ABSTRACT

Background: *Nelumbo nucifera* seeds are medicinally important due to the presence of phytochemicals with strong antioxidant potential. Extraction of these phytochemicals has been a major problem faced by the researchers. 

Objective: The purpose of the study was to optimize the process conditions for extraction of phenolic antioxidants from *N. nucifera* seed.

Methods: Process variables for microwave assisted extraction of phenolic antioxidants from *N. nucifera* seed flour were optimized by response surface methodology using a multi-factorial central composite design based on five levels of each of four input variables including X1: particle size (in terms of mesh No.), X2: microwave treatment period (MTP), X3: solvent concentration (ISC) and X4: extraction period (EP). Total extract yield was calculated as Total extractable components (TEC) and the extracts were analyzed for total phenolic content (TPC) and total antioxidant activity (TAOA).

Results: Data analysis showed a significant increase (p<0.05) in TEC, TPC and TAOA in response to increase in the levels of selected factors. Optimum levels of extraction variables to achieve maximum level of response parameters were found to be: 125.96, 2.45 min, 55.21% and 3.87 h for TEC, 61.73, 1.66 min, 80.08% and 4.98 h for TPC and 60.89, 2.37 min, 44.98% and 1.123 h for TAOA. 

Conclusion: The extraction of phenolic antioxidants from *N. nucifera* seed flour is significantly increased in response to an increase in mesh No. (decrease in particle size), MTP, SC and EP but up to a certain limit. The decrease in extraction yield and antioxidant activity at higher levels of studied factors may be attributed to the possible decomposition of phenolic compounds due to prolonged duration of microwave heating and prolonged extraction time.

Key words: *Nelumbo nucifera* seed flour, Microwave assisted extraction, Antioxidant activity, Response surface methodology, Central composite design, Phenolic content.

Correspondence:
Mr. Haq Nawaz,
Department of Biochemistry, Bahauddin Zakarya University, Multan-60800, PAKISTAN.
Phone no: 92-61-9210391; Mobile no: 92-300-6373150
E-mail: haqnawaz@bzu.edu.pk
DOI: 10.5530/fra.2017.1.10

INTRODUCTION

Increasing demand of natural antioxidants as compared to synthetic antioxidants in the field of food and pharmaceutical industries in last two decade has attracted the attentions of researchers to explore new, cheap, reliable, effective and frequently available sources of natural antioxidants. Plant materials, particularly the underutilized vegetative crops and waste biomass from food processing industries, have been proved to be valuable sources of natural antioxidants which would be advantageous over synthetic ones to overcome the problems of oxidative damage to live stock and human health.¹³ Phenolic compounds are generally considered as the non nutritional components present in plant foods which are taken in diet and play surprising role in protection against oxidative damage. These compounds, owing to their redox potential, free radical scavenging capacity, hydrogen donating ability, reducing and metal ion chelating properties, possess strong antioxidant characteristics and reduce the oxidative stress induced by imbalanced generation and removal of reactive oxygen and nitrogen species.⁴⁻⁷ Various studies have been reported which have proved that plant phytochemicals are potent antioxidants and show wide biological activities including anti-diabetic, anticancer, antiproliferative antiobesity and antimicrobial activity.⁹⁻¹⁰

Increase in the extraction yield and recovery of phenolic compounds from plant biomass at economical costs is the major concern of the day in bioprocess technology. Several studies have been reported regarding the method development and optimization of extraction of bioactive phenolic compounds from plant matrices. Particle size of plant material, extraction temperature, nature and concentration of extracting solvent and extraction period are some important factors which significantly affect the extraction process of phenolic compounds from plant tissues. Several techniques such as ultrasonication, microwave irradiation, gamma irradiation, super critical fluid extraction and soxhlet extraction have been employed to assist the extraction procedure and increase the extraction yield.¹¹⁻¹⁴ Solvent extraction has been found to be cost saving and easy to handle method of phenolic extraction. Solvent polarity is important factor for the selective extraction of bioactive compounds of a particular nature. Among the series of polar and nonpolar organic solvents hydroalcoholic solvent combinations are commonly used for extraction of phenolic compounds.¹⁵⁻¹⁶ It is, however, still demanding to explore new and more efficient extraction methods and optimize the existing methods to increase the bioavailability, extraction yield and recovery of phenolic antioxidants from underutilized food materials and waste biomass.

