Multi-response optimization of an extraction procedure of rosmarinic acid from *Prunella vulgaris* L. and antioxidants using

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ABSTRACT

Prunella vulgaris L. (PV) have been reported to have a variety of important biological activities. A process for rosmarinic acid (RA) extraction - antioxidants from PV was performed to obtain the highest extraction yield, strongest antioxidant activity and optimized by a multi-response optimization process. A three-level three-factor Box-Behnken design (BBD) was performed as response surface methodology (RSM) with desirability function (D) to attain the optimal extraction parameters. The rosmarinic acid extraction rate was determined by UV-Vis method with linear line $Y = 2.1033x + 0.1108$, $R^2 = 0.9966$. The DPPH and $ABTS^{\bullet+}$ scavenging percentage was used to represent the antioxidant ability. The maximum D value of 0.251, along with the maximum yield (6.025%), %RA (0.115%), scavenging percentage DPPH (IC_{50}) (84.975 µg/ml) and ABTS (TEAC) (0.207) were achieved after an ethanol concentration of 60°, an extraction time of 60 min, using an extraction temperature of 50°C.

INTRODUCTION

Prunella vulgaris L. is a perennial herbaceous of the Lamiaceae family, growing in the Northeastern Asia region. The dried fruit – spike of *P. vulgaris* is occasionally used in Vietnamese folk medicine for treatment of sore throat, fever and wound healing. Furthermore, the methanol or water extracts of this remedy exhibits bioactive properties including anti – microbial, anti – viral, anti – cancer, anti – hyperglycemic, anti – oxidative, and anti – inflammation effects [2]. Previous phytochemical studies of P. *vulgaris* revealed the presence of triterpenoids, phenols, flavonoids, tannins, caffeic acid, the tannins, and anionic polysaccharide prunelline [1, 3]. Of them, rosmarinic acid and its structural analog as the major phenolic component in this remedy has been known to contributor to the therapeutic effects as well as the criterion for quality control. Recently, rosmarinic acid only showed a good antioxidant activity relative to quercetin in a non – cellular assay, but this activity was markedly attenuated in a cell – based assay [9]. In the consequence, other component for instance uronic acid, quercetin, and polysaccharide in extraction also were attributed to anti-oxidative properties. This effects were evaluated an important role in many human diseases including cancer, diabetic complications, heart disease, liver damage, autism and Alzheimer's disease, etc.

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On the other hand, the conventional solid-liquid extraction were widely apply for antioxidants with advantages such as cheap and easy to scale up (Mulinacci et al., 2011) [6]. Other techniques include ultrasound – assisted extraction (Rodríguez – Rojo, Visentin, Maestri, & Cocero, 2012) [5], and a supercritical fluid technology (Herrero et al., 2010) [7] are mainly employed to improve the process efficiency and final extracted quality.

Box – Behnken design (BBD) belong response surface methodology (RSM) is one of the most popular experimental designs due to efficient and flexible tools. BBD combination with desirability functions could be determined the simultaneous optimization of multiple responses (Ghafoor et al., 2009) [8].

Hence, the first study for P. *vulgaris* aim to apply conventional solid – liquid extraction method for achieving a high rosmarinic acid content and maximize the antioxidant ability of extraction. Next, the desirability function that simultaneously maximizes the antioxidants extraction and their concentrations in the final product was validated

MATERIALS AND METHODS

Materials

Fruit clusters of P. *vulgaris* (PV) were storage in National institute of medicinal materials (NIMM) (Quang Trung, Ha Noi).

It was authenticated by Dr. Nguyen Minh Ngoc (NIMM).

Chemicals and Apparatus

All chemicals and reagents used were of analytical reagent grade or better. Standard rosmarinic acid, rutin, quercetin and trolox were purchased from Sigma-Aldrich (USA). Zirconyl chloride octahydrate, 2,2´azinobis (3-ethylbenzothiazonline-6-sulfonate), 1,1-diphenyl-2-picrylhydrazyl.

