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Research paper

Statistical Mixture Design: Study of Solvent Performance in Temperature Controlled Microwave Assisted Extraction System on Antioxidant Properties of Nephrolepis biserrata (Schott.) Sw. frond Extract

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Abstract

Statistical simplex-centroid design mixtures of water, acetone, acetonitrile, ethanol were used to extract Nephrolepis biserrata (Schott.) SW. frond (NBF) using temperature controlled microwave extraction system (TCMES). The effects of different solvents and their mixtures on its antioxidant properties were studied. The result shows, the quality of extracted material is found to depend on the solvent proportions according to special cubic model of TPC, TFC and IC50. The binary and quaternary solvents are found to explain the superior extraction results as compared to single solvent for TPC, TFC and IC50.

Keywords: *Mixture Design, Nephrolepis biserrata (Schott.) Sw, TPC, TFC, IC50*

1. Introduction

Microwave-assisted extraction (MAE) is a relatively efficient extraction technique that utilizes microwaves for heating up the sample and solvent, rapidly and homogeneously. The principle of heating using microwaves is based on the direct action of the electromagnetic radiation on the molecules through ionic conduction and dipole rotation, resulting in heating [1]. Polar solvents with high dielectric constants tend to heat up more rapidly with microwaves than non-polar solvents with lower dielectric constants [2]. Thus, polar solvents or non-polar solvents diluted with water are frequently employed solvent systems with MAE.

MAE has been increasingly used for the rapid extraction of bioactive compounds from plant sources [3, 4]. Compared to solvent extraction, it has been shown to reduce solvent requirements and the extraction time [5, 6]. However, the study on antioxidant and physicochemical properties of *Nephrolepis biserrata* (Schott.) Sw, frond (NBF) using MAE has not been studied.The formatter will need to create these components, incorporating the applicable criteria that follow.

In this research, *Nephrolepis biserrata* (Schott.) Sw, frond (NBF) was analyzed. NBF is basically a ground fern, but is also commonly found as an epiphyte on oil palm. *Nephrolepis biserrata* has been reported as a skin remedy in order to cure blisters, boils, abscesses and sores [7, 8] found drimanes in the epicuticular wax of *Nephrolepis biserrata*. Drimane a group of terpenic compound which posses a wide variety of biological activities (antiallergic, antiviral, antifungal, antibacterial, antifeedant, insecticidal, cytotoxic, phytotoxic and others). There are 4.3 million hectares of oil palm in Malaysia which covered with these epiphyte ferns provide significant raw materials for the extraction purpose. Compare to other well-known medicinal plant that require a high cost of farming, epiphytes fern is a good alternative for a cheap source of phytochemical compounds.

The efficacy of microwave extraction of secondary metabolites from plant materials depends on their chemical nature. In the literature, binary solvent systems show better result compares mono solvent because it could extract all the antioxidants with their different polarities. Although the literature shows many studies on aqueous mixtures of pure organic solvents, no investigations of microwave extraction using these solvent systems were found using statistical mixture designs. In this work a simplex lattice mixture design involving water, acetone, acetonitrile and ethanol has been applied to the extraction of NBF as a plant material. The mixture design model was assessing the importance of the effects of solvents and their mixtures on total phenolic compound (TPC), total flavonoid compound (TFC) and DPPH radical scavenging activity (IC_{50}).

A. Design of Experiment

Statistical experimental design, also called design of experiment (DoE), is a well-established concept for planning and execution of informative experiments which can be used in many applications. These strategies were originally developed for the model fitting of physical experiments, but can also be applied to numeral experiments. The objective of DoE is the selection of the points

where the response should be evaluated. Most of the criteria for optimal design of experiments are associated with the mathematical model of the process. Mixture design is one of the design families instead of fractional factorial and central composite, which commonly used to study the relationship of factors on response.