*Nelumbo nucifera*, commonly known as lotus is an aquatic plant mainly found in China and subcontinent. Leaves, seeds and rhizome of *N. nucifera* are good source of nutritional and medicinal components.¹⁵⁻²² Previously, studies have been reported on the extraction and antioxidant properties of phenolic compounds from *N. nucifera* rhizome, seeds and leaves. Seed extracts have been found to be rich in a variety of naturally occurring phenolic compounds which possess certain biological activities including antioxidant, antiproliferative, antiarythmia, anti-inflammatory, anticancer, antiobesity, hypolipidemic, hepatoprotective, and immu-
nomodulating properties. Any progress in research on extraction optimization of phenolic compounds from N. nucifera would contribute significantly in making it a valuable competitor in food and pharmaceutical industry.

Microwave treatment has been found to affect the extraction yield and antioxidant properties of plant phytochemicals. Microwave assisted extraction of bioactive compounds from plant material is frequently used method of the day to increase the extraction yield. The microwave irradiation and extraction of phenolic compounds from various plant materials have been optimized using different methods. In present study the effect of four factors including particle size of flour, microwave treatment period, solvent concentration and extraction period on extract yield and antioxidant activity of phenolic compounds from N. nucifera seed flour was optimized using response surface methodology (RSM). RSM is a statistical technique which has got preference over conventional method of varying one parameter keeping others constant for use in multifactor optimization procedures. It has been proved to be the suitable methodology for optimization of multivariate processes because it neglects the inter-parameter effect on the optimal value. It has been extensively used in various fields of research to create response-surface models for optimization and prediction of changes in response variables as a function of changes in input variables.

MATERIALS AND METHODS

Dry mature seeds of N. nucifera were purchased from local market, cleaned and seed coat was removed manually. Seed plumule was ground in an electric grinder at low speed (1000 rpm) to minimize the temperature fluctuations beyond 35°C. The flour thus obtained was packed in air tight glass bottles and stored in dark at standard laboratory conditions until further processing.

Experimental design

The cumulative effect of four input variables including particle size (in terms of sieve mesh No.), microwave treatment period (MTP), solvent concentration (SC) and extraction time (EP) on extraction yield in terms of total extractable components (TEC), total phenolic content (TPC) content and Trolox equivalent total antioxidant activity (TAOA) of N. nucifera flour was optimized by response surface methodology (RSM) using a four factor five level rotatable central composite design (CCD). The coded levels of the selected variables were calculated as:

$$X_i = \left(\frac{\xi_i - \xi \bar{\xi}}{S_i}\right) \quad i = 1,2,\ldots,k$$

where $X_i$ is the coded value of an independent variable, $\xi_i$ is the specific location of independent variable, $\bar{\xi}$ is the center point and $S_i$ is the scale factor i.e. the difference between different levels of variables. The actual and coded levels of input variables are presented in Table 1.

The relationship of total extractable components, total phenolic content and total antioxidant activity of N. nucifera seed flour against the Mesh No., MTP, SC and EP was determined by developing a second order polynomial quadratic model. The suggested model finds the levels of input variables in region of optimal response. The study was done in phases based on CCD.

Sieving

The flour was sieved successively through micro screens of mesh No. 60, 80, 100, 120 and 140 meshes to obtain different levels of particle size. The distribution of particle size was done on the basis of sieve mesh No. As the particle size is inversely proportional to sieve mesh No., a gradual decrease in particle size is observed with a respective increase in mesh No. The range of particle size of flour obtained from selected sieves was as under (Sigma Aldrich 2014):

<table>
<thead>
<tr>
<th>Sieve mesh No.</th>
<th>Particle size (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>178-250</td>
</tr>
<tr>
<td>80</td>
<td>150-177</td>
</tr>
<tr>
<td>100</td>
<td>126-149</td>
</tr>
<tr>
<td>120</td>
<td>106-125</td>
</tr>
<tr>
<td>140</td>
<td>&lt;106</td>
</tr>
</tbody>
</table>

Microwave treatment of flour

The flour of various particle sizes (50g each) as obtained from the selected sieves was processed for microwave treatment at various levels of treatment period as selected by CCD. Microwave treatment was carried out in glass beakers using LG MH2043DRY WT 20L Microwave Oven. The operating conditions were selected as radiation intensity: at medium low power (200 W), sample mass per load: 5g, treatment duration: as selected by experimental design. The treatment was discontinued after each min for 30 sec and the flour was mixed thoroughly to avoid the burning of flour.