A UV-visible spectrophotometer (Varian Cary-100, Palo Alto, CA, USA) was used for the analysis of the RA content. The ELx808™ Absorbance Microplate Reader (USD) was used for the analysis of DPPH, ABTS free radical scavenging.

Extraction

The PV powder (05 g) was placed into a volumetric flask (250 ml), soaked with ethanol solvent (varying ethanol concentration from 40 to 80%, v/v; extraction temperature from 40 to 60°C; time from 30 to 90). The extract was filtered concentrated under reduced pressure to a dark syrup. The yield (%) was calculated as follows:

$$\mathbf{Y(\%) = \frac{W_i}{W_j} \times 100\%}$$

where Y (%) was the yield, Wi was the weight of extraction and Wj was the weight of raw material.

Determination of rosmarinic acid

Determination of rosmarinic acid species was investigated by the method of Mehmet Öztürk et al. An aliquot of 200 µl of the extract solution in ethanol was added to a test tube containing 4.6 ml of ethanol and 200 µl of zirconium (IV) oxide chloride solution. The absorbance was determined at 362 nm at room temperature, after 5 min. The concentrations of rosmarinic acid in the extracts were calculated according to the following equation that was obtained from the standard rosmarinic acid graph (Fig. 1). The rosmarinic acid extraction rate (%) was then calculated as follows:

$$\mathbf{RA(\%) = \frac{c}{W_j} \times 100\%}$$

where *RA (%)* was rosmarinic acid extraction rate, *C* was the weight of RA and W_j was the weight of raw material.

Determination of antioxidant activity

DPPH radical – scavenging assay

The antioxidant activity of scavenge DPPH radical species was investigated by the method of

Ronald et al. [4] with some modifications. The extracts of PV were dissolved in DMSO at different concentrations (125, 250, 500, 750, 1000 µg/ml). The 180 µl methanol solution of DPPH (150 µM/ml) was added to the 20 µl solution of sample with different concentrations of PV, respectively. The mixture was reacted at room temperature for 30 min under strict exclusion of light. After that, the absorbance was measured at 490 nm by The ELx808™ Absorbance Microplate Reader (USD). Also, Quercetin and rutin were used as reference standard. DPPH radical scavenging activity was calculated using formula:

$$\mathbf{I(\%) = \frac{(A_i - A_t)}{(A_i - A_j)} \times 100\%}$$

where A_i is the absorbance of DPPH solution without sample (180 µl DPPH + 20 µl DMSO); A_t is the absorbance of the test sample mixed with DPPH solution (180 µl DPPH + 20 µl sample) and A_j is the absorbance of the sample without DPPH solution (180 µl MeOH + 20 µl DMSO). The result was expressed as the half maximal inhibitory concentration (IC_{50}) of PV.

ABTS radical – scavenging assay

The antioxidant activity of scavenge ABTS radical species was investigated by the method described by Apak et al. with some modifications. $ABTS^{\bullet+}$ was produced by reacting 20 mM ABTS solution with 2.45 mM potassium persulphate solution and allowing the mixture to stand in dark at room temperature for 16 – 24h before use. The $ABTS^{\bullet+}$ solution was diluted with water to an absorbance of 0.70 (± 0.02) at 734 nm at a temperature of 25°C. Then, to perform the ABTS radical scavenging assay, the 195 µl solution of $ABTS^{\bullet+}$ was added to the 05 µl PV solution at concentration was 25 µg/ml. The mixture was reacted at room temperature for 06 min under strictly exclusion of light. After that, the absorbance was measured at 690 nm, with solvent as blank control. Additionally, Trolox was used as a positive control. ABTS radical scavenging activity was calculated using formula:

$$\mathbf{I(\%) = \frac{(A_i - A_t)}{(A_i - A_j)} \times 100\%}$$

where A_i is the absorbance of $ABTS^{\bullet+}$ solution without sample (195 µl $ABTS^{\bullet+}$ + 5 µl DMSO); A_t is the absorbance of the test sample mixed with $ABTS^{\bullet+}$ solution (195 µl $ABTS^{\bullet+}$ + 5 µl sample) and A_j is the absorbance of the sample without $ABTS^{\bullet+}$ solution (195 µl H_2O + 5 µl DMSO). The standard curve was linear between 0.4 and 6.4 µg/ml Trolox with the regression line (y = 14.086x + 9.8824, R2 = 0.9901) The results were expressed as µg/ml of concentration Trolox equivalent (TE) per µg/ml concentration extracts of PV (TEAC).