Mixture design is usually employed to investigate the relationship between the blend fraction matrix (factors) and the final blend product property matrix (response), given that the raw materials to be blended are already chosen. The response in mixture of experiments primarily depends on the mixing proportions. Ever since Scheffe devised a single-lattice and single-core design in 1958, the mixture design has developed a variety of methods. The characteristic feature of a mixture design is that the sum of mixture factors=100% ($\sum Xk = 1$). That meant these components (Xk), mixture factors, dependently of one another, and their proportions must be somewhere between 0 and 1.

2. Experimental

A. Reagents and solvents

Sodium hydroxide, α-tocopherol, butylated hydroxyl-anisole (BHA) were purchased from Sigma-Aldrich. Gallic acid, sodium carbonate, aluminium chloride, 1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin-ciocelteau, sodium nitrite, catechin, acetonitrile, acetone and EtOH purchased from Merck Germany. All the reagents were of analytical grade.

B. Apparatus and instruments

Temperature controlled microwave system (TCMCS), thermocouple Type K, Thermocouple data logger (TC-08, Pico Technology), rotary vacuum evaporator (Buchi, Germany), freeze drier (Cleanvac 8 BIOTRON, South Korea), cuvette, oven (Memmert, Germany), sonicator (Branson, USA), UV-Vis spectrophotometer (Thermo Scientific model Genesys 10S).

C. Fern Materials

The Nephrolepis biserrata (Sw.) Schott was collected on the palm oil trunk at a palm oil plantation in Seri Medan, Johor Malaysia. The voucher specimens labeled as KLU47725 have been deposited in the Herbarium of the Rimba Ilmu, University of Malaya for further reference.

D. Preparation of Microwave Assisted Extraction (MAE) Process of Epiphytes Fern

Plant material was extracted in a domestic microwave oven (Haier, model EA-180M) with modification. It had a rated power output of 700 watts with an operating frequency of 2450 MHz. In the modified microwave oven, the hole diameter was built up below 7 cm to ensure safety and to accommodate two necked vessels with fluid sealed stirring device. Nephrolepis biserrata (Sw.) Schott frond (NBF) was initially dried in the oven to reduce the moisture content at 10-11%. The sieved plant materials (16.5 g) were weighed and transferred into a reaction vessel where 500 mL of solvent was added. The glass vessel setup was equipped with glass connecters attached to a reflux condenser and a thermocouple for controlling the temperature. In order to increase the interactions among the materials, solvent and microwave radiation, the content of the reaction vessel was continuously stirred. The extraction temperature was kept at 60°C for 4 minutes of extraction times, and microwave power is 420 Watts. The sample was extracted using at a ratio of solid to liquid: 1:25. After extraction, the solution of extract was filtered using Whatman 41. The extracted solution was evaporated using a rotary evaporator. The sample was stored in a -20°C until further use in antioxidant property's analysis.

E. Antioxidant Properties Analysis

Determination of TPC

TPC of TCMAE water extracts was carried out using Folin–Ciocalteu reagent according to the reported method. The concentration of polyphenols was calculated from the calibration curve using gallic acid and the result were expressed in gallic acid equavalents (GAE mg/100gm dry weight material). Samples were analyzed in triplicates. Samples were analyzed in triplicates.

$$Total\ \ phenolic\ compound\ (TP) = \frac{C \times V \times df}{M} \quad (1)$$

Where:

C = gallic acid (µg/ml) (i.e 0-200 µg/mL)
V = volume of plant extract (mL)
M = sample weigh (mg)
df = dilution factor

Determination of TFC

TFC of TCMAE water extracts was determined by aluminium chloride colorimetric method by [9]. The flavonoid was calculated based on the catechin calibration curve and the result were expressed in catechin equivalents (mg CE/g dry weight material).