Extraction

The untreated flour (5 g) of N. nucifera seed at random particle size ranging from 60-120 mesh No. was extracted in 80% aqueous methanol (solid to solvent ratio 1:20 v/v) for 5 h. TEC were calculated as g/100 g dry wt.:

$$TEC (g/100 \text{ g dry wt.}) = \left(\frac{\text{Weight of extract}}{\text{Weight of sample}}\right) \times 100$$

The microwave treated flour (5 g) at various levels of particle size was extracted in aqueous methanol (solid to solvent ratio 1:20 v/v) at various levels of methanol concentration (% v/v) and extraction period as selected by CCD. The extracts were evaporated to dryness at 25±5°C under vacuum, weighed and TEC were calculated as above.

The crude methanolic extracts were stored at 4 ± 1°C in air tight sterile laboratory environment to minimize the chances of microbial contamination and growth throughout the study period. The samples were also protected from direct sunlight exposure throughout the study period in order to minimize the chances of photo-oxidation of antioxidant compounds.

Phytochemical and antioxidant analysis

TPC in crude methanolic extract (10 mg/100 ml) was estimated by previously described Folin-Ciocalteu method. TPC was calculated as g gallic acid equivalent/100 g dry wt using a linear regression equation obtained from standard curve of gallic acid ($R^2 = 0.9843$):

$$TPC (g/100 \text{ g dry wt.}) = \text{Abs. at 720 nm/18.284}$$

TAOA of extracts was determined by Phosphomolybdenum assay using the reported method (34). TAOA was calculated as Trolox equivalent g/100 g dry wt. using a linear regression equation obtained from standard curve of Trolox ($R^2=0.9795$):

$$\text{TAOA (g/100 g dry wt.) = Abs. at 695 nm/6.576}$$

Statistical analysis

The data was statistically analyzed by one way analysis of variance (ANOVA) using second order polynomial quadratic response surface model. The optimum levels of response variable as a function of input variables were predicted by creating polynomial regression equations.
The generalized polynomial regression model for prediction of variation in response variables is given as:

\[ Y_i = \beta_0 + \sum \beta_{i1}X_{i1} + \sum \beta_{i2}X_{i2} + \sum \sum \beta_{ij}X_{ij} \]

where \( Y_i \) is the predicted response, \( \beta_0 \) is the regression coefficient for main, \( \beta_{i1} \) for linear, \( \beta_{i2} \) for quadratic and \( \beta_{ij} \) for interaction effect of input variables \( Xi \) and \( Xj \).

The polynomial regression equation was used to obtain the predicted values of response variables which were plotted against the experimental ones to check the adequacy and validity of the suggested response-surface models. The degree of scatter of data points in response surface model, fairness of fit of regression equation and significance of estimated regression coefficient for each response were assessed by determining the coefficient of determination \( (R^2) \), adjusted coefficient of determination \( (R^{adj}) \) and lack of fit \((F\text{-value})\) at a probability \( p<0.05 \) respectively. Coefficient of variation \( (CV) \) and adequate precision were determined to check the reliability and precision of experiments. For graphical expression of variability in responses as a function of input variables, the three-dimensional plots were constructed between response and independent variables. The optimum levels of input variables in a region of optimal responses were found by numerical optimization of data at maximum desirability. The development of experimental design, data analysis and optimization procedure was carried out using statistical software, Design Expert 10.0 (Stat-Ease, Inc.).

**RESULTS**

In an initial experiment phenolic antioxidants of native N. nucifera seed flour were extracted in 80% methanol for 5 h and analyzed for TEC, TPC and Trolox equivalent TAOA. TEC, TPA and TAOA were found to be 15.90 ± 2.23, 0.68 ± 0.14 and 8.32 ± 1.67 g/100 g respectively (Table 2). In a subsequent experiment the experimental conditions for extraction of phenolic antioxidants from microwave treated flour of N. nucifera seed were optimized using RSM. The experimental values of TEC, TPA and TAOA of extracts obtained at random levels of extraction variables as selected by experimental design are presented in Table 3. TEC, TPC and TAOA ranged from 10.79 to 22.47, 0.18 to 0.99 and 5.11 to 16.01 with mean ± standard deviation 17.47 ± 1.61, 0.55 ± 0.09 and 10.35 ± 1.45 g/100 g dry wt. respectively. The results obtained at various combinations of extraction variables were found to be statistically different \( (p<0.05) \).

