Optimization of the extraction of phenolic compounds from *Cyclosorus extensa* with solvents of varying polarities

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**ABSTRACT**

The leaves of *Cyclosorus extensa* are used in the preparation of rice beer in Assam, India. The optimal conditions of time and temperature of fermentation for extraction of bioactive compounds from the dried leaves were obtained using response surface methodology. The central composite rotatable design was used and 13 experimental runs based on two-factor-five-level design were generated and performed for each of the solvents. The independent variables were extraction time (12 and 48 h) and temperature (25 and 55°C). The responses studied were total polyphenol content, radical scavenging activity, antibacterial activity, and antifungal activity. The analysis of variance of the test data was performed and the sequential sum of squares, F-value, \(R^2\), and adjusted \(R^2\) were deduced. The predicted models for all the response variables were adequately fitted to the observed experimental data (\(p \leq 0.001\)). The maximum extraction of bioactive compounds under the optimum conditions of extraction temperature and time for hexane, ethyl acetate, methanol, and distilled water were found to be 25°C for 29.43 h, 28.28°C for 41.27 h, 43.95°C for 29.61 h, and 55.00°C for 48.00 h, respectively. It was also observed that the solubility of the polyphenols was higher in methanol, followed by ethyl acetate, and the highest antibacterial activity against *Escherichia coli* was shown by the ethyl acetate extracts.

**Introduction**

Polyphenols are secondary metabolites produced by higher plants and they have potential health benefits on human health, mainly as antioxidants, antiallergic, anti-inflammatory, anticancer, antiinflammatory, and antimicrobial agents.\(^{[1]}\) The impact of bioactive compounds derived from plants in preventing or controlling pathogenic microbes is enormous. Many reports are available on the use of plant-derived phenolics in the control of human pathogenesis.\(^{[2–6]}\) Plant phenolics frequently occur in conjugation with glycosides and are usually located in the cell vacuolar structures, and solvent extractions are the most commonly used procedures to extract and liberate those.\(^{[7]}\) The yield of the liquid–solid extraction is significantly influenced by the type of solvents with varying polarities, solvent proportion, extraction time, temperature, pH, liquid/solid ratio, and particle size as well as by the chemical composition and physical characteristics of the samples.\(^{[8]}\) The efficiency of extraction is basically a function of process conditions, and role of each factor in the mass transfer process may either be positive or negative. Each solvent system may show different behavior toward each material.\(^{[9]}\)

Rice beer is an indigenous beverage prepared in Assam and various plants are intensively used in the preparation process. Many of these plants also possess medicinal properties and affect the quality of rice beer.\(^{[10]}\) In our previous study,\(^{[11]}\) it was observed that out of these plants, *Cyclosorus extensa* possesses high content of various polyphenols and these phenols were found to have substantial antioxidant properties. It is a commonly growing fern and is widely distributed all over Asia. Three new coumarin derivatives, three new furanocoumarins, and a novel dioxocane derivative have also been isolated from the closely related species of *Cyclosorus interruptus*.\(^{[12]}\)

Response surface methodology (RSM) is a multivariate equation solving technique which uses a collection of mathematical and statistical methods to evaluate relationships between a group of quantitative independent variables and one or more responses. Operation variables that may or may not have significant effect in the main response can be identified and optimized using RSM.\(^{[13–15]}\) In one of our earlier studies,\(^{[16]}\) RSM has been successfully applied in the optimization of process parameters for the fermentation of rice beer and the same conditions were applied to prepare beer from cassava and plantain. Literature survey revealed that no work till now has been reported on the correct choice of solvent as compared with the extraction conditions for obtaining phenolic compounds from *C. extensa*. Hence, in the present study, an attempt was made to standardize these parameters through optimization of time and temperature using four different solvents using RSM based on the response of total phenolic content, antioxidant, antibacterial, and antifungal properties.

