Optimization of the solvent extraction of bioactive polyphenolic compounds from aquatic fern *Azolla microphylla* using response surface methodology

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Central composite design
Antioxidant activity
*Azolla microphylla*
Polyphenolic compounds

Abstract

Response surface methodology (RSM) based on a central composite design (CCD) was employed to optimize the experimental conditions for extraction of bioactive polyphenolic compounds from aquatic fern *Azolla microphylla*. The effects of three independent parameters, namely, methanol concentration (X₁: 60-85%), extraction temperature (X₂: 55-80°C) and extraction time (X₃: 45-100 min) were investigated to optimize the extraction yields of total phenolic (TPC) and flavonoid (TFC) contents, and compounds showing major antioxidant properties, particularly DPPH, FRAP (Ferric reducing antioxidant power) and ABTS (2',2'-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt) activities. Data were analyzed by using Design Expert (version 8.0.7.1, Stat-Ease, Inc., Minneapolis, MN, USA) statistical analysis software. The optimum extraction conditions were obtained at methanol concentration (X₁): 84.85-85%, extraction temperature (X₂): 55°C and extraction time (X₃): 99.76-100 minutes, respectively. Under this condition, the optimum yields of TPC and TFC are 2167.03-2160.44 mg gallic acid equivalents (GAE)/g and 46.11-43.02 mg rutin equivalents (RU)/g of extract. The corresponding antioxidant activities are 80.06-76% DPPH, 84-80.54% ABTS, and FRAP value of 56.65-50.48 µg mol (Fe (II))/g. The experimental results were well matched with the predicted results. Through reversed phase-high performance liquid chromatography (RP-HPLC), rutin and quercetin were identified in the optimally obtained extract of *Azolla microphylla*. This procedure can be helpful in the food and pharmaceutical industry in studying the optimization of extraction of high quality bioactive products from natural sources.

Introduction

Floating aquatic macrophytes are defined as plants that float on surface of the water body, generally not dependent on the soil or water depth (Watanabe and Berja, 1983). Fast growing free floating aquatic fern *Azolla* species is distributed in tropic and temperate fresh water worldwide (Saunders and Fowler, 1993). The genus *Azolla*, discovered by J. B. Lamark as early as 1783, belongs to the *Salvinaceae* family of the order *Salviniales* (Svenson, 1944). *Azolla* species are traditionally used as a bio-fertilizer, animal feed, water purifier, biological herbicide and for concentration of nutrients and heavy metals from flood waters (Becerra et al., 1990; Arora and Singh, 2003). The genus *Azolla*, discovered by J. B. Lamark as early as 1783, belongs to the *Salvinaceae* family of the order *Salviniales* (Svenson, 1944). *Azolla* species are traditionally used as a bio-fertilizer, animal feed, water purifier, biological herbicide and for concentration of nutrients and heavy metals from flood waters (Becerra et al., 1990; Arora and Singh, 2003). It has been reported that *Azolla microphylla* is rich in essential amino acids, vitamins, proteins, polyphenols, sugar, anthroquinone glycosides and steroids (Abraham and Vidhu, 2012). Numerous investigations have proved that *Azolla microphylla*, when fed to dairy cattle, pigs, ducks and chickens, results in increased milk production, increase of weight of dairy cattle, pigs, ducks and broiler chickens (Van Hove, 1989; Nikkah and Motaghi-Talah, 1992).

Many researchers indicated that secondary metabolites of the plant contain bioactive compounds such as polyphenols, glycosides and steroids (Harborne, 1973). Among them polyphenols are particularly attractive for various pharmacological properties such as antioxidants (Heim et al., 2002; Gould and Lister, 2006), anti-microbial (Kouam et al., 2007), anti-allergic (Lyons Wall and Samman, 1997), hepatoprotective (Sannigrahi et al., 2009) and anti-proliferative activity on tumor cells (Fang et al., 2010; Al-Taweel et al., 2012).