**DISCUSSION**

Plants are rich source of phenolic compounds which possess antioxidant potential. N. nucifera seeds are good source of phenolics and other

### Table 1: Actual and coded levels of selected input variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>Actual Levels</th>
<th>Equation for coding</th>
<th>Coded levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>X1: Particle size (Mesh No.)</td>
<td>60, 80, 100, 120, 140</td>
<td>( X_1 = (\text{Mesh No.} - 100)/20 )</td>
<td>-2, -1, 0, 1, 2</td>
</tr>
<tr>
<td>X2: Microwave treatment period (min)</td>
<td>0.5, 1, 1.5, 2, 2.5</td>
<td>( X_2 = (\text{Microwave treatment period} - 1.5)/0.5 )</td>
<td>-2, -1, 0, 1, 2</td>
</tr>
<tr>
<td>X3: Solvent concentration (%)</td>
<td>20, 40, 60, 80, 100</td>
<td>( X_3 = (\text{Solvent concentration} - 60)/20 )</td>
<td>-2, -1, 0, 1, 2</td>
</tr>
<tr>
<td>X4: Extraction period (h)</td>
<td>1, 2, 3, 4, 5</td>
<td>( X_4 = (\text{Extraction period} - 3)/1 )</td>
<td>-2, -1, 0, 1, 2</td>
</tr>
</tbody>
</table>

which consists of 30 experimental points with \( n_f = 16 \) factorial points, \( na = 8 \) axial point and \( nc = 6 \) centre points.

### Table 2: TEC, TPC and TAOA of crude methanolic extracts of native N. nucifera seed flour

<table>
<thead>
<tr>
<th></th>
<th>Experimental value (g/100 g dry wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEC</td>
<td>15.90 ± 2.23</td>
</tr>
<tr>
<td>TPA (gallic acid equivalent)</td>
<td>0.68 ± 0.14</td>
</tr>
<tr>
<td>TAOA (Trolox equivalent)</td>
<td>8.32 ± 1.67</td>
</tr>
</tbody>
</table>
Table 3: Experimental values of TEC, TPC and TAOA of crude methanolic extracts of N. nucifera seed flour at selected levels of input variables by CCD