**Experimental**

**Materials**

Leaves of *C. extensa* (Blume) Ching were collected from the botanical gardens of Tezpur University campus, Assam, in
June 2013. Taxonomical identification of the collected plant species was done in the Department of Botany, Darrang College, Tezpur, Assam. Based on the traditional uses of the plants for starter cake making, only the young and tender leaves were selected for analysis. The chemicals and solvents used for analysis were of high purity analytical grade and obtained from Sigma-Aldrich (USA), E. Merck (Germany), and HiMedia (India). The mold Aspergillus niger MTCC 281 and bacteria Escherichia coli MTCC 40 were kindly provided by the Department of Food Engineering and Technology, Tezpur University, Assam.

**Drying and grinding of plant materials**

The plant materials were washed with distilled water (H_2O), cut into small pieces, and dried in a tray dryer (BDI-51, Labotech, India) at 45°C with continuous air flow until a constant weight of the plant samples was obtained. The dried plant materials were ground in a laboratory blender into fine powder and then sieved through a mesh of size 50 US mesh. The powdered samples were immediately transferred to airtight containers and stored at 4°C until it is further used.

**Experimental design**

In the following equation of RSM [Eq. (1)], x_1 and x_2 are independent variables and the dependent response is denoted by y.

\[ y = f(x_1, x_2) + \varepsilon. \]  

(1)

Here \( \varepsilon \) is the experimental error term, which represents any measurement error on the response as well as other type of variations not counted in \( f \). In case of a curve in the response surface, a higher degree polynomial called a second-order model is used and is given as Eq. (2).\(^{[17]}\)

\[ y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i<j}^{n} \beta_{ij} x_i x_j + \sum_{i=1}^{n} \beta_i x_i^2 + \varepsilon \]  

(2)

The central composite design (CCD) is the most popular design for fitting a second-order model. It consists of factorial points (2^k), central points (n_c), and axial points (2k). Here, the values of n_c, the number of center point replications, can be chosen, so that the CCD can acquire certain desirable properties.\(^{[12,18]}\) The total number of design points in a CCD is given by Eq. (3). The CCD is said to be rotatable, if the precision of the estimated response surface at some point x depends only on the distance from x to the origin and not on the direction. When the rotatable design is rotated about the center, the variance of \( \hat{y} \) will remain same.\(^{[19]}\)

\[ n = 2^k + 2k + n_c \]  

(3)

Each of the variables is taken at two levels meaning that each variable has a low and high numeric value. A coded numeric value of −1 and +1 is assigned to represent the variable’s low and high values, 0 for the center points and \( \pm \gamma \) for the axial points. The central point or zero point may be defined as the region where the optimal conditions are supposedly met.\(^{[14]}\) When the response data are obtained from the test work, a regression analysis is performed to determine the coefficients of the response model, their standard errors, and significance. In addition to the constant (\( \beta_0 \)) and error (\( \varepsilon \)) terms, the response model incorporates linear terms in each of the variables, squared terms in each of the variables, and first-order interaction terms for each paired combination. Thus, for the two variables under consideration, the response model is given by Eq. (4).

\[ y = (\beta_0 + \varepsilon) + \sum_{i=1}^{2} \beta_i x_i + \sum_{i=1}^{2} \sum_{j=i+1}^{2} \beta_{ij} x_i x_j \]  

(4)

The \( \beta \) coefficients, which should be determined in the second-order model, are obtained by the least square method. In general, Eq. (4) can be written in matrix form as given in Eq. (5).

\[ Y = \beta X + \varepsilon \]  

(5)

where \( Y \) is defined to be a matrix of measured values and \( X \) to be a matrix of independent variables. The matrices \( \beta \) and error \( \varepsilon \) consist of coefficients and errors, respectively. The solution of Eq. (5) can be obtained by the matrix approach [Eq. (6)].

\[ \beta = (X^T \cdot X)^{-1} \cdot X^T \cdot Y \]  

