x*	Retention time (min)
1.	Isomaltose	$C_{12}H_{22}O_{11}$	342.11621	341.108	0.86
2.	Mannotriose	$C_{18}H_{32}O_{16}$	504.16903	503.1613	0.85
3.	Stachyose	$C_{24}H_{42}O_{21}$	666.2219	665.2155	0.85

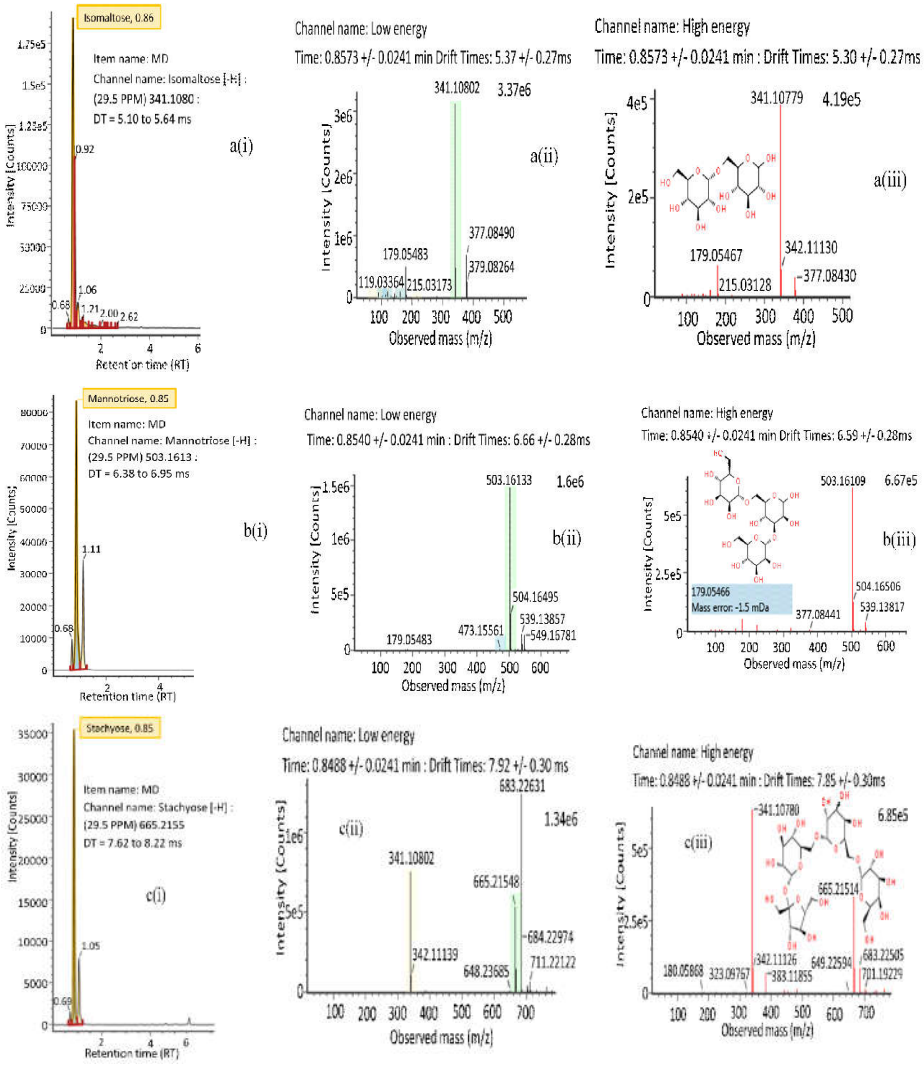

Figure 2. (i) UPLC-QTOF/MS chromatograms, (ii) mass spectra (MS/MS) at low collision energy and (iii) mass spectra (MS/MS) at high collision energy of (a) isomaltose, (b) mannotriose and (c) stachyose.

GC-MS/MS identification of fatty acids and other compounds in the optimized MDSE

Figure 3 and Table 7 portray 12 selected compounds found in extract of Medjool seeds using 80% EtOH. The relative concentration of each compound can be indicated using the peak area percentage. The saturated fatty acids primarily identified within optimized MDSE include pentanoic acid (valeric acid), dodecanoic acid (lauric acid), tetradecanoic acid (myristic acid), hexadecanoic acid (palmitic acid) and octadecanoic acid (stearic acid), which were commonly found in all date seed variants studied thus far [18]. Lists of saturated fatty acids determined in the current study co-revealed some of the fatty acid composition within the 8.42% of total fat extracted from Medjool date seed powder, as shown in Table 3. Numerous reports have discussed that date seeds are a rich source of oleic acid, which is followed by another 11-14 other fatty acids discovered in date seeds such as lauric, myristic, palmitic, linoleic, stearic, capric, arachidic, gadoleic, behenic, tricosylic, lignoceric, linolenic, palmitoleic, margaric, pentadecanoic and myristoleic acid [18-24]. Research suggests that the medium-chain fatty acids present in date seeds have been shown to have a positive impact on the gastrointestinal tract of poultry, promoting the growth of beneficial bacteria and improving nutrient absorption [46]. The fatty acids present in MDSE portrayed its suitability for the use in developing anti-inflammatory pharmaceutical formulations, albeit not as active ingredients but rather as coadjuvants that can improve the transdermal permeation of non-steroidal anti-inflammatory drugs [47]. On the other hand, the detection of valeric acid in MDSE indicates its potential use as a carbon source for producing bioplastics [48]. This aligns with the trend of utilizing agricultural and industrial waste for the production of eco-friendly and biodegradable plastics. Curzerenone, a natural sesquiterpene lactone, extracted primarily from *Cucurma* sp. [49] and *Lindera pulcherrima,* has been documented to possess both antioxidant and antibacterial activities [50], and similar compound has been identified from the current GC-MS/MS analysis of MDSE.