In order to extract the bioactive polyphenolic compounds from *Azolla microphylla*, the polar-solvent extraction method was often used. Many parameters such as particle size, extraction solvent, solvent concentration, extraction temperature and time etc. have significantly influenced the extraction yield (Wu et al., 2007; Gong et al., 2012). However, no work has so far been reported on the optimization of extraction of bioactive compounds from them.
Extraction yields of TPC, TFCs and those showing antioxidant properties are influenced by multiple parameters, namely, solvent concentration, extraction temperature and extraction time, etc., inherently appear to be unrelated to each other. Under this situation, a statistical method of optimization seems to be very useful. Response surface methodology (RSM) is one of such statistical tools (Bezerra et al., 2008; Wijanggaard and Brunton, 2010). Response surface methodology can be defined as a statistical method that uses quantitative data from appropriate experiments to determine and simultaneously solve multivarient equations (Gan and Latiff, 2011).

The objective of the present study was to develop and validate an extraction method for the enhanced recovery of total phenolic and flavonoids and biomolecules showing antioxidant scavenging activities using response surface methodology. In the current study optimization of five levels (-1.682,-1, 0, +1, +1.682) three factors central composite design (CCD) was employed to examine the optimum conditions with respect to extraction yields of TPCs, TFCs and antioxidant compounds for different combination of extraction variables of Azolla microphylla sample. The sensitivity of extraction of the biomolecules with respect to three independent parameters such as methanol concentration (%), extraction temperature (˚C) and time (minutes) were investigated.

**Materials and Methods**

**Plant material and sample preparation**

*Azolla microphylla* fern were purchased from Vivekananda Kendra-NARDEP (Natural Resources Development Project), Vivekanandapuram, Kanyakumari, Tamilnadu, India. It was washed several times with tap water, dried in shade for 72 h and the whole plant was ground to a fine powder. The powder was passed through 60 mesh size screen and kept in airtight desiccator for further extraction experiments.

**Chemicals**

2, 4, 6-tripyridyl-s-triazine (TPTZ), Folin-Ciocalteu’s phenol reagent (FCR) and Gallic acid of Himedia laboratories Pvt. Ltd. Mumbai, India; 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2’-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diaminonium salt (ABTS) of Sigma–Aldrich, MO, USA; Aluminium chloride, Sodium carbonate, Sodium hydroxide, Ferric chloride and analytical grade solvents of Merck, Mumbai, India were used in the study.

**Preliminary selection of appropriate extraction solvent**

The objective of the preliminary experiment was to select the best solvent based on the highest polyphenolic content from the extraction of aquatic fern *Azolla microphylla*. Four different solvent systems, namely, methanol, acetone, ethyl acetate and n-hexane were examined. Each solvent extraction was done with constant solvent concentration (60% v/v in water) at fixed values of extraction temperature (80˚C) and time (60 minutes).

**Extraction of Polyphenolics and Antioxidants**

Extraction of polyphenolic compounds and antioxidants from *Azolla microphylla* was carried out by the pre-selected solvent based on the results of preliminary selection experiments for the choice of appropriate solvent. The extraction was carried out by using 2 g of powdered sample of *Azolla microphylla*. The sample was transferred to a 100 mL conical flask containing 60 mL miscible liquid-liquid mixture of pre-selected solvent and water. The cotton plugged conical flask was placed on a constant temperature water bath equipped with shaking arrangement. Sets of experiments were carried out by varying methanol concentration (60-85%) in methanol-water mixture, temperature (55-80˚C) and time (45-100 minutes). Values of operating parameters were set according to the CCD table. Each extract, obtained by filtration of content of conical flask through Whatman No: 4 filter paper was analyzed for TPC, TFC and antioxidant activities.