Measurement of DPPH radical scavenging activity (IC50)

The radical scavenging activity of epiphytes species was estimated according to the procedure modified by [10]. IC50 value was determined from the plotted graph of scavenging activity against the concentrations of TCMCS water extracts of each species epiphytes fern samples, which is defined as the concentration of extract causing 50% inhibition of absorbance. Triplicate measurements in different concentration were carried, such that a 50% fall in absorbance of the DPPH can be calculated. The IC50 value of each extract was measured and compared with the corresponding α-tocopherol and butylated hydroxyl anisole (BHA).

3. Result and Discussion

To fit the response function and experiment data, regression analysis was performed and the model was evaluated by (ANOVA) for dependent variable or response, including their interaction with each other on the response. The result of antioxidant properties in terms of TPC, TFC, IC50 as per the experimental plan were input into the Design Expert software for further analysis following the steps outlined previously. Examination of the fit summary output revealed that the special cubic model was statistically significant for the TPC, TFC, IC50,. Therefore, this model was used to represent each of the responses for further analysis. ANOVA

A. Analysis for Antioxidant Properties

In order to ensure a good model, test for significant of regression model, test for significance on individual model coefficients and test for lack of fit needed to be performed. An ANOVA table is commonly used to summarize the tests performed. Table I shows the ANOVA table for the response special cubic model for TPC, TFC and IC50. were observed based on the value of F-ratio. The larger the magnitude of the F-value and the smaller the P-value, the more significant is the corresponding coefficient [11]. The lack of fit can also be said to be insignificant.

The value of "Prob. >F" for the special cubic model of TPC, TFC, IC50, are less than 0.05, which indicates that the model is significant. It also indicates that the term in the all models has a significant effect on the response. The significant factors This is desirable, as we want a model that fits. R2 value is the first criteria to see the appropriateness of the model from the determination coefficient. It also reveals the total variation of the observed values of activity about its mean [12]. As the show in Table 1, the R2 value calculated for TPC, TFC, IC50, are 0.969, 0.972 and

0.971 respectively, reasonably close to 1, which is acceptable. It implies that about more than 98% of the variability in the data is explained by the model. The predicted R2 is in reasonable agreement with the adjusted R2. The adjusted R2 value is particularly useful when comparing models with a different number of terms. This comparison is, however, done in the background when model reduction is taking place. Adequate precision compares the range of the predicted values at the design points to the average prediction error. Adequate precision value is well above 4 which indicate adequate model discrimination. In this case, the value of TPC, TFC, IC50, is well above 4.

Table i: ANOVA table of antioxidant properties (TPC, TFC and IC50)

Response variables	Sum of square	Degree of freedom	Mean square	F value	Prob.	
TPC-Special cubic						
Regression	18263.4	13	1404.88	21.38	0.0007	significant
Residual	394.35	6	65.73			
Lack of fit	171.85	1	171.85	3.86	0.1066	not significant
Pure error	222.5	5	44.5			
Cor total	18657.75	19				
R^2						0.9689
Adj R^2						0.9331
Adeq precisior						12.062
TFC-Special cubic						
Regression	1197.46	13	92.11	26.19	0.0003	significant
Residual	21.1	6	3.52			
Lack of fit	7.32	1	7.32		0.1643	not significant
Pure error	13.79	5	2.76			
Cor total	1218.56	19				
R^2						0.9727
Adj R^2						0.9506
Adeq precisior						16.045
IC_{50}.Special cubic						
Regression	$5.13x10^{-7}$	11	$4.66x10^{-6}$	58.85	<0.0001	significant
Residual	$6.34x10^{-5}$	8	79262.71			
Lack of fit	$3.47x10^{-5}$	5	$1.15x10^{5}$	2.02	0.23.2	not significant
Pure error	$2.87x10^{-5}$	5	57379.43			
Cor total	$5.19x10^{-5}$	19				
R^2						0.9718
Adj R^2						0.961
Adeq precisior						21.428

B. Effect of Solvent Composition on Antioxidant Properties

Table II shows the effects of the solvent proportion of the average value of antioxidant properties (TPC, TFC and IC50) of NBF extract. It was observed that the TPC, TFC and IC50 were greatly influenced by the different solvent proportion. By comparing the average of the TPC, in general, can notice that the both values increased in the following order, pure solvents (47.48 mg GAE/g), binary solvent mixtures (50.17 mg GAE/g) and quaternary solvent mixtures (63.22 mg GAE/g) respectively. The binary solvent mixture shows higher TFC value following by pure and quaternary.

