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Seeds of Health: Maximising Antioxidant Potential from *Phoenix dactylifera* L. Medjool Date Seeds via BBD Optimisation and UPLC-QTOF/MS Metabolite Identification

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ABSTRACT

The growth in date production industries resulted in a significant accumulation of date palm wastes, especially the seeds, which poses environmental issues and escalates processing and storage expenses. Appropriate characterisation of this product would mitigate these issues while adding commercial value through beneficial nutraceutical and pharmaceutical applications. Current research highlights the ultrasonication process parameters required for extracting the maximum yield of antioxidant activity from *Phoenix dactylifera* Medjool seeds utilising Response Surface Methodology (RSM). A Box-Behnken design has been adopted to determine the impact of 3 extraction variables, namely ethanol (EtOH) concentration (X_A), ultrasonication duration (X_B), and temperature (X_C), on antioxidant activity evaluated through hydroxyl radical ($OH^{\bullet}$) scavenging activity. The results portrayed that the experimentally obtained value was consistent with the predicted output, affirming the reliability of the model employed for optimising the extraction methodologies. The extraction at 79% EtOH, for 45 min, at 40°C

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provided the highest antioxidant activity recorded at 87.40% ± 1.29. Two compounds, namely, 1-O-caffeoyl-β-D-glucopyranoside and genistein-7,4'-di-O-β-D-glucoside, categorised to the group of caffeic acid and flavonoid derivatives supported the higher antioxidative response obtained. Our study indicates a suitable technique for extracting Medjool date seeds phytocompounds of therapeutic benefits, applying a modern statistical tool (RSM), which offers a more cost-effective and less labour-intensive optimisation process compared to conventional methods. The outcome also presents bioactive phytocompounds from Medjool date seeds that strengthen the role of this candidate for nutraceutical purposes.

Keywords: Antioxidant, Box-Behnken design (BBD), date seeds, hydroxyl radical scavenging, *Phoenix dactylifera*, response surface methodology (RSM), UPLC-QTOF/MS.

INTRODUCTION

Oxidative stress, arising from an imbalance between oxidants and antioxidants or endogenous defence mechanisms within the human body, has been linked to the etiology of various ailments. This includes heart diseases, carcinoma, diabetes, stroke, Parkinson's disease, Alzheimer's dementia, inflammation, arthritis, muscle degeneration, and hyperlipidaemia. This process typically gets initiated with the generation of reactive oxygen species (ROS), particularly free radical species such as hydroxyl radicals ($OH^{\bullet}$), superoxide ($O_2^{\bullet-}$), alkoxy ($RO^{\bullet}$), and peroxy ($RO_2^{\bullet}$). Oxygen derivatives that are not free radicals, such as hydrogen peroxide (H_2O_2), singlet oxygen (O_2), and hypochlorous acid (HOCl), are also classified as ROS as they can serve as precursors or substrates that lead to the formation of highly reactive free radicals (Patel et al., 2018). These ROS arise from cellular metabolism and exogenous agents, which in turn contribute to the destabilisation and breakdown of cell membranes and set the stage for health challenges (Babior, 2000).

Concurrently, the idea of boosting the self-defence system by consuming synthetically manufactured antioxidants such as propylene glycol (PG), butylated hydroxyanisole (BHA), butylated hydroxyl toluene (BHT), and tert-butylhydroquinone (TBHQ) in food has seen to raise the concerns over its safety and other adverse effects (carcinogenicity) (Singh et al., 2023; Taghvaei & Jafari, 2015). As a result, these factors escalated the quests for exploring novel, effective and cost-effective natural sources of antioxidants, supplanting the artificial alternatives in nutraceuticals, dietary supplements, pharmaceuticals, and cosmetic products.

For centuries, botanical sources have served as the cornerstone of traditional medicinal practices, which continuously introduce innovative therapeutic remedies to mankind up to date. One such plant is the date palm (*Phoenix dactylifera* L.), among the oldest cultivated species, which has been extensively grown in the Arabian Peninsula, North Africa, and the Middle East regions for thousands of years (Chao & Krueger, 2007). This dioecious, perennial, monocotyledonous fruit tree belongs to the *Arecaceae* family (subfamily