Experimental design

A three – variable, three – level Box – Behnken design (BBD) was employed in this optimization study based on the results of preliminary experiments. Ethanol concentration (v/v, X_1), extraction temperature (°C, X_2) and extraction time (min, X_3), were the independent variables selected to be optimized for the extraction of P. *vulgaris*. The response variables were the extraction yield (Y_1), rosmarinic acid extraction rate (Y_2), ABTS (TEAC) (Y_3) and DPPH (IC_{50}) (Y_4). The coded and uncoded (actual) levels of the independent variables are given in Table 1.

Statistical Analysis and Optimization

The parameters of the response equation and analysis of variance (ANOVA) were performed by Design Expert Software (Version 11). Linear function and second order polynomial model used to fit the response to the independent variables is shown below, respectively:

$$Y = \beta_0 \sum_{i=1}^{k} \beta_i X_i + \varepsilon$$

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum\sum_{i<j}^{k} \beta_{ij} X_i X_j + \varepsilon$$

where k is the number of variables, β_0 is the constant term, β_i, β_j, β_{ij} represents the coefficients of the linear parameters, X_i, X_j represents the variables, and ε is the residual associated to the experiments.

The statistical significance for each term in the polynomial was evaluated by computing the F-value at a probability p of 0.05. The regression coefficients were then used to make statistical calculations and generate contour maps from the regression models. However, for multi – response, a desirability function approach can be used to transformed several response variables into a desirabitity function, which can be optimized by univariate techniques. A modified desirability approach, proposed by Derringer is defined as:

$$\mathbf{D} = (\mathbf{d_1 d_2 ... d_k})^{1/k}$$

where d_k is an individual desirability function for each of the k responses and D is the overall desirability. Then, the optimal setting is determined by the following, which is described previously.

$$d_k = \begin{cases} 0 & Y_k \le Y_{k-min} \\ \left[\dfrac{Y_k - Y_{k-min}}{Y_{k-max} - Y_k}\right]^r & Y_{k-min} < Y_k < Y_{k-max} \\ 1 & Y_k \ge Y_{k-max} \end{cases}$$

where Y_k is the response value, $Y_{k\text{-min}}$ is the minimum acceptable value for response k, $Y_{k\text{-max}}$ is the maximum acceptable value for response k, and r is a weight used to determine scale of desirability and equals 1 in this work.

RESULTS AND DISCUSSION

Identification

In the presence of zirconium (IV) ions, rosmarinic acid gives a light – yellow colour, which comes from the complexation of acid with zirconium (IV) ions. Fig. 1 illustrates the comparison of rosmarinic acid (line 1) and rosmarinic acid – zirconium (IV) ion complex (line 2) spectrum. Rosmarinic acid has absorption band (band A occurring at 332.5 nm) as indicated in Fig. 1. After the addition of Zr^{4+} ions the absorption band A at 332.5 nm shifted to at 362 nm (band A'). The UV – Vis absorption spectrum of the complex occurred between rosmarinic acid and zirconium (IV) ions at different concentrations (62.5, 125, 250, 500, 1000 µM) are given in Fig. 2. The standard calibration curve of rosmarinic acid – Zr^{4+} that was shown in Fig. 2 was calculated from these data. The absorptivity coefficient was calculated from the regression line (y = 2.1033x + 0.1108, R^2 = 0.9966) for the rosmarinic acid – Zr^{4+} complex.