Pure acetone and acetonitrile presented the lowest TFC value, whereas maximum values were obtained with a mixture of water and acetonitrile. The experimental results of TPC and TFC were in accordance with previous studies, which reported that mixture of solvent system was more useful and favorable in the extraction of phenolic compounds from plant samples as compared to the mono-solvent system [13-16].

Note that from Table II, the average value of DPPH scavenging shows a single solvent have higher reading, followed by the binary and quaternary solvent. A lower value of IC50 indicates a higher antioxidant activity. It is interesting to notice that highest antioxidant activities were obtained with quaternary solvent.

Compared to other solvent combination, MAE using a mixture quaternary solvent were found to highest result of TPC and IC50 and good result in TFC. Microwave heating is also referred to as dielectric heating [17]. The ability of solvent to absorb energy in a microwave cavity is related to the high dielectric properties of the solvent, thus resulting in higher reaction rates [18, 19]. Water, indeed, possesses a much higher dielectric constant than acetonitrile, ethanol and acetone. The molecules or atoms comprising the dielectric exhibit a dipole movement. This movement generates friction inside the dielectric and the energy is dissipated subsequently as heat [20]. A higher proportion of high dielectric properties, solvent like water resulted in a more rapid temperature increase and better extraction efficiency.

Table Ii:. Average Amount Of Tpc, Tfc And Ic_{50} In Different Solvent Proportion

	TPC mg GAE/g	TFC mg CE/g	IC_{50} µg/mL
single solvent	47.47	11.60	2537.66
binary	50.17	17.62	1709.42
quaternary	63.22	14.37	1520.34

In addition, the interaction of dielectric properties of solvent with electromagnetic radiation in the microwave range results in energy absorbance. This depends on the relaxation times of the molecules in the material, which in turn depends on the nature of the functional groups, the volume of the molecule and the number of carbon atoms in the molecule. [21] and [22] were found that, a number of carbons in the molecule decrease, the dielectric constant increases. Single acetonitrile and ethanol possess a number of carbons, resulted higher dielectric constant, observed good TPC, TFC and IC50 result as compared to single acetone.

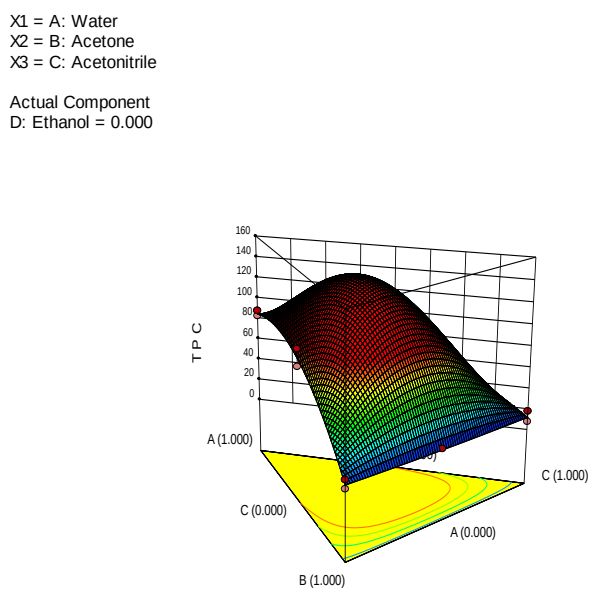
Though as stated by prior research, a heating process was required to dissolve some of the phytochemicals in order to provoke the full dissolution of compounds. During extraction, the heating rate should not be higher due to degradation of phytochemicals by super heating effect. The mass heat capacity (cup) of the solvent which influences the amount of energy absorbed during microwave heating [23] also should be considered during extraction process. Acetone had higher heat capacity value as compared to other solvent. Nevertheless, the extraction process

was fixed in 60°C which near to acetone boiling points. In this situation, burning effect should cause lower result of TPC, TFC and IC50 in single solvent acetone.