Table 7. Phytochemical compounds in the optimized extract of MDSE using 80% EtOH solvent.

Peak No.	Identified name	Molecular formula	Retention time, Rt (min)	Peak area (%)	Base peak m/z
1	Octadecanoic acid (stearic acid)	$C_{19}H_{3}6O_3$	7.897	0.145	44.1
2	Pentanoic acid (valeric acid)	$C_{19}H_{30}O_3$	8.14	5.125	190.8
3	Dodecanoic acid (lauric acid)	$C_{12}H_{24}O_2$	8.717	4.815	73
4	Curzerenone	$C_{15}H_{18}O_2$	9.369	0.258	44
5	Isospathulenol	$C_{15}H_{24}O$	9.666	0.290	44.1
6	Tetradecanoic acid (myristic Acid)	$C_{14}H_{28}O_2$	10.973	2.832	44.1
7	2,6-Octadien-1-one, 3,7-dimethyl-1-phenyl-, (E)-	$C_{16}H_{20}O$	12.440	2.528	44.1
8	Hexadecanoic acid (palmitic acid)	$C_{16}H_{32}O_2$	13.289	1.305	44.1
9	9,9-Dimethoxybicyclo[3.3.1]nona-2,4-dione	$C_{11}H_{16}O_4$	15.607	1.643	44.1
10	Ethyl oleate	$C_{20}H_{38}O_2$	16.001	0.514	44.1
11	Ethanethioic acid, S-(2-methyl-5-benzoxazolyl) ester	$C_{10}H_9NO_2S$	31.970	2.866	206.7
12	1,4-Bis(trimethylsilyl)benzene	$C_{12}H_{22}Si_2$	35.390	17.656	206.7

Compared to other studies, the overall yield of some fatty acids obtained from the MDSE was relatively low, which may be attributed to the use of polar solvent during the ultrasonication process. This is due to the low solubility of the lipids in the polar solvent contributing to the lower relative concentrations of the recovered fatty acids. The extraction process may also have imposed some limitations, leading to the detection of oleic acid as ethyl oleate, an ester resulting from the condensation of oleic acid and EtOH used during ultrasonication. However, the present

investigation posits that a considerable amount of fatty acids were also proven to be extracted when an aqueous polar solvent at high EtOH concentration were used, despite other common studies that usually utilizes non-polar solvents to yield higher beneficial fatty acids. This may be also associated to the ultrasonic waves created during UAE that were able to extract these acids due to the larger cavitation formed in the system.

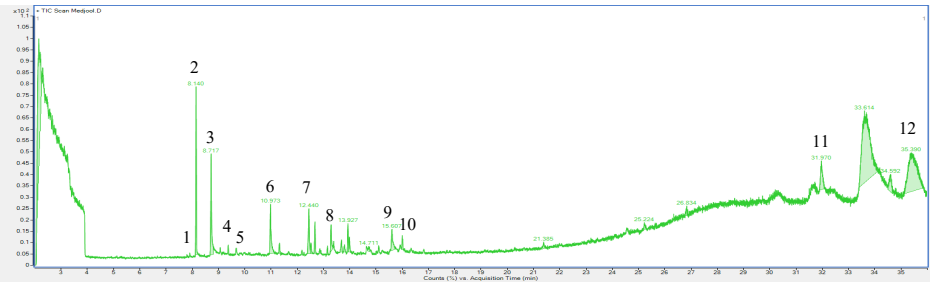

Figure 3. GC-MS/MS chromatograms of the optimized MDSE extracted with 80% EtOH solvent.

CONCLUSION

This investigation corroborates the efficacy of employing RSM to optimize the UAE extraction conditions (EtOH concentration, time, and extraction temperature) for achieving optimal enhanced $ABTS^{\bullet+}$ scavenging activity from MDSE. The RSM results unequivocally demonstrate the significant influence of EtOH concentration as a key independent variable on the antioxidant activity. However, the impact of sonication time and temperature were found to be insignificant for the responses. The optimized conditions (80% EtOH concentration, 44 min and 57 °C) result in an experimental value which aligns well with the predicted value. Analysis using GC-MS/MS and UPLC-QTOF/MS unveiled the presence of phytocompounds, including saturated fatty acids and saccharides, indicating MDSE's potential as a source of functional antioxidants with reasonable radical scavenging activity. The optimization of UAE holds immense desirability in maximizing the retrieval of antioxidants from MDSE, which renders them exceptionally valuable for industrial applications. This study strongly recommends further research to quantify and isolate individual significant antioxidative compounds from MDSE, thus fortifying the impetus to incorporate these waste products in the development of nutraceutical products.

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