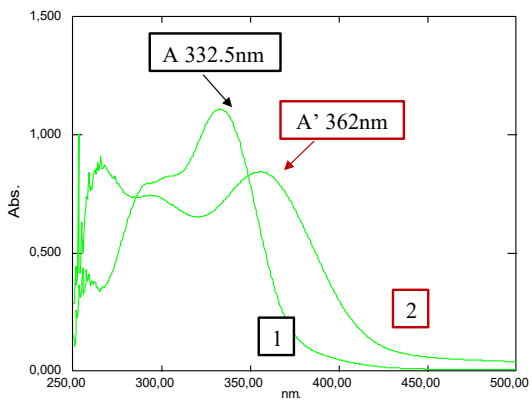

Figure 1. Rosmarinic acid (line 1) and rosmarinic acid – zirconium (IV) ion complex (line 2) spectrum

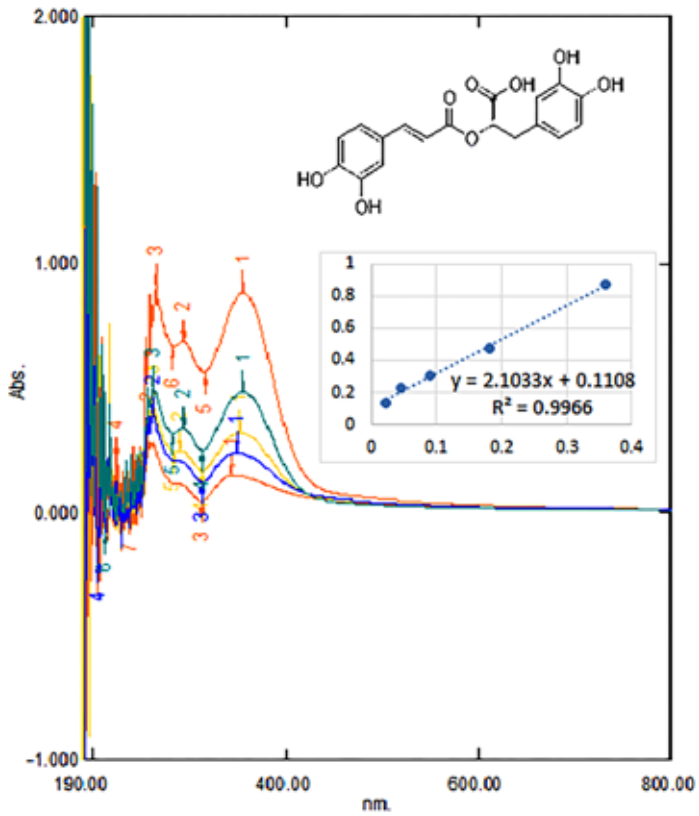

Figure 2. The calibration curve and UV – vis spectra of standard RA in ethanol with different concentrations (62.5 – 1000 μM)

Statistical Analysis and Model Fitting

The operational parameters were optimized using Box – Behnken design combined with response surface methodology.

Table 1. Box–Behnken design and observed responses

Run	X_1 (%)	X_2 (°C)	X_3 (min)	Yield (%)	Rosmarinic acid extraction rate (%)	DPPH (IC_{50}) (μg/ml)	ABTS (TEACg/g)
1	60	40	90	4.81	0.04	66.17	0.28
2	60	60	90	7.61	0.29	53.76	0.35
3	80	40	60	3.45	0.08	84.87	0.20
4	80	50	90	3.86	0.03	89.04	0.16
5	60	50	60	5.63	0.10	78.53	0.19
6	80	60	60	3.97	0.02	57.09	0.22
7	40	50	30	7.07	0.05	71.67	0.24

8	40	60	60	8.40	0.09	51.80	0.32
9	80	50	30	3.23	0.04	84.16	0.20
10	60	60	30	5.53	0.17	87.02	0.20
11	40	40	60	6.77	0.13	62.11	0.32
12	60	40	30	4.27	0.11	66.44	0.23
13	40	50	90	7.94	0.09	34.28	0.40
14	60	50	60	6.27	0.11	77.93	0.19
15	60	50	60	6.31	0.11	79.13	0.19

Table 2. Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of extraction parameters