Coryphoideae). The species name "*dactylifera*" originates from the Greek word "dactylus," meaning "finger," and the Latin "ferous," meaning "bearing" (Ashraf & Hamidi-Esfahani, 2011). Among the thousands of date varieties cultivated around the world, Medjool dates are particularly famous for their large fruit size, vibrant orange-yellow flesh, and exceptional taste and texture, making them a popular choice in the Islamic world, especially during Ramadan. Medjool dates are also known by several other names, including 'Medjhool,' 'Medjehuel,' 'Mejhul,' 'Majhoul,' and 'Mejhoul.' These dates are believed to have originated from Morocco's Tafi Lalett Valley (Errachidia) (Nixon, 1950). Genetically, Medjool dates belong to a primary group of North African date varieties, alongside other well-known types such as 'Deglet Noor' (from Algeria/Tunisia) and various Egyptian cultivars (El-Assar et al., 2005). For years, the focus has been primarily on the fruit, yet the seeds of Medjool date also deserve attention for their potential health benefits, especially as a rich source of antioxidants. These seeds, comprising 10% w/w of total date fruit weight, are often discarded in the industry and are capable of implicating serious environmental concerns if unattended. Beyond their culinary use, date seeds have been traditionally used in ethnobotanical practices for medicinal purposes, such as in Turkey, where they are ground into herbal coffee called "Hurma coffee" to enhance memory (Sekeroglu et al., 2012). Meanwhile, in Algeria, date seeds are commonly used in folk remedies to treat conditions like weakness and gout, and they are also believed to help boost lactation in breastfeeding women (Selmani et al., 2017). These seeds harbour a wealth of bioactive antioxidative compounds, primarily the phenolic acids such as derivatives of hydroxylated benzoic acid (protocatechuic acid, p-hydroxybenzoic acid, gallic acid, and vanillic acid), along with cinnamic acid derivatives (ferulic acid, caffeic acid, m-coumaric, p-coumaric acid, and o-coumaric acid) while rutin, catechin, quercetin, luteolin, and kaempferol were the prominent flavonoids (Al-Farsi & Lee, 2008; Bouhlali et al., 2020). Importantly, without appropriate and optimised extraction conditions, the valuable functional phytocompounds within date seeds may be irrevocably lost.

The valorisation of these agricultural by-products aims to increase their economic and functional value, as they were once limited to use as animal feed. Recently, seeds of date have gained interest as promising sources of bioactive compounds with potential applications in food, pharmaceuticals, and cosmetics. However, many of these by-products remain underutilised due to the absence of effective extraction methods. Several innovative extraction methods have been developed for isolating antioxidant secondary metabolites, with ultrasonic-assisted extraction (UAE) standing out as one of the preferred green technologies that meet modern extraction standards (Shirzad et al., 2017). UAE offers a straightforward, cost-effective, conserves energy, and efficient alternative that not only optimises yield and preserves the quality of extracts, but also scales well to meet industrial demands (Pagano et al., 2021; Rao et al., 2021). On the other hand, it

is essential to acknowledge that extraction conditions play a crucial role in maximising yields and deciding the type and structures of the extracted compounds. Various factors must be carefully considered when using extraction methods, such as the choice and ratio of solvents, extraction temperature, duration, and the solid-to-liquid ratio (Ghasemi et al., 2024). These variables are key to ensuring the full recovery of target compounds while minimising the risk of chemical alterations.

When selecting a reliable mathematical and statistical approach to optimise analytical procedures, Response Surface Methodology (RSM) is widely employed, particularly in the food and medical fields, for refining extraction processes (Fattahi & Rahimi, 2016). RSM offers significant advantages by minimising the number of experimental trials needed while still assessing the relative significance of multiple variables and their interactions, making the process more time and labour-efficient. Among the available Designs of Experiments (DoE), the Box-Behnken Design (BBD) was selected due to its specific suitability for modelling second-order responses, which are the focus of most RSM studies (Ahmed et al., 2022). Additionally, BBD requires only three levels for each factor to construct a second-order regression model, making it a practical choice for this investigation.

The present study focusses on applying RSM, a valuable technique for optimising the antioxidant potential through hydroxyl radical scavenging assay from Medjool date seeds. These seeds were subjected to varying ultrasonic-assisted extraction (UAE) parameters, namely EtOH concentration (50-80%), duration or time of ultrasonication (30-90 min) and extraction temperature (40-70°C). A Box-Behnken design was employed at three-factor and three-level modes.

METHODS

Chemicals and Reagents

Analytical graded chemicals such as ethanol (EtOH), 1,10-phenanthroline monohydrate, ferrous (II) ammonium sulphate hexahydrate, phosphate buffer solution (0.1 M, pH 7.4), and 30% hydrogen peroxide were utilised in this research.

Materials and Chemicals

Date fruits (Medjool) originating from Palestine were locally purchased from licensed vendor, depitted, and cleansed under running water to deter the adherent fleshy tissue. The seeds were then subjected to oven drying at 45°C for 24 hours. Approximately 500 g of seeds were retrieved following the drying process, where these seeds were ground into a powdered form using a heavy-duty crusher and grinder. To achieve uniform particle size, the resulting powder was carefully sifted through a 1-2 mm sieve, ensuring homogeneity.

Ultrasound-assisted Extraction (UAE)

The technique of ultrasound-assisted extraction (UAE) was conducted using an ultrasonic water bath machine (Elmasonic S60 kHz, Elma Hans Schmidbauer GmbH, Singen, Germany). For each run, Medjool date seeds powder (2 g) was added to 100 mL of extraction solvent following the Box-Behnken experimental design outlined in Table 1. Prior to the extraction process, the samples were centrifuged and filtered through a vacuum filtration unit using Whatman No. 1 filter paper. The filtrate solvent was then removed by vacuum drying in a rotary evaporator at 45°C, ensuring the chemical composition and physical properties of the extract were preserved. The resulting crude extracts of Medjool date seeds (MSE) were stored in sealed glass containers at a temperature of -20°C.