(6)

where \( X^T \) is the transpose of the matrix \( X \) and \( (X^T \cdot X)^{-1} \) is the inverse of the matrix \( X^T \cdot X \).\(^{[20]}\)

To minimize energy cost of the extraction process, time and temperature are important parameters to be optimized. With the increase in working temperature, extraction is enhanced; due to increase in both solubility of solute and the diffusion coefficient, but beyond a certain value, phenolic compounds may be denatured.\(^{[21]}\) The experimental runs which were performed according to the CCRD design were for the two identified design independent variables, namely, extraction time in hours (\( \beta_1 \)) and extraction temperature in°C (\( \beta_2 \)), and for all the four solvents, namely, hexane (Hex), ethyl acetate (EA), methanol (MeOH), and \( \text{H}_2\text{O} \). The extreme values of the variables were \( X_1 \) (12 and 48 h) and \( X_2 \) (25 and 55°C). The central values of the variables were \( X_1 \) (30 h) and \( X_2 \) (40°C). The total number of experiments generated was 13 [Eq. (1)]. The responses studied were total polyphenol content, radical scavenging activity (RSA), antibacterial activity (ABA), and antifungal activity (AFA). The statistical software Design Expert Ver. 6.0.11 (Stat-Ease Inc., Minneapolis, MN) was used for design of experiments, regression and graphical analyses of the data obtained, and statistical analysis of the model to evaluate the analysis of variance.

**Extraction procedure**

Dried powdered samples (10 g) were taken in 250-mL Erlenmeyer flasks and 150 mL of each of the solvents, namely, Hex, EA, MeOH, and \( \text{H}_2\text{O} \) was added to it. The flasks were shaken in an incubator shaker (Excella E24 R, NBS, USA) at 150 rpm and maintained at the specified conditions of time and temperature as given by the design. Following this, the mixture was filtered through four layers of muslin cloth and then centrifuged at 1,000 g for 10 min and further filtered through Whatman No. 1 filter paper in a vacuum-assisted filtration unit. The removal of chlorophyll from the polar extracts was done by washing with carbon tetrachloride twice in a separating funnel.
Estimation of total phenolic compounds

The total phenolic compounds (TPC) in the extracts were analyzed by the method of Slinkard and Singleton[22] using Folin–Ciocalteu reagent. A standard curve of gallic acid was prepared in 1–10 µg/mL and the TPC was expressed in milligram gallic acid equivalent per 100 mL.

Estimation of radical scavenging activity

The free RSA was measured as per the method of Brand-Williams et al[23]. The extracts were mixed with 0.1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution and left for 30 min in the dark after which the absorbance (A1) was measured at 517 nm. The reading for a similar set which contained H2O in place of the extracts was taken as control (A0). The RSA was calculated percent inhibition relative to control using Eq. (7).

\[ \% \text{RSA} = \frac{(A0 - A1)}{A0} \times 100 \]  

Estimation of antibacterial activity

The bacterium E. coli MTCC 40 was used as test organisms for assessment of ABA. Cultures (100 µL, 24 h old) with the count adjusted to 10⁶ CFU/mL were spread on the EMB agar plates. Two equidistant wells were made in all the plates with a sterile cork borer. A volume of 100 µL of the extracts was added to each well. The solvents alone were used as the negative control. Diffusion of the extracts was allowed at room temperature for 1 h in a laminar air flow work chamber. The plates were incubated for 48 h at 37°C and observed for the inhibition of bacterial growth and measured in terms of the mean diameter of zones of inhibition (ZOI) around the wells.[24]

Estimation of antifungal activity

The mold A. niger MTCC 281 was used as test organism for assessment of AFA. Using a cork borer, 10 mm diameter circles of 72-h-old culture of Fusarium oxysporum (10⁶ CFU/g) were dug out and inoculated on the center of PDA plate and spread evenly over the plate using a sterile cotton swab. Wells were dug and extracts were added in the same manner as for ABA. Incubation was done for 96 h at 25°C and inhibition was expressed in the same manner as ABA.[25]