Source		Model	Lack of Fit	Pure Error	R^2	C.V%	Adjusted R^2
Yield (%)	SS	37.63	2.16	0.2926	0.9389	8.31	0.9223
	df	3	9	2			
	MS	12.54	0.2394	0.1463			
	F-value	56.38	1.64				
	p-value	< 0.0001	0.4361				
RA(%)	SS	0.0632		0.0001	0.9992	5.23	0.9942
	df	12		2			
	MS	0.00053		0.0000			
	F-value	202.67					
	p-value	0.0049					
DPPH (IC_{50})	SS	3419.51		0.7200	0.9998	0.8621	0.9985
	df	12		2			
	MS	284.96		0.3600			
	F-value	791.55					
	p-value	0.0013					
ABTS (TEAC)	SS	0.0706	0.0013	0.0000	0.9815	6.65	0.9482
	df	9	3	2			
	MS	0.0078	0.0004	0.0000			
	F-value	29.47					
	p-value	0.0008					

The extraction yield PV

As seen in Table 1, the results showed that the extraction yield (Y_1) ranged from 3.23% to 8.4%. A regression analysis (Table 2) was carried out to fit mathematical models to the experimental data aiming at an optimal region for the responses studied. Predicted response Y for the extraction yield could be expressed by the following first degree equation in terms of coded values:

$$\mathbf{Y_1 = 5.67 - 1.96X_1 + 0.7772X_2 + 0.5166X_3}$$

The analysis of variance (ANOVA), goodness-of-fit and the adequacy of the regression model were summarized in Table 2. The high model F-value and the low P-value indicates the level of confidence of the selected model. A good model performance with the correlation coefficient R^2 = 0.9389 and the relationship extraction parameters such as ethanol concentration, temperature and time. The reliability of the model from the variance analysis and the related coeficient examined: the p-value < 0.0001 for yield suggests that was highly statistically significant. The value of R^2 reflects the proportion of variation in the response attributed to the model rather than to random error. The regression coefficient R^2 of the extraction yield was 0.9389 > 0.75, the model was compatible with the experiment, saying 93.89% of the change in extraction yield was due to the influence of extraction parameters such as ethanol concentration, temperature and time; only 6.11% of the variation was due to unidentified factors (random errors). The linear coefficients (X_1, X_2, X_3) were all significantly correlated with the extraction yield ($p < 0.01$).

Response surfaces were plotted by the Design Expert software to explain the interactions of the variables for the maximum response. The corresponding three-dimensional response surfaces are shown in Figure 3. Each figure shows the effects of two factors at a time on the extraction yield while all other factors were kept at average level. The extraction yield was very low at low extraction temperature and time, and increased as extraction time and extraction temperature increased. Ethanol concentration was opposited, the extraction yield was decreased when ethanol concentration was increased.

The rosmarinic acid extraction rate (RA%)

As seen in Table 1, he rosmarinic acid extraction rate (Y_2) ranged from 0.02% to 0.29%. The data were analyzed by multiple regression analysis to get the following second-order polynomial equation:

$$\mathbf{Y_2 = 0.1091 - 0.0151X_1 + 0.0758X_2 + 0.0121X_3 - 0.0056X_1X_2 - 0.0113X_1X_3 + 0.0473X_2X_3 - 0.0643X_1^2 + 0.0367X_2^2 + 0.0060X_3^2 - 0.1032X_1^2X_2 - 0.0030X_1^2X_3 - 0.0160X_1X_2^2}$$

ANOVA results of the quadratic model presented in Table 2 show The high model F-value (202.67), p-value < 0.05 and a high R^2 of 0.9992 that the model can adequately describe the response surface of RA%. It can be seen from Table 2 that all the linear coefficients ((X_1, X_2, X_3), quadratic term coefficients (X_1^2, X_2^2) and cross product coefficients (X_1X_3, X_2X_3) were significant model terms, with p-value < 0.05.

According to Figure 3, RA% was low at low ethanol concentration, and increased as ethanol concentration increased until a peak value was reached, further increasing the ethanol concentration led to a decreased RA%. RA% increased as the extraction time and extraction temperature increased.