Table 1
The BBD suggested conditions and experimental responses obtained using UAE

Run	Independent variables			
	X_A	X_B	X_C	Y
	EtOH concentration (%)	**Duration (min)**	**Extraction temperature (°C)**	**Hydroxyl radical scavenging activity (%)**
1	80	60	40	88.50
2	50	30	55	66.52
3	65	60	55	76.32
4	50	60	40	66.10
5	65	90	70	82.50
6	80	30	55	78.36
7	50	90	55	59.77
8	65	90	40	69.62
9	80	60	70	85.09
10	65	60	55	78.20
11	65	30	70	76.08
12	80	90	55	81.62
13	65	60	55	77.49
14	65	60	55	76.81
15	50	60	70	74.51
16	65	30	40	79.80
17	65	60	55	75.32

Hydroxyl Radical Scavenging Activity

Antioxidant activity possessed by compounds within MSE was evaluated through the hydroxyl radical (OH•) scavenging assay following a modified method of Mukhopadhyay et al. (2016). To perform the assay, a 3 mM solution of 1,10-phenanthroline was prepared using phosphate buffer (0.1 M, pH 7.4), while a ferrous ammonium sulphate solution with the same concentration was prepared in water. In a multiwall plate, 50 µL of MSE extract at a concentration of 1 mg/mL was combined with 50 µL of ferrous ammonium sulphate. Subsequently, 50 µL of 0.01% H_2O_2 was added to initiate the reaction. The resulting solution was placed in darkness at room temperature for 5 min. Following incubation, 50 µL of 1,10-phenanthroline was added, thoroughly mixed, and further incubated for a duration of 10 min at room temperature. The mixture's absorbance was then recorded at 510 nm with the aid of a microplate reader. The blank solution, containing only ferrous ammonium sulphate, water, and 1,10-phenanthroline, exhibited the highest absorbance. Additionally, an extra blank reagent was prepared, consisting of only 1,10-phenanthroline and the absorbance of this blank was subtracted from the absorbance of all the treatment samples. The scavenging activity of MSE compounds towards hydrogen peroxide was calculated using the provided formula Eq. [1]:

$$\%\ H_2O_2\ Scavenging\ Activity = \frac{A_T}{A_B} \times 100, \qquad [1]$$

where A_T is the absorbance of a solution containing the MSE, while A_B is the absorbance of a blank.

Response Surface Methodology Design (RSM)

The present study used a Box-Behnken Design (BBD), utilising a three-level, three-factor full factorial design (3^3) to access the impact of each independent variable: X_A (EtOH concentration, 50-80%), X_B (ultrasonication duration, 30-90 min), and X_C (temperature, 40-70°C) on the response variable (hydroxyl radical scavenging activity) associated with UAE. The variables were coded at three levels (-1, 0, 1), as shown in Table 2, with a sum of 17 experimental runs suggested and conducted as outlined in Table 1. The levels regarding the independent variables were selected and slightly modified based on the optimised values and ranges reported in previous studies. These studies detailed the use of RSM optimisation for ultrasonication-assisted extraction from various date seed varieties and pitaya seeds (Afifi et al., 2017; Alshammari et al., 2024; Niroula et al., 2024; Zulkifli et al., 2020). Subsequently, the acquired data was analysed using RSM to determine the optimal processing condition for each independent variable. The RSM outputs, such as contour

and 3D graphic surface plots, were retrieved to visualise the optimum and most influential variables. The influence of the extraction parameters on hydroxyl radical scavenging values was examined using a second-order polynomial equation Eq. [2], derived from RSM:

$$Y = \alpha_0 + \alpha_1 X_A + \alpha_2 X_B + \alpha_3 X_C + \alpha_{11} X_A^2 + \alpha_{22} X_B^2 + \alpha_{33} X_C^2 + \alpha_{12} X_A X_B + \alpha_{13} X_A X_C + \alpha_{23} X_B X_C \quad [2]$$

where *Y* represents the response variable (hydroxyl radical scavenging activity); extraction variables include, X_A (EtOH concentration), X_B (duration), and X_C (extraction temperature); X_A^2, X_B^2, and X_C^2 defining the square effects; interaction terms were represented by X_AX_B, X_AX_C, and X_BX_C; α_0 stands for the constant coefficient of the model; α_1, α_2, α_3 referring to linear effects; α_{11}, α_{22}, α_{33} directing quadratic effects, and α_{12}, α_{13}, α_{23} indicating interaction effects.