**Measurement of total polyphenolic content (TPC)**

TPC was estimated spectrophotometrically (Varian Cary 50 UV-Spec) using Folin-Ciocalteu’s phenol reagent (FCR) according to the method described by Singleton and Rossi (1965) with slight modifications. Approximately 0.3 mL extracts were mixed with 1.8 mL of Folin-Ciocalteu’s reagent and allowed to stand at room temperature for 5 minutes, and then 1.2 mL sodium carbonate (7.5%, w/v) solution were added to the mixture. The blank sample was prepared by replacing 0.3 mL of extract with 0.3 mL of distilled water. After standing for 60 minutes at room temperature, absorbance was measured at 765 nm using spectrophotometer. Gallic acid was used as standard and results were expressed as mg gallic acid equivalents (GAE)/g sample.

**Measurement of total flavonoid content (TFC)**

The content of flavonoids was determined using the aluminium chloride method described by Siddhuraju and Becker (2003) with slight
modifications. 1 mL of the extract followed by 0.3 mL of 5% (w/v) sodium nitrite solution and 4 mL of 80% (v/v) methanol were mixed for 5 minutes, and subsequently 0.3 mL of 10% (w/v) aluminium chloride solution was added and mixed. After 6 minutes, 3 mL of 1 µL sodium hydroxide solution was added. Immediately, the volume of reaction mixture was made up to 10 mL with distilled water. The mixture was thoroughly vortexed and the absorbance was measured at 510 nm. Based on the standard curve prepared with rutin, the concentrations of TFC in the extracts were expressed as mg rutin equivalents (RU)/g samples.

Determination of antioxidant capacity

% DPPH Scavenging assay

The 2, 2-diphenyl-1-picrylhydrazil (DPPH) free radical scavenging activity of the extracts was determined following the protocol suggested by Ramadan et al. (2003) with some modifications. Aliquot of each extract (0.1 mL) was added to 3 mL of ethanolic solution of DPPH (0.1 µM). The mixture was shaken vigorously and allowed to stand for 30 minutes in the dark, and the absorbance was measured at 517 nm against a blank. The capability to scavenge the free radical DPPH in percentage of sample (%DPPHSC) was calculated using the formula;

\[
%\text{DPPHSC} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100
\]

Where \(A_0\) = absorbance of the control; \(A_1\) = absorbance of the sample.

%ABTS Scavenging assay

ABTS\(^\bullet\) radical scavenging activity assay was carried out by the method of Re et al. (1999) with some modifications. The ABTS\(^\bullet\) was generated by the reaction between 7 mM ABTS (2, 2’-Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diaminonm salt) solution and 2.45 mM potassium persulphate solution incubated in the dark at room temperature for 16 h. Before use, the absorbance at 734 nm was adjusted to 0.700 (±0.0020) by dilution with ethanol. 3 mL of the ABTS solution was mixed with 0.1 mL of the extracts and mixed vigorously. The reaction mixture was incubated for 6 min and the absorbance was determined at 734 nm by a UV-Vis spectrophotometer. A standard curve was obtained by using rutin in 80% ethanol. The % ABTS which was scavenged (% ABTSSC) was calculated using the formula;

\[
\%\text{ABTSSC} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100
\]

In this study, equation (3) can be converted into the following equation according to the value of three variables.
Table 1. Experimental range of coded and actual values of central composite design (CCD)

<table>
<thead>
<tr>
<th>Independent variable(s)</th>
<th>Symbol</th>
<th>Coded Values</th>
<th>Actual Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol concentration (%)</td>
<td>X₁</td>
<td>32.16</td>
<td>54</td>
</tr>
<tr>
<td>Extraction Temperature (°C)</td>
<td>X₂</td>
<td>46.48</td>
<td>55</td>
</tr>
<tr>
<td>Extraction Time (minutes)</td>
<td>X₃</td>
<td>26.25</td>
<td>45</td>
</tr>
</tbody>
</table>

\[ Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_{12} + \beta_{13} X_{13} + \beta_{23} X_{23} + \beta_{11} X_{11} + \beta_{22} X_{22} + \beta_{33} X_{33} \]  

Where \( Y \) is the dependent variables (TPC, TFC, %DPPH, %ABTS and FRAP), \( \beta_0 \) is the model constant, \( \beta_i, \beta_j, \beta_{ij} \) are the model coefficients, \( X_i \) and \( X_j \) are coded value of the independent variables, and \( \varepsilon \) is the error. Additional confirmation experiments were subsequently conducted to verify the statistical experimental analysis.