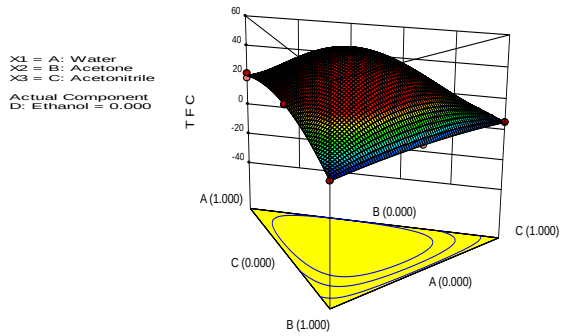
Solvent extractions are the most part frequently used procedures to practice extracts from plant materials due to their simplicity of use, effectiveness, and extensive applicability. It is known that the quantitative and qualitative results of extraction depend on the type of solvents with changeable polarities and sample to solvent ratio as well on the chemical composition and physiochemical characteristics of the plant extract. The
dissolution of phytochemical is governed by the chemical nature of the plant sample, as well as the polarity of the various solvents used in this study. Hence a suitable combination of solvent is important in order to get a better result.

C. Surface Plot of Antioxidant Properties

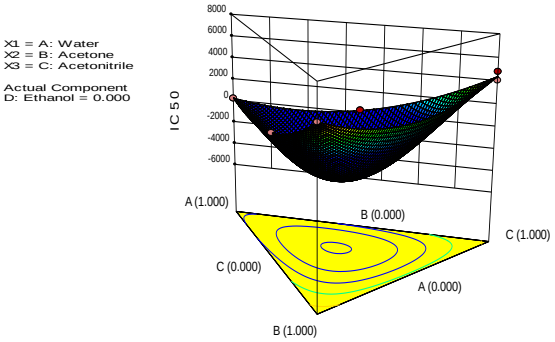
The three dimensional surface plots demonstrate the effect of solvent mixture on TPC, TPC and IC50 and they are depicted in Fig I (a), (b) and (c) respectively. Surface plots were then generated at each of the water, acetone and acetonitrile increased along the line from the centroid (1.00:1.00:1.00) toward its vertex (1.00:0.00:0.00), while the ethanol held constant.



(a)



(b)



(c)

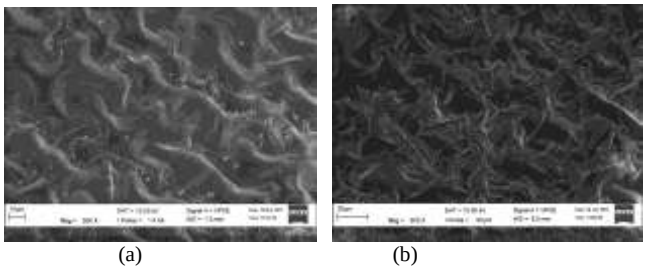
Fig 1:. Surface plot of (s) TPC (b) TFC (c) IC_{50}

The effect of antioxidants on DPPH is thought to be due to their radical scavenging and hydrogen donating ability which could provide as free radical inhibitors or scavengers. Through this assay, it was observed that the violet color of DPPH was reduced to a pale yellow color due to the abstraction of the hydrogen atoms from antioxidant compound [24]. The more antioxidants occurred in the extract, the more DPPH reduction will occur. High reduction of DPPH is related to the high scavenging activity performed by the particular sample as reported by Ghafar [25]. Since IC50 is a measure of inhibitory concentration, a lower IC50 value would reflect greater antioxidant activity of the sample.