Results and discussion

Statistical analysis and model fitting

The combination of temperature and time for extraction set by the design and the responses of TPC, RSA, ABA, and AFA obtained for the whole experiment are shown in Table 1. The statistical data representing the analysis of variance were obtained. The sequential sum of squares, F-value, the corresponding coefficient of determination (R²), and adjusted coefficient of determination (adj. R²) are also presented. Variance and regression analysis were performed to fit the suggested quadratic models and investigated the statistical significance of model factors. The adequacies of the models were investigated by the F-values and corresponding p values of the regression models. It was observed that the predicted models for all the response variables were adequately fitted to the observed experimental data (p ≤ 0.001). The effect of linear, squared, and interaction terms of each response variable was also obtained. The accuracy of the fitness of the models was also judged by the lack of fit values for each response and observed no lack of fits (p > 0.05) in any response model. Nonsignificant lack of fit tests also suggested that quadratic models were best fitted for the extraction of bioactive compounds using four different solvents. Fitness of quadratic models was ascertained by computing the R² and adj. R² values. All these values are provided as supplementary files 1 and 2. Except for the TPC of Hex extracts, the R² values for all the other responses were above 90%. The difference between the R² and adj. R² values was less than 0.2, implying there are no insignificant terms added to the models.[16] Thus, results revealed that the models can establish optimum condition for the extraction of bioactive compounds from the leaves of C. extensa using Hex, EA, MeOH, and H₂O.

### Table 1. Response sheet for CCRD experimental design with process variables and experimental results for extraction with different solvents.

| Run | Temperature (°C) | Time (h) | TPC (mg/100 mL) | RSA (%) | ABA (mm) | AFA (mm) |
|-----|-----------------|----------|-----------------|---------|----------|---------|
|     | Hex | EA | MeOH | H₂O | Hex | EA | MeOH | H₂O | Hex | EA | MeOH | H₂O | Hex | EA | MeOH | H₂O |
| 1   | 25  | 12 | 0.04 | 0.35 | 0.62 | 0.13 | 25.38 | 40.30 | 60.79 | 66.13 | 12.00 | 14.67 | 12.67 | 12.67 | 12.00 | 15.13 | 12.14 | 14.50 |
| 2   | 40  | 30 | 0.00 | 0.56 | 0.99 | 0.20 | 19.33 | 47.80 | 65.69 | 60.28 | 12.23 | 13.50 | 13.25 | 13.00 | 13.00 | 12.84 | 15.50 | 11.50 |
| 3   | 18.79 | 30 | 0.11 | 0.41 | 0.53 | 0.09 | 24.21 | 40.84 | 59.66 | 68.38 | 13.67 | 14.33 | 12.67 | 12.33 | 13.50 | 16.04 | 14.00 | 13.50 |
| 4   | 40  | 30 | 0.01 | 0.55 | 0.97 | 0.16 | 18.76 | 47.11 | 65.43 | 60.21 | 11.98 | 13.56 | 13.06 | 13.20 | 13.06 | 15.50 | 15.00 | 11.63 |
| 5   | 40  | 30 | 0.05 | 0.62 | 0.93 | 0.19 | 19.56 | 47.95 | 64.84 | 59.32 | 12.71 | 14.13 | 12.98 | 13.20 | 13.00 | 15.13 | 12.00 | 14.50 |
| 6   | 25  | 48 | 0.10 | 0.44 | 0.43 | 0.18 | 17.94 | 50.21 | 61.47 | 67.24 | 12.00 | 14.00 | 12.00 | 12.00 | 12.48 | 14.35 | 14.50 | 12.50 |
| 7   | 40  | 30 | 0.01 | 0.63 | 0.90 | 0.20 | 19.74 | 47.53 | 65.34 | 60.15 | 12.05 | 13.78 | 12.86 | 12.94 | 13.08 | 15.14 | 15.55 | 11.25 |
| 8   | 61.21 | 30 | 0.03 | 0.46 | 0.82 | 0.29 | 23.32 | 52.86 | 65.55 | 63.78 | 14.87 | 13.50 | 12.00 | 13.67 | 13.47 | 12.14 | 15.50 | 16.00 |
| 9   | 55  | 48 | 0.06 | 0.39 | 0.79 | 0.26 | 22.49 | 55.52 | 66.79 | 64.07 | 13.33 | 15.00 | 12.00 | 13.33 | 12.25 | 12.01 | 11.50 | 14.00 |
| 10  | 55  | 12 | 0.00 | 0.42 | 0.42 | 0.29 | 23.81 | 56.22 | 69.81 | 64.28 | 12.33 | 12.67 | 11.68 | 13.00 | 12.00 | 14.14 | 15.50 | 14.50 |
| 11  | 40  | 4.54 | 0.00 | 0.36 | 0.38 | 0.21 | 24.28 | 46.43 | 68.38 | 62.56 | 11.23 | 13.67 | 12.14 | 12.33 | 11.50 | 14.48 | 11.00 | 13.74 |
| 12  | 40  | 30 | 0.00 | 0.52 | 0.82 | 0.20 | 18.34 | 47.02 | 66.54 | 60.25 | 11.88 | 13.94 | 12.76 | 13.06 | 12.84 | 14.29 | 15.00 | 11.50 |
| 13  | 40  | 55.46 | 0.1 | 0.39 | 0.75 | 0.23 | 15.57 | 56.15 | 66.53 | 65.13 | 12.00 | 14.33 | 12.00 | 12.33 | 12.04 | 11.08 | 11.00 | 11.00 |