**Statistical data analysis**

All the experimental data collected from the extraction process were analyzed by using design expert (version 8.0.7.1, Stat-Ease, Inc., Minneapolis, MN, USA) software. Strength of analysis was assessed by one way analysis of variance (ANOVA). The optimal extraction conditions were analyzed by three dimensional (3D) response surfaces and contour plots.

**Results and Discussion**

In Figure 1 total polyphenolic content of the extract has been plotted as a function of solvent of extraction. It shows that the highest extraction of polyphenolic compounds has been possible with methanol, compared to other solvents. Therefore, for further experimental studies, methanol has been used as the solvent of extraction. In the development of optimization using response surface method, the effects of three independent variables i.e., methanol concentration (\( X_1: 60-85\% \)), extraction temperature (\( X_2: 55-80^\circ C \)) and time (\( X_3: 45-100 \) min) on TPC, TFC and antioxidant activities (%DPPH, %ABTS, FRAP) were investigated. The experimental design of five levels and three variables using central composite design with 20 runs of extraction were predicted and experimental results are shown in Table 2. Among the 20 experiments including 6 replicates (Table 2), it was observed that the yield of TPC and TFC contents ranged from 1663.09-6829.07 mg gallic acid equivalents (GAE)/g and 13.24-92.61 mg rutin equivalents (RU)/g, respectively. The range of three antioxidant activities of %DPPH: 30.02-80.06%, %ABTS: 32.1-84% and FRAP: 11.49-56.65 \( \mu \)g mol Fe (II)/g were recorded under the experimental conditions. The highest level of TPC (6829.07 mg gallic acid equivalents (GAE)/g) and TFC (92.61 mg rutin equivalents (RU)/g) were obtained with 60% methanol, at 80°C for 45 min and 93.52% methanol, at 67.50°C for 72.50 min, respectively. The maximum antioxidant potential (%DPPH: 80.06%, %ABTS: 84% and FRAP: 56.65 \( \mu \)g mol Fe (II)/g) were measured at 55% methanol, at 55°C for 100 min. Therefore, an optimal condition for the extraction of TPC, TFC and antioxidant scavenging activities were: methanol concentration (\( X_1 \)): 84.85-85%, extraction temperature (\( X_2 \)): 55°C and extraction time (\( X_3 \)): 99.76-100 minutes.

**Fitting the models**

The yields of TPC, TFC and the antioxidant activities (%DPPH, %ABTS, FRAP) in *Azolla* extracts obtained from all the experiments are listed in Table 2. The experimental results were used to find the coefficients of the second-order polynomial equation and Table 3 shows the results of fitting quadratic models with the data. The result of analysis of variance (ANOVA) shows the significance of the coefficients of the models. The significance of each coefficient was determined using the F-test in Table 3. The corresponding variables would be more significant if the F-value becomes greater and the p-value becomes smaller (Atkinson and Donev, 1992). The p-values were used as an important tool to check the significance of the interactions of the variables. A p-value less than 0.05 indicated that the coefficient was statistically significant. The F-value (\( F = 7.58 \)) and p-value (\( p = 0.0221 \)) also implied that the model was significant. The fitness and adequacy of the model was judged by the determination of multiple regression coefficients (R²) and the significance of lack-of-fit. The second–order polynomial equation for the fitted quadratic models for TPC, TFC, %DPPH, %ABTS, FRAP in coded variables are given in equation (5)-(9).