DPPH radical-scavenging assay

As seen in Table 1, IC_{50} ranged from 34.28 to 89.04 µg/ml. The following second-order polynomial equation:

$$Y_3 = 78.53 + 16.81\,X_1 + 2.04X_2 - 8.38X_3 - 4.37X_1X_2 + 10.57X_1X_3 - 8.25X_2X_3 - 6.56X_1^2 - 8.00X_2^2 - 2.18X_3^2 - 11.56\,X_1^2X_2 + 0.2549X_1^2X_3 - 9.80X_1X_2^2$$

ANOVA results of the quadratic model presented in Table 3 show a high R^2 of 0.9998 and a low C.V. value of 0.8621%, demonstrating that the model can adequately describe the response surface of DPPH scavenging percentage. The high model F-value (791.55) and low p-value ($p = 0.0013$) suggested the results were highly statistically significant and had a good fit of the model. It can be seen from Table 3 that all the linear coefficients (X_1, X_2, X_3), quadratic term coefficients (X_1^2, X_2^2, X_3^2) and cross product coefficients (X_{12}, X_{13}, X_{23}) were significant model terms, with p-value < 0.05. Moreover, the coefficient of $X_1^2X_3$ was found non-significant ($p > 0.05$).

According to Figure 3, IC_{50} increased as ethanol concentration and extraction temperature increased until a peak value was reached, further increasing the ethanol concentration and extraction temperature led to a decreased IC_{50}. Similar trends were observed for the effects of extraction temperature and extraction time.

ABTS radical-scavenging assay

The following second-order polynomial equation:

$$Y_4 = 0.1906 - 0.0630X_1 + 0.0086X_2 + 0.0420X_3 + 0.0042X_1X_2 - 0.0489X_1X_3 + 0.0241X_2X_3 + 0.0293X_1^2 + 0.0459X_2^2 + 0.0275X_3^2$$

The model-value of 29.47 and the associated lower p-value ($p = 0.0008$) implied the model was highly statistically significant. The value of R^2 reflects the proportion of variation in the response attributed to the model rather than to random error. The model has shown a good fit with the high R^2 value and adjusted determination coefficient (R^2_{adj}) of 0.9815 and 0.9482, respectively. The results indicated that the linear coefficients (X_1, X_3), quadratic term coefficients (X_2^2) and cross product coefficients (X_1X_3, X_2X_3) were all significantly correlated ($p < 0.01$).

According to Figure 3, TEAC increased as ethanol concentration and extraction time increased. TEAC decreased as extraction temperature increased until a mininum value was reached, further increasing the extraction temperature led to an increased TEAC.

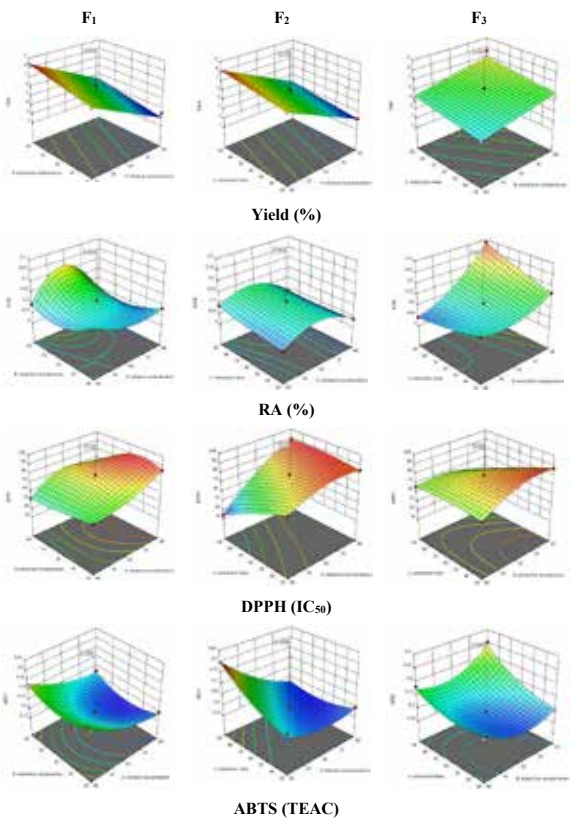

Figure 3. Response surface (3D) showing the effect of extraction parameters on the response variables: (F_1) ethanol concentration and temperature; (F_2) ethanol concentration and extraction time; (F_3) extraction temperature and time

Optimization Analysis

We take the maximum value and the minimum were showed in table 3.