CCRD, central composite rotatable design; TPC, total phenolic compounds; RSA, radical scavenging activity; ABA, antibacterial activity; AFA, antifungal activity; Hex, hexane; EA, ethyl acetate.
Effect of the process variables on various responses of the extracts

The values of the coefficients for TPC, RSA, ABA, and AFA for Hex, EA, MeOH, and H₂O extracts were used for constructing a final predictive equation, neglecting the nonsignificant cross terms (Supplementary file 2). To determine the optimal levels of variables for obtaining the maximum TPC, RSA, ABA, and AFA for all the extracts, three-dimensional surface plots were constructed according to these predictive equations which are illustrated in Figures 1–4 for Hex, EA, MeOH, and H₂O, respectively.

For all the solvents under study, it was observed that all the response values increased up to a certain level with increase in both time and temperature, keeping the other variables constant. In case of Hex, the TPC increased with increase in time and reached up to 0.4 mg/100 g and it was maximum (0.6 mg/100 g) at 30 h and 40°C. An increase in temperature also led to a gradual increase in the TPC. The RSA and ABA also increased with increasing time and reached up to 18% and 12 mm ZOI, respectively. The RSA and ABA also increased gradually with increase in temperature. The AFA reached up to 12.5 mm ZOI at a fixed temperature and the maximum AFA (13 mm ZOI) was obtained at 30 h and 40°C. In case of EA, the TPC increased up to 0.4 mg/100 g at fixed temperature and the maximum TPC of 0.6 mg/100 g was at 30 h and 40°C. The RSA, ABA, and AFA values increased up to 50%, 14 mm ZOI, and 13 mm ZOI, respectively, at a fixed temperature. Similarly, the increase in temperature at fixed time also led to a gradual increase in all the responses. In MeOH, the TPC reached 0.4 mg/100 g at fixed temperature with a maximum value of 0.9 mg/100 g at 30 h and 40°C. RSA increased with the increase in time and reached 62%. The ABA increased up to 12 mm ZOI at a fixed temperature and reached the maximum of 12.5 mm ZOI at 30 h and 40°C. The AFA also reached up to 14 mm ZOI at a fixed temperature. Similarly in H₂O, the TPC, RSA, ABA, and AFA increased till 0.15 mg/100 g, 66%, 12 mm ZOI, and 12 mm ZOI, respectively, at fixed temperature. An increase in temperature at fixed time led to a gradual increase in all the responses.