\[
\begin{align*}
TPC &= 2497.26 - 730.71X_1 - 2.29X_2 - 603.30X_3 + 205.49X_1X_2 - 161.66X_1X_3 + 1202.13X_2X_3 - 205.5X_1^2 - 54.20X_2^2 - 161.66X_3^2 \quad (5) \\
TFC &= 22.35 + 1.18X_1 + 8.34X_2 + 4.02X_3 - 14.58X_1X_2 - 0.39X_1X_3 + 1.54X_2X_3 + 17.18X_1^2 + 2.31X_2^2 - 2.87X_3^2 \quad (6) \\
\%DPPH &= 32.37 - 3.20X_1 + 4.42X_2 + 3.46X_3 - 16.07X_1X_2 + 4.97X_1X_3 - 5.11X_2X_3 + 11.66X_1^2 + 3.46X_2^2 - 0.12X_3^2 \quad (7) \\
\%ABTS &= 35.88 - 3.87X_1 + 4.35X_2 + 3.38X_3 - 17.07X_1X_2 + 5.55X_1X_3 + 4.90X_2X_3 + 12.03X_1^2 + 3.69X_2^2 - 0.22X_3^2 \quad (8) 
\end{align*}
\]
Table 2. Central Composite Design with experimental responses and predicted responses

<table>
<thead>
<tr>
<th>Run</th>
<th>TPC (mg GAE/g)</th>
<th>FRAP (µmol TE/g)</th>
<th>Ext. Temp. (°C)</th>
<th>Methanol Conc. (%)</th>
<th>TFC (µg Fe(II)/gm)</th>
<th>Polynomial Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>88</td>
<td>39</td>
<td>45</td>
<td>45</td>
<td>73.5</td>
<td>FRAP = 14.39 + 5.77X₁ – 2.97X₂ + 2.25X₃ – 9.63X₁X₂ + 3.48X₁X₃ – 2.44X₂X₃ + 6.63X₁² + 0.64X²² – 0.85X₃² (9)</td>
</tr>
<tr>
<td>2</td>
<td>26</td>
<td>10.2</td>
<td>55</td>
<td>55</td>
<td>67.5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>39</td>
<td>11.2</td>
<td>65</td>
<td>65</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>45</td>
<td>12.2</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

All the experiments were repeated three times

Analysis of the model

TPC

From the Table 3 it may be inferred that the variables with the largest effects on extraction yield of TPC are the quadratic term of methanol concentration (X₁^2), linear term of methanol concentration (X₁) and linear term of extraction temperature (X₃). The results show that the effects of methanol concentration and extraction temperature are significant (p < 0.05) on the yield of polyphenolics in the extracts of Azolla microphylla obtained through solvent extraction process using methanol. The effect of extraction time is relatively less. The correlation coefficient (R²) of the predicted model regarding TPC extraction was 0.7990 with p-value of lack of fit was 0.0221. This signifies that the model is a considerably fitting one. Equation (5) shows the relationship between TPC yield and extraction parameters.

Figure 2.A and 2.B is a 3D response surface and the contour plot showing the significant effect of extraction temperature and extraction time on the yield of TPC. The supporting data have also been provided in Table 2. Results in Table 2 show that highest yield of TPC was obtained at methanol concentration 60%, extraction temperature 80°C and time 45 minutes. At a lower region of extraction temperature (55-80°C) and extraction time (45-100 min) would give a higher TPC yield (≥5882.25 mg GAE/g) as compared to higher extraction temperature and time.

TFC

It can be seen from the ANOVA table and model equation that quadratic term of methanol concentration (X₁^2) has the largest effect on the extraction of TFC from Azolla microphylla, followed by the linear term of extraction temperature (X₃) and interaction term of methanol concentration and extraction temperature (X₁X₃). The results (Table 3) show that the effects of methanol concentration, extraction temperature and their interaction term (X₁X₃) are significant (p < 0.05). All other terms are not significant (P > 0.05). The response surface analysis of TFC also demonstrated high regression coefficient value R² = 0. 8369 and p-value for lack of fit was < 0.0001. Thus the model equation (6) showing the relationship between TFC yield and extraction parameters is valid.