Table 3. The maximum value and the minimum of the response variables

	the maximum value	the minimum value
Yield (%)	8.4	3.23
RA extraction rate (%)	0.29	0.02
DPPH (IC50) (µg/ml)	89.04	34.28
ABTS (TEACg/g)	0.4	0.16

A one-sided transform of yield (d_1), rosmarinic acid extraction rate (d_2), DPPH scavenging percentage (IC_{50}) (d_3), ABTS (TEAC) (d_4) were obtained as follows:

$$d_1 = \begin{cases} 0 & Y_k \le 3.23 \\ \left[\frac{Y_k - 3.23}{8.40 - Y_k}\right] & 3.23 < Y_k < 8.40 \\ 1 & Y_k \ge 8.40 \end{cases}$$

$$d_2 = \begin{cases} 0 & Y_k \le 0.02 \\ \left[\frac{Y_k - 0.02}{0.29 - Y_k}\right] & 0.02 < Y_k < 0.29 \\ 1 & Y_k \ge 0.29 \end{cases}$$

$$d_3 = \begin{cases} 0 & Y_k \le 34.28 \\ \left[\frac{Y_k - 34.28}{89.04 - Y_k}\right] & 34.28 < Y_k < 89.04 \\ 1 & Y_k \ge 89.04 \end{cases}$$

$$d_4 = \begin{cases} 0 & Y_k \le 0.16 \\ \left[\frac{Y_k - 0.16}{0.4 - Y_k}\right] & 0.16 < Y_k < 0.4 \\ 1 & Y_k \ge 0.4 \end{cases}$$

The overall desirability *D* is calculated as:

$$D = \sqrt[4]{d_1 d_2 d_3 d_4}$$

By using *D* as the new response, the optimum values of selected variables can be obtained through regression analysis. In this study, the optimal conditions for highest *D* (with a *D* value of 0.251) were: extraction time of 60 min, extraction temperature of 50°C and ethanol concentration of 60%. The corresponding maximum yield, rosmarinic acid extraction rate, DPPH (IC_{50}) and ABTS (TEAC) were 5.674%, 0.109%, 78.526 µg/ml and 0.191 respectively.

Verification of the Predictive Model

To confirm the suitability of the model equation, confirmation experiments were conducted under the optimized conditions as follows: extraction time of 60 min, extraction temperature of 50°C and ethanol concentration of 60%. Under these conditions, the experimental yield of RAMP was 6.025%, rosmarinic acid extraction rate was 0.115, DPPH (IC_{50}) was 84.975 µg/ml and ABTS (TEAC) was 0.207, which matched well with the predicted values of 5.675%; 0.109%; 78.526 µg/ml; 0.191, respectively. This confirmed that the model was adequate for optimization of the extraction PV. As a result, RSM coupled with D approach was considered to be an accurate and decisive tool for predicting the maximum extraction yield, rosmarinic acid extraction rate, and highest antioxidant activity of extract of PV.

CONCLUSIONS

Process extraction has been optimized for effective extraction of PV with high antioxidant activity. The maximum D value of 0.251, along with the maximum yield (6.025%), %RA (0.115%), scavenging percentage DPPH (IC_{50}) (84.975 µg/ml) and ABTS (TEAC) (0.207) were achieved after an ethanol concentration of 60°, an extraction time of 60 min, using an extraction temperature of 50°C. These values were further validated by confirmatory experiments to see the efficacy of the model predictability and found to be in good agreement with the predicted values. Compared to other extraction methods, both the extraction yield and antioxidant activity obtained was favorable and the method appeared to be time-saving and of high efficiency. These results demostrated that is an appropriate and effective extraction technique for PV.

LEGENDS TO TABLE AND FIGURES

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