Table 2
Independent extraction variables subjected to BBD optimisation and its response

Independent variables	Levels			Dependent variable (Y)	Goal
	-1	0	1		
EtOH concentration (%) (X_A)	50	65	80	Hydroxyl radical scavenging activity (%)	Maximised
Duration (min) (X_B)	30	60	90		
Extraction temperature (°C) (X_C)	40	55	70		

Analysis of variance (ANOVA) was conducted for the response variable, with a significance level set at $p < 0.05$, to determine the significant factors in the model while fitting the data to the mathematical models. The adequacy of the models was assessed using criteria, including model analysis, F-value, lack-of-fit test, and comparing R^2 values (actual-R^2 and adjusted-R^2). According to Jumbri et al. (2015), a regression model with an R^2 value greater than 0.9 defined the response model possessing strong fitness. Additionally, the significance of the corresponding variables is deduced by larger F-values and smaller *p*-values, indicating higher significance. Design Expert Software (version 13, Stat-Ease Inc., Minneapolis, MN, USA) was accessed for the experimental data analysis.

Validation of the Model

The optimal condition suggested by RSM was validated for the maximum extraction of hydroxyl scavenging antioxidant potential according to the values retrieved, utilising the option of desirability. Consequently, the validation experiment was performed in triplicates.

The experimental result was matched with the model's forecasted value, which authenticates the model's validity. The percentage (%) difference between the predicted and experimental data was calculated as follows Eq. [3]:

$$\%\ Difference = \frac{(Predicted\ value - Experimental\ value)}{Predicted\ value} \times 100 \qquad [3]$$

Bioactive Phytocompound Identification – UPLC-QTOF/MS

Separation of phytocompounds within the optimised MSE was completed using ultra-high-performance liquid chromatography (UPLC-QTOF/MS). At the same time, the Waters Acquity ultra-performance LC system (Waters, Milford, MA, USA) was used for analysis. Chromatography separation performed on MSE used specific operational criterias, utilising an Acquity UPLC HSS T3 column with dimensions of 100 mm × 2.1 mm × 1.8 µm. . The sample injection volume was set to 1 µL, with the flow rate maintained at 0.6 mL/min. Negative ionisation mode was utilised during the operation. The mobile phase consisted of formic acid (solvent A) and acetonitrile (solvent B), with a gradient elution programmed as follows: 1% B and 99% A at 0 minutes, maintained until 0.5 minutes; transitioning to 35% B and 65% A at 16 minutes; 100% B at 18 minutes; and re-equilibrating to 1% B and 99% A by 20 minutes. Detection was carried out using a Waters Vion IMS QTOF system (Milford, MA, USA). Data collection utilises the mass-to-charge ratio (m/z) range between 50 to 1500, using high-definition mass spectrometry elevated energy (HDMSE) with a scan rate of 0.1 s/scan. Collision energies (CE) stay constant for scans at low energy at 4 eV and gradually increase to 40 eV approaching high-energy scans.

RESULTS AND DISCUSSION

Fitting the Model

The empirical model was assessed through ANOVA (Table 3) to determine its significance and suitability. In general, the relevance of the associated variables becomes more significant with greater F-values and smaller *p*-values. The Fischer variation ratio (F-value) serves as a valid statistical measure of the extent to which the parameters explain the variation in the data relative to its mean (Beeler et al., 2024). The statistical analysis, including the *p*-value and F-value (with a 95% confidence interval) of the current study, indicated a highly significant model ($p < 0.0001$; $F = 90.61$) was developed from this study. This suggests that there is merely a 0.01% probability that such a large F-value resulted from noise within the model. Adequate precision serves as a measure of the signal-to-noise ratio. Our findings achieved the acceptable minimum threshold of greater than 4 (Chen et al., 2023), a desirable limit that indicates the model was fit. Additionally, an insignificant lack of fit was observed for the model ($p > 0.6426$), suggesting that the model adequately

Table 3
Analysis of variance (ANOVA) tabulated for the fitted quadratic polynomial model of Y (hydroxyl scavenging activity)

Source	Hydroxyl radical scavenging activity (Y)				
	dF[a]	Mean square	SS[b]	F-value	p-value
Model	9	91.98	827.83	90.61	< 0.0001
X_A (EtOH Conc.[c])	1	555.31	555.31	547.03	< 0.0001
X_B (Duration)	1	6.53	6.53	6.44	0.0388
X_C (Temperature)	1	25.10	25.1	24.72	0.0016
X_AX_B	1	25.06	25.06	24.69	0.0016
X_AX_C	1	34.88	34.88	34.36	0.0006
X_BX_C	1	68.92	68.92	67.89	< 0.0001
X_A^2	1	14.48	14.48	14.27	0.0069
X_B^2	1	48.84	48.84	48.12	0.0002
X_C^2	1	53.90	53.90	53.10	0.0002
Residual	7	1.02	7.11		
Lack of fit	3	0.7443	2.23	0.611	0.6426
Pure error	4	1.22	4.87		
R^2	0.9915				
$R^2_{Adjusted}$	0.9805				
CV (%)	1.33				
Adequate Precision	36.5388				
Mean	76.04				
Std. dev.[d]	1.01				