<table>
<thead>
<tr>
<th>Std.</th>
<th>Runs</th>
<th>X&lt;sub&gt;1&lt;/sub&gt; (Mesh No.)</th>
<th>X&lt;sub&gt;2&lt;/sub&gt; (MTP (min))</th>
<th>X&lt;sub&gt;3&lt;/sub&gt; (SC (%))</th>
<th>X&lt;sub&gt;4&lt;/sub&gt; (EP (h))</th>
<th>TEC (g/100 g dry wt.)</th>
<th>TPC (g/100 g dry wt.)</th>
<th>TAOA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>1'</td>
<td>100</td>
<td>1.5</td>
<td>60</td>
<td>3</td>
<td>18.47</td>
<td>0.45</td>
<td>9.50</td>
</tr>
<tr>
<td>27</td>
<td>2'</td>
<td>100</td>
<td>1.5</td>
<td>60</td>
<td>3</td>
<td>18.47</td>
<td>0.45</td>
<td>9.50</td>
</tr>
<tr>
<td>16</td>
<td>3</td>
<td>120</td>
<td>2.0</td>
<td>80</td>
<td>4</td>
<td>20.84</td>
<td>0.57</td>
<td>10.97</td>
</tr>
<tr>
<td>17</td>
<td>4</td>
<td>60</td>
<td>1.5</td>
<td>60</td>
<td>3</td>
<td>18.47</td>
<td>0.83</td>
<td>8.69</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>80</td>
<td>1.0</td>
<td>40</td>
<td>4</td>
<td>16.07</td>
<td>0.35</td>
<td>7.02</td>
</tr>
<tr>
<td>11</td>
<td>6</td>
<td>80</td>
<td>2.0</td>
<td>40</td>
<td>4</td>
<td>19.09</td>
<td>0.42</td>
<td>9.27</td>
</tr>
<tr>
<td>15</td>
<td>7</td>
<td>80</td>
<td>2.0</td>
<td>80</td>
<td>4</td>
<td>20.50</td>
<td>0.84</td>
<td>12.68</td>
</tr>
<tr>
<td>20</td>
<td>8</td>
<td>100</td>
<td>2.5</td>
<td>60</td>
<td>3</td>
<td>22.47</td>
<td>0.64</td>
<td>9.10</td>
</tr>
<tr>
<td>6</td>
<td>9</td>
<td>120</td>
<td>1.0</td>
<td>80</td>
<td>2</td>
<td>16.07</td>
<td>0.84</td>
<td>10.38</td>
</tr>
<tr>
<td>22</td>
<td>10</td>
<td>100</td>
<td>1.5</td>
<td>100</td>
<td>3</td>
<td>19.52</td>
<td>0.96</td>
<td>14.91</td>
</tr>
<tr>
<td>8</td>
<td>11</td>
<td>120</td>
<td>2.0</td>
<td>80</td>
<td>2</td>
<td>19.15</td>
<td>0.63</td>
<td>10.59</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>80</td>
<td>2.0</td>
<td>80</td>
<td>2</td>
<td>18.57</td>
<td>0.88</td>
<td>15.19</td>
</tr>
<tr>
<td>29</td>
<td>13'</td>
<td>100</td>
<td>1.5</td>
<td>60</td>
<td>3</td>
<td>18.47</td>
<td>0.45</td>
<td>9.50</td>
</tr>
<tr>
<td>21</td>
<td>14</td>
<td>100</td>
<td>1.5</td>
<td>20</td>
<td>3</td>
<td>11.96</td>
<td>0.18</td>
<td>6.04</td>
</tr>
<tr>
<td>26</td>
<td>15'</td>
<td>100</td>
<td>1.5</td>
<td>60</td>
<td>3</td>
<td>18.47</td>
<td>0.45</td>
<td>9.50</td>
</tr>
<tr>
<td>25</td>
<td>16'</td>
<td>100</td>
<td>1.5</td>
<td>60</td>
<td>3</td>
<td>18.47</td>
<td>0.45</td>
<td>9.50</td>
</tr>
<tr>
<td>12</td>
<td>17</td>
<td>120</td>
<td>2.0</td>
<td>40</td>
<td>4</td>
<td>18.61</td>
<td>0.27</td>
<td>9.81</td>
</tr>
<tr>
<td>1</td>
<td>18</td>
<td>80</td>
<td>1.0</td>
<td>40</td>
<td>2</td>
<td>17.01</td>
<td>0.35</td>
<td>7.68</td>
</tr>
<tr>
<td>10</td>
<td>19</td>
<td>120</td>
<td>1.0</td>
<td>40</td>
<td>4</td>
<td>13.38</td>
<td>0.28</td>
<td>11.06</td>
</tr>
<tr>
<td>19</td>
<td>20</td>
<td>100</td>
<td>0.5</td>
<td>60</td>
<td>3</td>
<td>12.49</td>
<td>0.43</td>
<td>5.11</td>
</tr>
<tr>
<td>18</td>
<td>21</td>
<td>140</td>
<td>1.5</td>
<td>60</td>
<td>3</td>
<td>20.28</td>
<td>0.72</td>
<td>16.01</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td>80</td>
<td>1.0</td>
<td>80</td>
<td>2</td>
<td>17.98</td>
<td>0.70</td>
<td>12.18</td>
</tr>
<tr>
<td>13</td>
<td>23</td>
<td>80</td>
<td>1.0</td>
<td>80</td>
<td>4</td>
<td>16.10</td>
<td>0.79</td>
<td>13.70</td>
</tr>
<tr>
<td>14</td>
<td>24</td>
<td>120</td>
<td>1.0</td>
<td>80</td>
<td>4</td>
<td>17.84</td>
<td>0.99</td>
<td>15.48</td>
</tr>
<tr>
<td>24</td>
<td>25</td>
<td>100</td>
<td>1.5</td>
<td>60</td>
<td>5</td>
<td>19.20</td>
<td>0.42</td>
<td>10.94</td>
</tr>
<tr>
<td>23</td>
<td>26</td>
<td>100</td>
<td>1.5</td>
<td>60</td>
<td>1</td>
<td>10.79</td>
<td>0.64</td>
<td>8.80</td>
</tr>
<tr>
<td>2</td>
<td>27</td>
<td>120</td>
<td>1.0</td>
<td>40</td>
<td>2</td>
<td>11.99</td>
<td>0.25</td>
<td>8.25</td>
</tr>
<tr>
<td>30</td>
<td>28'</td>
<td>100</td>
<td>1.5</td>
<td>60</td>
<td>3</td>
<td>18.47</td>
<td>0.45</td>
<td>9.50</td>
</tr>
<tr>
<td>4</td>
<td>29</td>
<td>120</td>
<td>2.0</td>
<td>40</td>
<td>2</td>
<td>18.57</td>
<td>0.36</td>
<td>10.27</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>80</td>
<td>2.0</td>
<td>40</td>
<td>2</td>
<td>16.24</td>
<td>0.35</td>
<td>9.38</td>
</tr>
</tbody>
</table>