3D response surfaces and the contour plots shown in Figure 3.A and 3.B illustrate the effects of change in methanol concentration and extraction temperature on the yield of TFC. In Table 2 shows that the highest amount of TFC yield was obtained at methanol...
Table 3. Analysis of variance (ANOVA) for the quadratic polynomial mode

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>2100.14</td>
<td>5</td>
<td>420.03</td>
<td>18.26</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Pure Error</td>
<td>1559.16</td>
<td>12</td>
<td>129.93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>3659.30</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The coefficient of determination (R²) of the model was 0.9998.

*Degrees of freedom.

Table 4. Verification of individual experimental data and predicted values under optimum conditions

<table>
<thead>
<tr>
<th>Y (Experimental)</th>
<th>Y (Predicted)</th>
<th>Relative error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.01</td>
<td>4.75</td>
<td>5.13</td>
</tr>
<tr>
<td>4.75</td>
<td>4.59</td>
<td>3.51</td>
</tr>
<tr>
<td>4.59</td>
<td>4.43</td>
<td>3.46</td>
</tr>
</tbody>
</table>

*The coefficient of determination (R²) of the model was 0.9998.

Antioxidant activities (%DPPH, %ABTS and FRAP)

From the ANOVA table (Table 3) and the model equations (eqn. (7)-(9)) it is evident that the quadratic term of methanol concentration (X1) and interaction term of methanol concentration and extraction temperature (X1X2) have significant effect for all three antioxidant effects. However, Table 3 and the respective model equations also indicate that in addition to X1²X2 and X1X2, % DPPH activity is significantly influenced by other interaction terms, namely, X1X2, X2X3. Similarly, quadratic term X1² and interaction term X1X2 influence %ABTS and linear term of extraction temperature (X3) has significant (p < 0.05) effect on FRAP. While the regression coefficient value (R²) of the models in %DPPH, %ABTS and FRAP are 0.9306, 0.9295 and 0.8998, respectively, p-value for lack of fit were < 0.0001, < 0.001 and 0.0041, respectively. The high values of concentration 93.52%, extraction temperature 67.5°C and time 72.5 minutes. Lower region of methanol concentration (60-85%) and extraction temperature (55-80°C) would give a higher TFC yield (78.75 mg RU/g) as compared to higher methanol concentration and extraction temperature.

Antioxidant activities (%DPPH, %ABTS and FRAP)

The experimental results showed that the methanol concentration, extraction temperature, and extraction time have significant effect on antioxidant or antioxidant activities. Similar to other cases, extraction time has no significant effect on %DPPH, %ABTS and FRAP. The maximum yields of antioxidant or antioxidant activities are %DPPH: 80.06%, %ABTS: 84% and FRAP: 56.65 µg mol Fe(II)/g.

Validation of the model

The suitability of validation experiments were carried out to check the reliability of the optimization result. Table 4 shows the verification experiment under optimum conditions based on each individual response with predicted and experimental values. The experimental results showed that the methanol concentration, extraction temperature, and extraction time have significant effect on antioxidant or antioxidant activities.
time had significant effects on the yields of bioactive polyphenolic compounds.

The verification experiment was conducted under optimum conditions based on combination of responses and small deviations were observed as compared to predicted values. Optimal conditions based on combination of responses were: methanol concentration of 84.85-85%, extraction temperature of 55°C and extraction time of 99.76 - 100 minutes. Under this condition while the experimental values of TPC, TFC, %DPPH, %ABTS, and FRAP were 2165.88 - 2167.03 mg gallic acid equivalents (GAE)/g, 45.40-46.11 mg rutin equivalents (RU)/g, 78.88-80.06%, 83.63-84% and 54.66-56.65 µg mol Fe (II)/g, respectively, predicted values of TPC, TFC, %DPPH, %ABTS, and FRAP were 3086.29-3088.51 mg gallic acid equivalents (GAE)/g, 48.51-48.48 mg rutin equivalents (RU)/g, 70.14-70.26%, 73.95-74.07% and 48.96-49.04 µg mol Fe (II)/g, respectively. This model implied that there was a good fit between the experimental value and those predicted by the regression model.