[a]Degree of freedom; [b]Sum of squares; [c]EtOH concentration; [d]Standard deviation

explained the observed response. A model's quality could be measured through the use of a determination coefficient (R^2). A high R^2 value implies that the quadratic model applied parallelly adjusted to the investigated results (Zhang et al., 2023). The R^2 value (ideally nearing 1.00) for the hydroxyl radical scavenging antioxidant activity was determined to be 0.9915, which was very closely matched to the adjusted R^2 value of 0.9805, thereby confirming the reliability of the model. Referring to the adjusted R^2, only less than 2% of the overall variation remains unexplained by the model. These findings support the likelihood of replicating the data with a high degree of agreement between the observed response and the expected value, as described by Akçay and Anagün (2013). The low coefficient of the variation value (CV < 10%) for hydroxyl radical scavenging activity at 1.33% demonstrated that the models had a preferable accuracy. Ideally, a reduced CV demonstrates limited variability in the mean values, signifying greater precision, reliability, and consistency in the experimental outcomes (Wu et al., 2020). Following this, a second-order polynomial equation (quadratic model) resulted from the regression analysis of the data for hydroxyl

radical scavenging response, as illustrated in Eq. [4]:

$$Y = 76.83 + 8.33X_A - 0.9038X_B + 1.77X_C - 1.85X_A^2 - 3.41X_B^2 + 3.58X_C^2 + 2.50X_AX_B - 2.95X_AX_C + 4.15X_BX_C \quad [4]$$

Implication of Extraction Conditions towards Hydroxyl Radical Scavenging Activity

The migration of bioactive compounds from the plant matrix to a solvent can be achieved by modulating their diffusion coefficients through methods such as ultrasonication, suitable extraction temperature, duration of treatment, and solvent concentration, which holds a notable impact on solvent polarity (Wang et al., 2008). The choice of using UAE, the green extraction procedure, arises from the fact that sonicator-based extraction efficiently isolates non-lipid phytocompounds, particularly antioxidants (Sanou et al., 2023). Furthermore, the utilisation of ultrasound proves advantageous due to its ability to disrupt plant cell structures, thereby releasing cellular contents into the extraction medium (Chemat et al., 2017). Among the three key effects of ultrasound (thermal, mechanical, and cavitation), sonic cavitation is the most predominant force in UAE, and it facilitates the mass transfer of bioactive compounds (Das et al., 2022). It works by generating acoustic cavitation in the medium, where minuscule bubbles adhere to solid particles, and upon collapsing, these bubbles rupture cell structures by producing microjets that target cell surfaces, increasing pressure, and accelerating the diffusion of cell components into the solvent (Zahari et al., 2020). In this study, EtOH was chosen as the extraction solvent due to its reduced toxicity relative to other polar organic solvents. In relation to the antioxidant assay performed, while hydrogen peroxide is not a free radical, it reacts with metals such as iron and copper in physiological systems, producing highly reactive hydroxyl radicals (Fenton reaction) that pose significant toxicity to living tissues (Ofoedu et al., 2021). Hence, this assay mimics the production of hydroxyl ROS in our body system and the need to scavenge these free radicals using antioxidants to prevent oxidative stress and destruction towards cells and organs.

Across all the 17 experimental runs in Table 1, the extraction process yielded approximately 9.59-17.05% of extract, with the highest yield observed in Run 3 and the lowest in Run 9. Referring to the ANOVA tabulation (Table 3), it displayed a very highly significant ($p < 0.0001$) linear effect for X_A, EtOH concentration, followed by X_c, temperature ($p < 0.01$) and at a satisfactory level for X_B, duration ($p < 0.05$). These findings highlight that the concentration of EtOH has the most significant impact on the extractability of hydroxyl radical scavenging compounds from MSE. Following closely are temperature and ultrasonication duration. The interaction effects of all variables were also proven to be significant in the decreasing order from X_BX_C, X_AX_C, and X_AX_B. Additionally, smaller *p*-values were also obtained for all quadratic coefficients (X_A^2, X_B^2, and X_C^2). Thus, this

simplified the presence of significant positive linear, interaction, and quadratic effects of all variables tested on the response (hydroxyl radical scavenging activity).

In relation to the constructed regression model, a 2D-contour line (Figure 1a(i), 1b(i) and 1c(i)) and 3D-response graphs (Figure 1a(ii), 1b(ii), and 1c(ii)) were generated for the response by varying two factors within the experimental range while keeping the third-factor constant. The maximum ability to scavenge hydroxyl radicals was achieved under the conditions following experimental run 1 (88.50%), with an EtOH concentration of 80%, ultrasonication duration of 60 min, and a temperature of 40°C. However, the lowest scavenging activity (59.77%) was observed in the experimental run 7, conducted with 50% EtOH for 90 min, and at 55°C, respectively.