Mean ± STD 17.47±1.61 0.55±0.09 10.35±1.45

*Center point: Mean value of six parallel replicates was repeated at each of center point.

compounds which show strong antioxidant activity. Various extracts of N. nucifera seeds have been found to contain 0.56–20.12 g gallic acid equivalent/100 g dry wt. The present results indicate that a valuable amount of crude methanolic extract with significant value of phenolic compounds and antioxidant activity is obtained from native and microwave treated N. nucifera seeds flour. Present results for TPC covered the range: 0.313–0.469 g catechin equivalent/100 g dry wt. The significance and adequacy of the response surface model were measured in terms of F-value (lack of fit) and probability value at 5% significance level (p ≤ 0.05). The variation in corresponding variables with relatively larger F-values and smaller p-values were considered more significant. The measurement of F-value and p-values for main effects indicated that the model is significant for TEC (F = 5.31, p = 0.0013), TPC (F = 11.69, p < 0.0001) and TAOA (F = 5.58, p < 0.001). The values of coefficients of estimate (18.47, 0.45 and 9.50) with positive significance level (p ≤ 0.05) with positive signs suggest that each of the studied parameter is increased in response to increase in the levels of extraction variables. However, mixed response for linear, quadratic and interaction terms was shown by each parameter against the input variables. Mesh No. showed non-significant linear effect on each response and a significant positive quadratic effect on TPC and TAOA. MTP showed a significant linear negative effect on TEC and non-significant quadratic effects on each response. A significant linear

Response surface analysis and optimization of results

The data were statistically analyzed by applying response surface models to find the levels of four input factors in the optimal region of extraction yield and antioxidant activity of phenolic antioxidants of N. nucifera seed flour. The significance and adequacy of the response surface model were considered more significant. The measurement of F-value and p-values for main effects indicated that the model is significant for TEC (F = 5.31, p = 0.0013), TPC (F = 11.69, p < 0.0001) and TAOA (F = 5.58, p < 0.001). The values of coefficients of estimate (18.47, 0.45 and 9.50) with positive signs suggest that each of the studied parameter is increased in response to increase in the levels of extraction variables. However, mixed response for linear, quadratic and interaction terms was shown by each parameter against the input variables. Mesh No. showed non-significant linear effect on each response and a significant positive quadratic effect on TPC and TAOA. MTP showed a significant linear negative effect on TEC and non-significant quadratic effects on each response.
Table 4: Response surface analysis of data showing main, linear, interaction and quadratic terms and regression coefficients