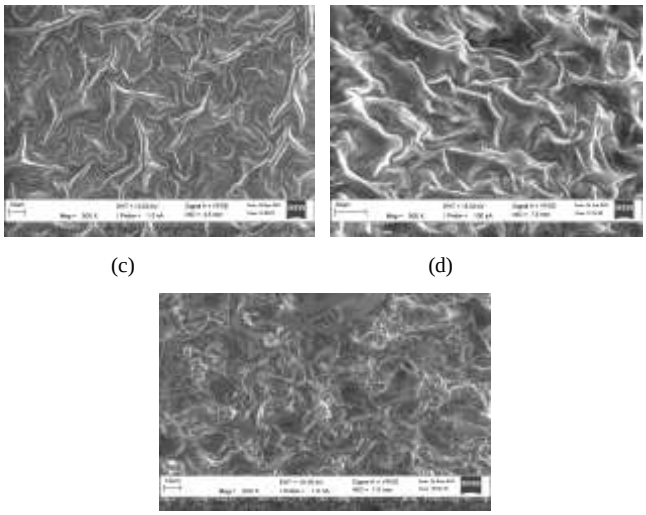
D. Effect of TCMA Extraction on NBF Microstructure under Various Solvent

Temperature controlled microwave-assisted extraction is a fast extraction process where microwave energy is delivered efficiently to materials through molecular interaction with the electromagnetic field and offers a rapid transfer of energy to the extraction solvent and raw plant materials [26]. The direct interaction of microwave with solvent also results in the rupture of the plant cells and release of intracellular products into the solvent quickly [27].

For TCMAE, the choice of extraction solvent takes into account not only its ability to absorb microwave energy but also solubility for target component. The mechanism of the extraction process with the solvent system was performed by scanning electron microscopy. As shown in Figure 2, the plant matrix before and after treatment, also with high dielectric properties to lowering dielectric properties, solvent, the correlation was found between the values of antioxidant properties with the change in plant matrix surface. Higher the recovery of antioxidant properties shows greater the damage to the plant matrix surface which allows the migration of the compounds out of the matrix. Microwave heating of the matrix should lead to the destruction of the macrostructure of the matrix, thereby increasing the surface available for the extraction solvent.



(a) (b)



(c) (d)

(e)

Fig 2.: The surface of plant matrix at various solvent a) before extraction b) water, c) acetone, d) acetonitrile, e) ethanol. Magnification 500x.

SEM image shows that the water extracts had more damage as compared to be other solvent. The presence of the excess amount of water can cause excess thermal stress due to rapid heating of the solution and increase in swelling of plant material. Based on the high dielectric constant of water, the free water content of the sample induces a very rapid and concentrated heating during the microwave extraction process. The resulting pressure increase may result in the rupture of membranes in the sample matrix enabling increased the contact surface area between the plant matrix and the solvent and various constituents to be released.

4. Conclusion

Mixture design was determined for antioxidant properties (TPC, TFC and IC50) as a function of the extraction solvent compositions. The special cubic model for TPC, TFC and IC50 showed no significant lack of fit at the 95% confidence level. The highest recovery for TPC, TFC and IC50 was obtained with quaternary, binary and quaternary solvent respectively. The mechanism of the enhanced extraction by microwave was discussed by observing cell destruction of NBF material after TCMAE treatment by scanning electron microscopy. The results showed that the plant materials were significantly destroyed, especially for binary solvent which contained water as solvent due to the cell rupture after TCMAE treatment. It needs to be noted here, solvent with polar molecules, which can very efficiently absorb microwave energy, plays an important role in the TCMAE process. Thus, binary and quaternary combination with high polar solvent was determined to be appropriate extraction solvent for further study.

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