Optimization of parameters

The independent variables were optimized numerically using statistical software Design Expert, Ver. 6.0.11. Less time and temperature for extraction would result in incomplete extraction; whereas more time and temperature would lead to waste of time and energy. For this purpose, the goals for the variables, i.e., extraction time and temperature were kept in range and all the response parameters were set at maximum. The optimal conditions, predicted values, and experimental values for various responses are shown in Table 2. Numerical analysis revealed that in case of Hex, extraction temperature of 25°C for a period of 29.43 h gave an optimized extraction condition with maximum TPC of 0.06 mg/100 mL, RSA of 21.51%, ABA of 12.67 mm, and AFA of 13.18 mm, all with a combined desirability of 0.582. When the experiment was actually performed under the optimized conditions, all the parameters were however found to be lower than the respective predicted values. In case of EA, extraction temperature of 28.28°C for a period of 41.27 h gave an optimized extraction condition with maximum TPC of 0.51 mg/100 mL, RSA of 47.73%, ABA of...
Figure 2. Effect of time and temperature on the: (a) total phenolic content (TPC), (b) radical scavenging activity (RAS), (c) antibacterial activity (ABA), and (d) antifungal activity (AFA) of the ethyl acetate (EA) extracts.

Figure 3. Effect of time and temperature on the: (a) total phenolic content (TPC), (b) radical scavenging activity (RAS), (c) antibacterial activity (ABA), and (d) antifungal activity (AFA) of the methanolic (MeOH) extracts.
13.95 mm, and AFA of 13.19 mm, all with a combined desirability of 0.497. Also in this case, all the parameters were found to be higher than the respective predicted values under actual optimized experimental conditions. In case of MeOH, extraction temperature of 43.95°C for a period of 29.61 h gave an optimized extraction condition with maximum TPC of 0.93 mg/100 mL, RSA of 66.23%, ABA of 12.89 mm, and AFA of 15.47 mm, all with a combined desirability of 0.810. Also in this case, all the parameters were higher than the respective predicted values under actual optimized experimental conditions. In case of H2O, extraction temperature of 55.00°C for a period of 48.00 h gave an optimized extraction condition with maximum TPC of 0.26 mg/100 mL, RSA of 63.99%, ABA of 13.36 mm, and AFA of 13.93 mm, all with a combined desirability of 0.677. However, under actual experimental optimized conditions, TPC and RSA were lower, while ABA and AFA were higher than the respective predicted values.

**Effect of the solvents and optimized extraction conditions on TPC**

The solubility of all the phenolics, and thereby their tendency to get transferred or diffused into a given solvent is governed by thermodynamics, is also described by the activity-coefficient factor. [26,27] The experimental data correlating the solubility of total phenolics in the four different solvents with varying temperature can also be correlated by the modified Apelblat equation [28,29] which is shown in Eq. (8), where \( w \) is the mass fraction solubility of phenolics and \( T \) is the absolute temperature. The parameters \( A, B, \) and \( C \) are the parameters of the equation and can be obtained by fitting the experimental solubility data.

\[
\ln(w) = A + B/(T/K) + C\ln(T/K) \quad (8)
\]

As evident from the experimental data, the solubility of the phenols was more in MeOH, followed by EA, H2O, and