Hence, the response surface model may be applied effectively to optimize the process of solvent extraction of bioactive polyphenolic compounds from aquatic fern *Azolla microphylla*.

**High performance liquid chromatographic analysis of polyphenolic compounds**

After investigation of the optimum conditions, the polyphenolic extract was analyzed by using RP-HPLC (Shimadzu, LC-8A, Japan). The extract was filtered through membrane filter (Millipore, USA) and injected (10 µl) with the BDS Hypersil RP-C18 column (Thermo, 5 µm, 120Å, 250 mm × 4.6 mm) at column temperature 25°C. The mobile phase, composed of 1:1 mixture of 70% (v/v) methanol in water and 1% formic acid, was used at a flow rate of 1 mL/min for elution. The elute was monitored at 280 nm by UV detector. Two peaks were detected and compared with the standards. The Chromatographic peaks indicated that compound-1 and compound-2 had the same retention time (Rt: 2.8 and 3.4min) as the standard flavonoids, namely rutin and quercetin.
Conclusions

A statistical analysis based on central composite design by response surface methodology was successfully employed to optimize the extraction parameters of bioactive polyphenolic compounds from *Azolla microphylla*. The results showed that the independent parameters (methanol concentration, extraction temperature and extraction time), and quadratic terms of methanol concentration, extraction temperature, and the interaction terms involving methanol concentration, extraction temperature and extraction time had significant effects on the yield of bioactive polyphenolic compounds. Thus all three parameters have important contribution for the maximization of yield of all biomolecules extracted for *Azolla microphylla*. The validity of the model was proven by fitting the values of the observed experimental values and by carrying out experiments using the predicted values. The optimum conditions ensuring maximum yield of TPC, TFC and antioxidant activities were obtained at methanol concentration of 85% v/v, extraction temperature of 55°C and extraction time of 100 minutes. Rutin and quercetin were the major flavonoid components present in the plant. It may be expected that the optimization results will be helpful for designing of extraction processes of the biomolecules, under study, from *Azolla microphylla* and similar herbs on industrial scale.

Acknowledgements

The authors are grateful to The Secretary, Vivekananda Kendra-NARDEP (Natural resource development project), Vivekanandapuram, Kanyakumari, Tamilnadu for providing the Azolla microphylla plant.

References


Table 5. Verification of experimental and predicted values under optimum conditions based on combination of responses

<table>
<thead>
<tr>
<th>Run</th>
<th>MEOH conc (%)</th>
<th>Ext Temp. °C</th>
<th>Ext. Time min</th>
<th>TPC (mg GAE/gm)</th>
<th>TFC (mg RU/gm)</th>
<th>ABTS sc (%)</th>
<th>DPPH sc (%)</th>
<th>FRAP (µg mol Fe(II)/gm)</th>
<th>Predicted value (Y&lt;sub&gt;2&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>85</td>
<td>55</td>
<td>100</td>
<td>2165.80</td>
<td>45.46</td>
<td>83.64</td>
<td>56.65</td>
<td>3106.29</td>
<td>48.96</td>
</tr>
<tr>
<td>2</td>
<td>83</td>
<td>55</td>
<td>100</td>
<td>2160.44</td>
<td>43.02</td>
<td>78.00</td>
<td>50.48</td>
<td>3104.48</td>
<td>47.87</td>
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<td>3</td>
<td>84.85</td>
<td>55</td>
<td>100</td>
<td>2160.44</td>
<td>43.02</td>
<td>78.00</td>
<td>50.48</td>
<td>3104.48</td>
<td>47.87</td>
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</tbody>
</table>

All the experiments were repeated three times

Figure 5. HPLC chromatographic profile of polyphenolic extract

Figure 6. (A) Chemical structure of Rutin. (B) Chemical structure of Quercetin.
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