<table>
<thead>
<tr>
<th>Source</th>
<th>TEC</th>
<th></th>
<th>TPC</th>
<th></th>
<th>TAOA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sum of Squares</td>
<td>Std. Error</td>
<td>F-Value</td>
<td>p-value</td>
<td>Sum of Squares</td>
<td>Std. Error</td>
</tr>
<tr>
<td>β0-Model</td>
<td>192.45</td>
<td>0.66</td>
<td>5.31</td>
<td>0.0013</td>
<td>1.37</td>
<td>0.037</td>
</tr>
<tr>
<td>β1-Mesh No. (mesh)</td>
<td>0.093</td>
<td>0.33</td>
<td>0.036</td>
<td>0.8526</td>
<td>0.021</td>
<td>0.019</td>
</tr>
<tr>
<td>β2-MTP (min)</td>
<td>84.71</td>
<td>0.33</td>
<td>32.75</td>
<td>&lt; 0.0001</td>
<td>1.50E-003</td>
<td>0.019</td>
</tr>
<tr>
<td>β3-SC (%)</td>
<td>40.59</td>
<td>0.33</td>
<td>15.69</td>
<td>0.0013</td>
<td>1.11</td>
<td>0.019</td>
</tr>
<tr>
<td>β4-EP (h)</td>
<td>23.34</td>
<td>0.33</td>
<td>9.02</td>
<td>0.0089</td>
<td>3.50E-003</td>
<td>0.019</td>
</tr>
<tr>
<td>β12</td>
<td>7.09</td>
<td>0.40</td>
<td>2.74</td>
<td>0.1186</td>
<td>0.043</td>
<td>0.023</td>
</tr>
<tr>
<td>β13</td>
<td>2.73</td>
<td>0.40</td>
<td>1.06</td>
<td>0.3205</td>
<td>1.06E-003</td>
<td>0.023</td>
</tr>
<tr>
<td>β14</td>
<td>0.54</td>
<td>0.40</td>
<td>0.21</td>
<td>0.6553</td>
<td>5.06E-004</td>
<td>0.023</td>
</tr>
<tr>
<td>β23</td>
<td>0.56</td>
<td>0.40</td>
<td>0.22</td>
<td>0.6488</td>
<td>0.020</td>
<td>0.023</td>
</tr>
<tr>
<td>β24</td>
<td>2.38</td>
<td>0.40</td>
<td>0.92</td>
<td>0.3528</td>
<td>9.51E-003</td>
<td>0.023</td>
</tr>
<tr>
<td>β34</td>
<td>1.80E-005</td>
<td>0.40</td>
<td>6.982E-004</td>
<td>0.9793</td>
<td>1.06E-003</td>
<td>0.023</td>
</tr>
<tr>
<td>β12</td>
<td>2.57</td>
<td>0.31</td>
<td>0.99</td>
<td>0.3350</td>
<td>0.15</td>
<td>0.017</td>
</tr>
<tr>
<td>β22</td>
<td>0.77</td>
<td>0.31</td>
<td>0.30</td>
<td>0.5928</td>
<td>4.80E-003</td>
<td>0.017</td>
</tr>
<tr>
<td>β32</td>
<td>9.97</td>
<td>0.31</td>
<td>3.85</td>
<td>0.0685</td>
<td>0.013</td>
<td>0.017</td>
</tr>
<tr>
<td>β42</td>
<td>17.08</td>
<td>0.31</td>
<td>6.60</td>
<td>0.0214</td>
<td>3.94E-003</td>
<td>0.017</td>
</tr>
<tr>
<td>Lack of Fit</td>
<td>38.80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pure Error</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R²</td>
<td>0.8322</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adj. R²</td>
<td>0.6756</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CV (%) &quot;</td>
<td>9.21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AP &quot;</td>
<td>9.544</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p≤0.05 indicates the significant variation in response

positive effect of SC was observed on each response. EP showed significant linear positive and quadratic negative effect on TEC only. The interaction effect of each factor was found to be non-significant on each response. Three dimensional response surface plots clearly represented the main, quadratic and interaction effects of extraction variables on TEC, TPC and TAOA of *N. nucifera* seed flour (Figure 1A-F, Figure 2A-F, Figure 3A-F respectively).

The variability of the model in the observed response values was determined by correlation coefficient ($R^2$). A value of $R^2$ closer to unity gives better prediction of the response and high significance of the model. The calculated values of $R^2$ indicated that more than 83% of the variability in TEC ($R^2=0.8322$), 91% in TPC ($R^2=0.9160$) and 83% in TAOA ($R^2=0.8389$) of *N. nucifera* as a function of selected variables could be explained by the suggested model. The values of adjusted $R^2$ (TEC: 0.6756, TPC: 0.8380, TAOA: 0.6886) also advocate the significance of the model. Coefficient of variation (CV) is a measure of standard deviation as a percentage of the mean. A relatively low value of CV (9.21-16.73) suggests a better precision and reliability of the experiments and claims a good reproducibility at suggested optimum levels of variables. Adequate precision measures the signal to noise ratio. A ratio greater than 4 indicates an adequate signal to navigate the design space. The high value of adequate precision (9.032-13.341) for the studied parameters suggests a better precision and reliability of the experiments.