**Table 2. Estimated optimum conditions, experimental value, and residual value.**

| Solvent | Parameter | Factor   | Response |
|---------|-----------|----------|----------|
|         | Temperature (°C) | Time (h) | TPC (mg/100 mL) | RSA (%) | ABA (mm) | AFA (mm) |
| Hex     | PV        | 25.00    | 0.06     | 21.51    | 12.67    | 13.18    |
|         | EV        | —        | 0.058    | 20.62    | 11.58    | 12.67    |
|         | RV        | —        | 3.33%    | 4.14     | 8.60%    | 3.87%    |
| EA      | PV        | 28.28    | 0.51     | 47.73    | 13.95    | 13.19    |
|         | EV        | —        | 0.55     | 49.57    | 14.39    | 14.07    |
|         | RV        | —        | 7.84%    | 3.86     | 3.15%    | 6.67%    |
| MeOH    | PV        | 43.95    | 0.93     | 66.23    | 12.89    | 15.47    |
|         | EV        | —        | 0.99     | 68.11    | 13.81    | 16.15    |
|         | RV        | —        | 6.45%    | 2.84     | 7.14%    | 4.40%    |
| H2O     | PV        | 55.00    | 0.26     | 63.99    | 13.36    | 13.93    |
|         | EV        | —        | 0.24     | 61.25    | 14.26    | 14.10    |
|         | RV        | —        | 7.69%    | 4.28     | 6.73%    | 1.22%    |

TPC, total phenolic compounds; RSA, radical scavenging activity; ABA, antibacterial activity; AFA, antifungal activity; Hex, hexane; PV, predicted value; EV, experimental value; RV, residual value.
Hex. The natural phenols possess a higher solubility preference to solvents with intermediate polarity (alcohols), rather than more polar (water) or less polar (Hex) solvents. This solubility preference can be attributed to the stereochemistry of phenols and the intermolecular forces which occur in between the molecules and the solvents, namely, the hydroxyl groups present in phenols can develop hydrogen bonds with the electronegative oxygen of the alcohols. The higher solubility in polar protic (MeOH) instead of polar aprotic solvents (EA) can be explained by the fact that the alcohols’ hydroxyl groups can develop hydrogen bonds with the oxygen atoms occurring inside phenolic molecules.\textsuperscript{30}

**Effect of the solvents and optimized extraction conditions on RSA**

DPPH is a stable free radical which has an unpaired valence electron at one atom of nitrogen bridge.\textsuperscript{31} and the sensitivity of DPPH radical is high enough to detect active ingredients at low concentrations and it can also accommodate many samples in a short period.\textsuperscript{32} Hence, its scavenging is considered as a good parameter in screening assays. Owing to the different antioxidant potentials of compounds with different polarities, both extraction yield and antioxidant chemical activity of extracts are strongly dependent on the solvent.\textsuperscript{33} As presented in Table 2, in the DHHP assay, the MeOH extracts possessed the highest RSA, followed by H\textsubscript{2}O, EA, and Hex. The overall antioxidant activities of extracts are contributed by the phenolic compounds,\textsuperscript{34} and a direct correlation was observed in between the phenolic content and the antioxidant activity for all the solvents. The antioxidant activity depends on the type and polarity of the extracting solvent and the isolation conditions, and this may be attributed to the different antioxidant activities of phenolic extracts.\textsuperscript{35}

Lafka et al.\textsuperscript{36} also reported the antioxidant activity of phenolic compounds from winery wastes by DPPH method and found that methanolic and ethanolic extracts possessed the highest RSA. The optimization study by RSM for extraction of antioxidants from black mulberry leaves by Radiokovi et al.\textsuperscript{8} found that ethanol (59.47%), temperature (59.92°C), and liquid/solid ratio of 20.73 mL/g yielded maximum TPC of 48.5 mg/g of dried leaves and minimum IC\textsubscript{50} value of 0.023 mg/mL for DPPH activity. Bouterfas et al.\textsuperscript{37} also performed the optimization study with Marrubium vulgare L. leaves and found that the maximum total phenolics (293.34 mg/g dry weigh) were obtained with 60% aqueous MeOH at 25°C for 180 min. Significant effect of various extracting solvents and temperatures on total phenolics and anthocyanin extracts was studied by Rababah et al.\textsuperscript{38} and found that MeOH and 60°C of extraction conditions were the best for extracting phenolic compounds from oregano, thyme, terebinth, and pomegranate.