Figures 1a (i) and 1a (ii) display the 3D and contour plots illustrating the relationship between hydroxyl radical scavenging activity with EtOH concentration and ultrasonication duration. Notably, as the EtOH concentration gradually increased while maintaining a constant sonication duration, the investigated response exhibited a gradual rise, ultimately reaching its peak at the highest EtOH concentration studied (80% EtOH). Mohamed Ahmed et al. (2020) reported a similar relationship of extraction conditions when studying the TPC content in *Solenostemma argel* Hayne leaves and also for seed-related extractions such as in Sapodilla fruit seeds, which reported 80% EtOH as the preferred solvent concentration for having high TPC value (Aquino et al., 2020). Increasing the sonication duration (while maintaining a constant EtOH concentration (80%) led to a marginal increase in the hydroxyl radical scavenging activity. Prior to numerous experimentations, the combination of EtOH and water demonstrated greater efficacy in extracting polyphenolic compounds compared to using a single solvent system. The enhanced efficacy was clearly attributed to the adjustment made to the polarity of the solvent, which often modifies the solvent's density, dielectric constant, and viscosity (Elboughdiri, 2018). Despite numerous research findings favouring EtOH concentrations of 50-62% are optimal for extracting high TPC, TFC, and antioxidant potential, including research on date seeds (Afifi et al., 2017; Alshammari et al., 2024; Luo et al., 2018; Zhang et al., 2024), this study advocates for a higher EtOH content of 80% as the preferred solvent concentration for extracting MSE phytocompounds with specific structural arrangements responsible for scavenging hydroxyl radicals. It is crucial to recognise that the antioxidant effectiveness of phenolic compounds depends highly on their specific structural makeup. Apart from this, elevated water content in the solvent was also known to enhance the contaminant solutes to be co-extracted. However, at the cost of reducing the extraction efficiency of phenolic active compounds (Rafi et al., 2020). This phenomenon could potentially account for the observed lower hydroxyl scavenging activity as depicted in the MSE when the content of water to EtOH ratio increased.

The linear increase in the antioxidant activity (hydroxyl scavenging) with increasing EtOH (up to 80%) was observed at low (< 46°C) and also as higher temperatures (64-70°C)