The polynomial regression equations were yielded by RSM in order to show an empirical relationship between the response and input variables. These equations include the coefficient for intercept, linear, interaction and quadratic effects. The influence of each factor on the response is shown by the sign and magnitude of the main effect. The response surface analysis indicated that the relationship between extraction variables and studied parameters of *N. nucifera* seed flour could be explained significantly by second order polynomial regression models.

The linear plot of predicted values of response variables against the experimental ones (Figure 4A-C) showed a good agreement with high values of correlation coefficients ($R^2=0.8322-0.9153$). The higher values

![Figure 1A-F: 3D response surface plots of TEC of *N. nucifera* seed flour at selected levels of input variables.](image)
Figure 2A-F: 3D response surface plots of TPC of *N. nucifera* seed flour at selected levels of input variables.
Figure 3A-F: 3D response surface plots of TAOA of *N. nucifera* seed flour at selected levels of input variables.
of $R^2$ prove the applicability of proposed model with good accuracy to study the effect of Mesh No., MTP, SC and EP on extract yield, of phenolic content and antioxidant properties of *N. nucifera* seed flour. The numerical optimization of process variables showed that maximum extraction yield (24.25 g/100 g dry wt.) could be achieved at particle size in terms of sieve Mesh No.: 125.96, MTP: 2.45 min, SC: 55.21% and EP 3.87 h (Figure 5A). Optimum level of extraction variables to achieve desired prediction of TPC (1.067 g/100g dry wt.) and TAOA (16.14 g/100g dry wt.) were found to be 61.73, 1.66 min, 80.08% and 4.98 h and 60.89, 2.37 min, 44.98% and 1.123 h respectively.

**CONCLUSION**

It is clear from RSM results that extraction of phenolic antioxidants from *N. nucifera* seed flour significantly increased in response to an increase in Mesh No. (decrease in particle size), MTP, SC and EP but up to a certain limit. An increase in the level of studied factors beyond the optimum values suggested by the applied statistical model results in a decrease in the response values. The decrease in extraction yield and antioxidant activity at higher levels of studied factors may be attributed to the possible decomposition of phenolic compounds due to prolonged duration of microwave heating, high methanol/water ratio and prolonged extraction time. The data would be significant contribution to the research in pharmaceutical and food science regarding the extraction of phenolic antioxidants.

**ACKNOWLEDGEMENT**

The authors are grateful to the Institute of Chemical Sciences, Bahaud-din Zakariya University, Multan, Pakistan for providing all the facilities regarding the present research work.

**CONFLICT OF INTEREST**

The research project was not funded by any funding agency and collaborated with any other institute. Therefore, there is no conflict of interest regarding this research work.
ABBREVIATIONS USED

AP: Adequate precision; CCD: Central composite design; CV: Coefficient of variance; EP: Extraction period; MTP: Microwave treatment period; RC: Regression coefficient; RSM: Response surface methodology; SC: Solvent concentration; TAOA: Total antioxidant activity; TEC: Total extractable components; TPC: Total phenolic content.

REFERENCES

3. Dubey NK. Plants as a Source of Natural Antioxidants [Internet]. CABI; 2014 (cited 2016 Mar 3).
8. Abbas ZK, Saggau S, Sakeran MI, Zidan N, Rehman H, Ansari AA. Phytochemical,
PICTORIAL ABSTRACT

- Extraction parameters of phenolic antioxidants from *Nelumbo nucifera* seed flour were optimized.
- Studied factors significantly affected the response parameters.
- Particle size, microwave treatment, solvent concentration and extraction period significantly affect the extraction of phenolic antioxidants from *Nelumbo nucifera* seed flour.
- Microwave treatment for 1.66-2.45 min was found to be effective for extraction.

ABOUT AUTHOR

Haq Nawaz: Is doctoral student in Institute of Chemical Sciences, Bahauddin Zakria University, Multan Pakistan. He is also working as Lecturer and Research supervisor at Department of Biochemistry of the same University. He is working in the field of biological sciences with special interest in Food Sciences Antioxidant chemistry and Clinical sciences.

Nawaz et al.: Phenolic Antioxidants of *Nelumbo nucifera* Seed