**Effect of the solvents and optimized extraction conditions on ABA and AFA**

Most strains of *E. coli* are commonly a part of the normal flora of the gut and are harmless, but some causes serious food poisoning.\textsuperscript{39} *A. niger* is a common food contaminant and causes a disease called black mold on certain fruits and vegetables. It is ubiquitous in soil and is commonly reported from indoor environments.\textsuperscript{40} All the extracts were found to have variable inhibitory activity against these two indicator microbes. Variability in potency is usually ascribed to differences in the relative amount and composition of phenolic compounds extracted with specific solvents.\textsuperscript{41} Phenolic extracts are believed to induce lesions in cell membranes, thereby initiating a series of events that lead to microbial cell death.\textsuperscript{42} It was observed that under the optimized conditions (Table 2), the highest ABA was found in EA extracts and AFA was the highest in MeOH extracts. In both the cases, H\textsubscript{2}O extracts exhibited the second highest activity. These results are favorably supported by an explanation that antimicrobial phytochemicals are soluble in moderate polar solvent\textsuperscript{43} and activity may be slightly weaker in aqueous extracts due to lower concentrations of compounds like tartaric acid esters and flavonols than in methanolic extracts.\textsuperscript{41} The phenomenon has been reported previously by Bassam et al.\textsuperscript{44} who found higher activity of methanolic extracts of aromatic herbs against a range of micro-organisms as compared to the aqueous extracts prepared in hot water.

Optimization of the process conditions for extraction of antibacterial compounds (against *Bacillus subtilis* and *E. coli*) with MeOH, EA, Hex, and H\textsubscript{2}O from 19 Malaysian flowering plants was performed by Abdullah et al.\textsuperscript{45} and EA extracts revealed the highest ABA, and the best conditions postulated from the optimization study were 9.58 h, 300 rpm, and 27.35°C. In another study, the optimum extraction conditions for antimicrobial activities of Nitelopsis obtusa and *Chara vulgaris* extracts against *Staphylococcus aureus*, *B. subtilis*, *E. coli*, *Proteus vulgaris*, *Saccharomyces cerevisiae*, and *Candida albicans* were found to be solid to solvent ratio 1:1.5, temperature 85°C, ethanol concentration 50%, extraction time 6 h for *N. obtusa*; and solid to solvent ratio 1:1.5, temperature 85°C, ethanol concentration 70%, extraction time 10 h for *C. vulgaris*.\textsuperscript{46} Palakawong et al.\textsuperscript{41} also optimized the extraction of phenolics with antimicrobial properties against *E. coli*, *Salmonella typhimurium*, *Listeria monocytogenes*, and *S. aureus* from mangosteen bark, leaf, and fruit pericarp and found the optimal conditions to be 60°C and 1:60 solute to solvent ratio.

**Conclusion**

Results revealed that the total phenolic content, antioxidant activity, and antimicrobial activity of the extracts from *C. extensa* leaves varied depending on the type of the solvent used. These results reinforced the importance of extraction conditions on total phenolic content, antioxidant activity, and antimicrobial activity as a function of changing polarities of the four solvents in the order of H\textsubscript{2}O>MeOH>EA>Hex. Analyses of the response surfaces performed eased to optimize the extraction conditions for bioactive compounds. The experimental data were satisfactorily described by the second-order polynomial model. The optimum conditions of extraction temperature and time for Hex, EA, MeOH, and H\textsubscript{2}O were 25°C for 29.43 h, 28.28°C for 41.27 h, 43.95°C for 29.61 h, and 55.00°C for 48.00 h, respectively. Increasing
temperature enhanced diffusivity and yields of bioactive compounds in extracts, however, when temperature was too high, the solvents decreased and the yields also decreased. A good correlation between total phenolic content and antioxidant activity was also observed. Except in case of ABA, the methanolic extract was found to have the highest activity for all the responses. These optimal conditions will be useful in future studies to perform the extraction of bioactive compounds with reduced time and also in the development of industrial extraction processes.

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