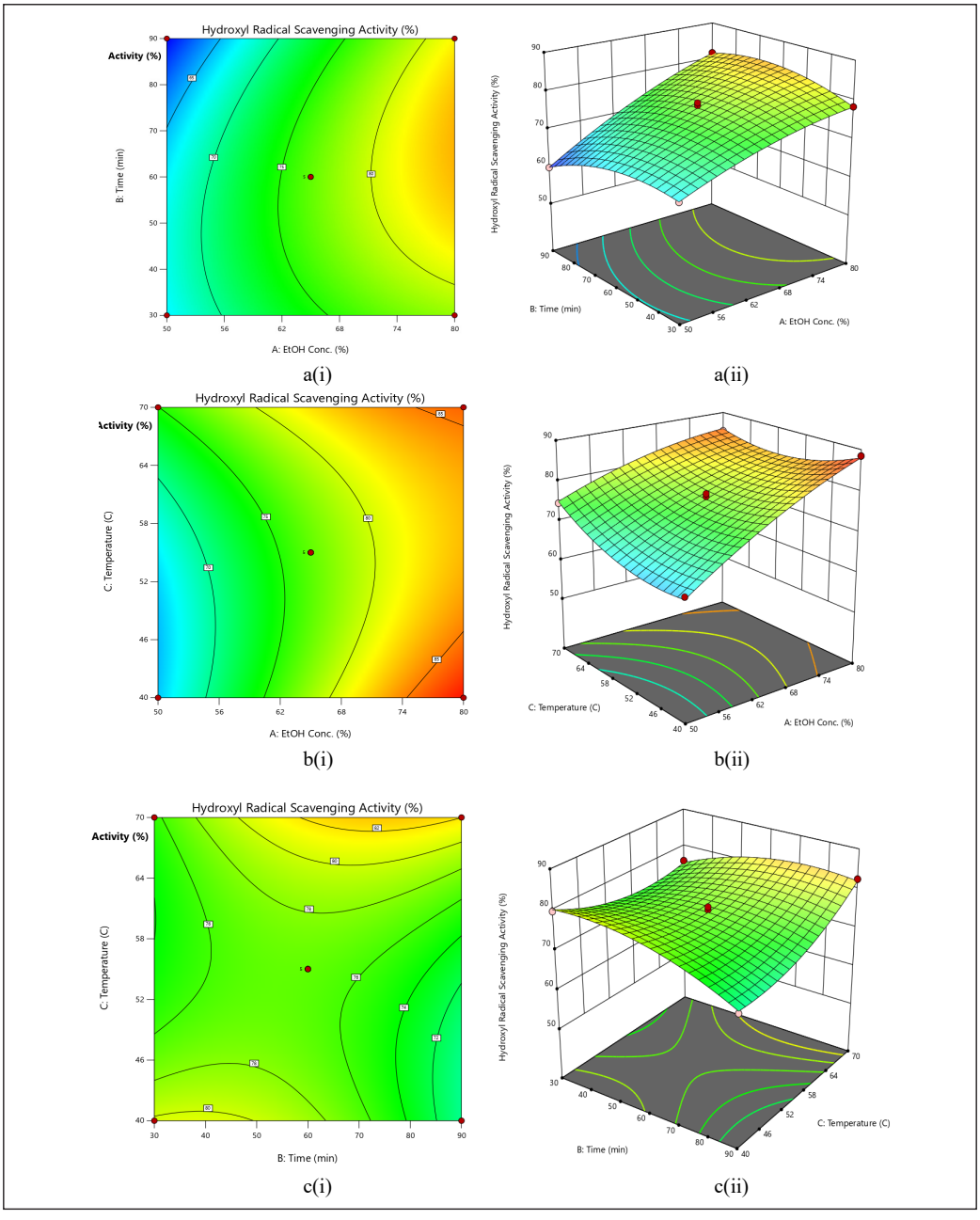

Figure 1. RSM generated contour plot (i) and 3D surface plots (ii) for hydroxyl radical scavenging (Y); (a) effect of EtOH concentration and duration (time); (b) effect of EtOH concentration and temperature; (c) effect of temperature and duration (time)

were approached (Figure 1b(i) and 1b(ii)). This result was supported by a study conducted on the effect of EtOH concentration and temperature on grape cane extraction, which demonstrated a similar trend (Karacabey & Mazza, 2010). Temperature holds a pivotal role in controlling the extraction process by weakening solvent viscosity and surface tension, as well as softening the tissues and disrupting the cell structures, particularly in fragmenting the cell wall and membrane (Zhang et al., 2024; Hossain et al., 2012). Moreover, the factor of heat also hydrolyses the bonds of bound phenolic compounds (phenol-protein or phenol-polysaccharide. These increase the solubility and diffusion coefficient, facilitating the mass transfer of polyphenolic compounds (Liu et al., 2022). The observed improvement in antioxidant activity from MSE with increasing temperature is likely attributed to the heat-induced disruption to the cell structures, which enhances the solubility and extraction of phenolic compounds from the intracellular matrix. Inversely, the enhanced hydroxyl scavenging ability utilising higher EtOH concentration but at lower temperatures may have been attributed to the fact that Medjool date seeds comprised antioxidants that were both heat-sensitive and insensitive. Therefore, maintaining high EtOH concentrations, regardless of whether the temperature is elevated or reduced, may prove effective in improving the extraction of antioxidant compounds from MSE, particularly those responsible for neutralising hydroxyl radicals. However, low temperatures will be preferred when relying on the cost-effectiveness and sustainable processing of date seeds for industries.

It appears that extending the ultrasonication duration (30-90 min) when the highest temperature was used (70°C) in the current study moderately increased the antioxidative potential. This elevates the exposure duration of date seed solids within the solvent at maximum temperature, resulting in the recovery of the highest levels of heat-insensitive antioxidants.

Response Optimisation and Verification of the Model

In this study, numerical optimisation of extraction conditions was conducted on the basis of the initial experimental outcomes, in which our target intended to attain maximum hydroxyl radical scavenging potential from MSE. The optimum extraction conditions suggested through RSM-guided optimisation utilising its desirability function at 0.981 were at 79% EtOH, 45 min, and 40°C, respectively, for achieving up to 87.40% of hydroxyl scavenging potential. Under these optimised conditions, the extraction process produced 11.19% ± 0.28 of extract. The results of the experimental response matched well with the predicted value, with a CV accounting for 0.64%, as shown in Table 4. Zakaria et al. (2021) deemed the experimental results to align with the predicted data only when the CV values were below 5%, which is consistent with the findings in this study. This clearly proves that the RSM model generated is well-fitted for the recovery of antioxidants from MSE via UAE while having a good correlation.

Table 4
Predicted and experimental response values for hydroxyl radical scavenging activity utilising optimised conditions

Response variable (Y)	Predicted value	Experimental value[a]	% Difference (CV)[b]
% Hydroxyl radical scavenging	87.96	87.40 ± 1.29	0.64

[a]Mean ± standard deviation of triplicates (n = 3); [b]CV: coefficient of variation

Functional Metabolite Identification from MSE - UPLC-QTOF/MS

The optimised EtOH extract of MSE was fractionated by chromatography for secondary functional metabolites. The compounds presumably in charge of the antioxidative activity discussed in Table 4 and Figure 1 in MSE were tentatively listed in Table 5 and attached with their UPLC-QTOF/MS chromatograms (Figure 2). These compounds belong to the group of bioactive caffeic acid and flavonoid derivatives. Plenty of compounds from similar groups have been reported in other scientific studies exploring Medjool date seeds (Khallouki et al., 2018; Salomón-Torres et al., 2019). However, this study introduces additional findings by identifying 1-O-caffeoyl-β-D-glucopyranoside and genistein-7,4'-di-O-β-D-glucoside also to be present in MSE.

1-O-caffeoyl-β-D-glucopyranoside was known to exhibit potent antioxidative activity, as reported by Deng et al. (2016) and Braham et al. (2005), investigated in cape gooseberry, *Physalis pubescens* L. and *Moricandia arvensis*, respectively. This correlated with the high antioxidative activity detected in our current study. The second compound listed as the genistein-7,4'-di-O-β-D-glucoside is classified as a flavonoid derivate. According to De la Parra et al. (2016), genistein is generally an important phytocompound used as an anti-cancer. This liaised with the report released by Subarmaniam et al. (2023), which listed genistein-7,4'-di-O-β-D-glucoside isolated in *A. paniculata* ethanolic extract exhibits anti-cancer potential. Alongside this, the same compound was reported to be found in *Saphoro japonica* L. and noted an excellent protective effect against erythrocyte hemolysis (Wang et al., 2019; Qi et al., 2007). These results revealed that flavonoid and caffeic acid derivatives are health-benefit-contributing compounds in Medjool date seeds, strengthening the arguments for the potential therapeutic usage of these seeds.

Table 5
Identified bioactive compounds from optimised MSE using UPLC-QTOF/MS corresponding to the antioxidative properties

Identified compound name	Classification	Molecular formula	Natural mass (Da)	Observed m/z	Retention time, RT (min)
1-O-caffeoyl-β-D-glucopyranoside	Caffeic acid derivative	$C_{15}H_{18}O_9$	342.09508	341.0869	5.92
Genistein-7,4'-di-O-β-D-glucoside	Flavonoid	$C_{27}H_{30}O_{15}$	594.15847	593.1521	10.14

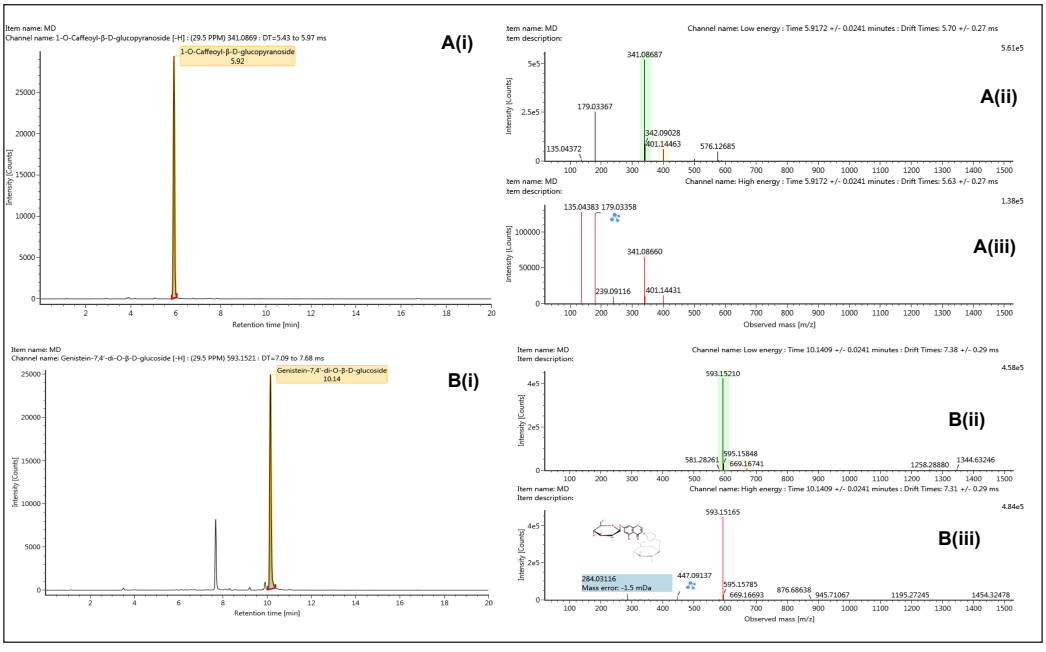

Figure 2. (i) UPLC-QTOF/MS chromatograms, (ii) low collision energy mass spectra (MS), and (iii) high collision energy mass spectra (MS), of: (A) 1-O-caffeoyl-β-D- glucopyranoside, (B) genistein-7,4'-di-O-β-D-glucoside identified from MSE

CONCLUSION

Optimisation of antioxidant activity (hydroxyl radical scavenging potential) from *P. dactylifera* Medjool seeds extracts was successfully determined, employing RSM. The optimised conditions for the maximum retrieval of hydroxyl scavenging were achieved at 79% EtOH with a lower temperature at 40°C, ultrasonicated for 45 min. Ethanol concentration and temperature were the crucial factors significantly affecting the antioxidant activity derived from MSE. The identification of functional antioxidative phytocompounds within the extracted Medjool date seeds signifies the scavenging activity observed. This investigation holds potential implications for the large-scale extraction of Medjool date seeds by boosting the extraction methodologies and producing nutraceutical and pharmaceutical-rich constituents.

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