Chapter

Optimization of the Extraction of Polyphenols and Antioxidant Capacity from *Byrsonima crassifolia* (L.) Kunth Fruit by Response Surface Methodology

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Abstract

The purpose of this research was to optimize the extraction conditions of polyphenols from murici (*Byrsonima crassifolia* (L.) Kunth) using the response surface methodology. Temperature (from 10 to 70°C), acetone concentration (from 10 to 100%), extraction time (from 0 to 160 min), and solid-liquid ratio (from 20 to 140 mg/mL) were investigated as independent variables in order to obtain the optimal conditions for extraction and to maximize the total phenolic content (TPC) and antioxidant activity (DPPH) of obtained extracts. Experimental results were fitted to the second-order polynomial model where multiple regression and analysis of variance were used to determine the fitness of the model and optimal condition for investigated responses. The solid-liquid did not interfere in the two responses. The results showed that for TPC extraction, the optimal conditions were obtained with an acetone concentration of 44%, a temperature of 29°C, and an extraction time of 51 min. For DPPH, the optimal conditions were the following: an acetone concentration of 45%, a temperature of 40°C, and an extraction time of 53 min. The use of such conditions allowed the maximum extraction of antioxidant murici at a lower cost of production, which may contribute to large-scale industrial applications and future pharmacological research.

Keywords: murici, total phenols, antioxidant activity, response surface methodology

1. Introduction

Byrsonima crassifolia (L.) Kunth, murici, is a species of fruit trees belonging to Malpighiaceae family, *Byrsonima* genus, which includes more than 150 species [1]. Generally, it occurs in the Amazon region and in the Brazilian Northeast [2].

Byrsonima crassifolia fruits are good sources of dietary fiber [3], vitamin C (84 mg 100 g^{-1}) [1], phenolic compounds, and carotenoids [4]. The main phenolics contained in murici include gallic acid and quercetin [5], and the main carotenoids,

lutein and zeaxanthin [6]. In addition, there are reports of trace quantities of catechin, epicatechin, rutin, and kaempferol [7].

Some investigations have demonstrated the potential antioxidant effect of murici leaves, fruits, and seeds *in vitro* [5, 7, 8] and *in vivo* [9, 10]. The extracts can also be used as an alternative for the treatment of diabetes, reducing blood glucose, cholesterol, and triglycerides [11].

On the other hand, research suggests that the increased consumption of foods rich in antioxidants compounds, as polyphenols, is associated with a reduction of chronic disease risk [12, 13]. Polyphenols are an important group of phytochemicals originated from the secondary metabolism of plants. They are involved in plant growth and reproduction, pigmentation, and as agents that offer resistance to pathogens and stress conditions [14]. Moreover, the use of natural antioxidants, as polyphenols, has also been increased in the food and pharmaceutical industry. This increase is justified because the food industry has sought to replace synthetic antioxidants since that some of them present toxic activity [15].

However, there is no standard and universally accepted method for extracting antioxidant compounds. The methodological problems are due to great diversity of compounds, which give them different physicochemical properties, and their high susceptibility to oxidation [16]. This challenge is even greater when one considers the polymerization patterns of polyphenols and the food matrix in which they are inserted [17].

Many methods concentrate on the extraction of polyphenols since they are substances abundant in foods and effective antioxidants. Usually, these methods are based on the use of solvents with different polarities and in the study of the binomial time and temperature. The final yield of the extraction will depend on the solubility of the phenolics in the solvent used, the degree of polymerization, and its interactions with other constituents of food [18]. Free polyphenols can be extracted with water or with polar solvents such as methanol and acetone. However, some polymers may require the use of acids or enzymes, such as glycosidase [17]. Moreover, the use of long times and very high temperatures interferes negatively in the yield of the polyphenols [19]. The opposite may occur, depending on the polyphenol type and the matrix studied.

In this perspective, the use of response surface methodology becomes a useful and valuable tool in the field of antioxidant extraction. The factorial planning allows the study of the interaction of the various factors that interfere in the extraction of the polyphenols, minimizing costs and maximizing extraction yield. The advantages still include reducing of the number of experiments/replicates, simultaneous analysis of results, optimization of more than one response, and calculation of experimental error [20].

Thus, the present study was designed to determine the optimum condition for maximizing polyphenolic antioxidant extraction from fruits of *Byrsonima crassifolia* (L.) Kunth). As far as we know, this is the first investigation concerning the effects of temperature, time, solvent, and solid-liquid ratio on extraction of murici antioxidants.

2. Material and methods

2.1 Plant materials

A total of 10 kg of fresh fruits of *Byrsonima crassifolia* (L.) Kunth were purchased in a single batch from a rural producer in Araioses, Maranhão, Brazil. We used 10 kg of starting material to obtain representative amounts of fresh fruits. Since 50% of

the fruits are composed by pits, the final weight of murici was 5 kg. The fruits were selected, washed, and kept frozen at −20°C until analysis.

2.2 Extraction procedures

For preparation of the extracts, the murici was weighed and homogenized with the solvent in turrax for 1 minute. The mixture was then subjected to magnetic stirring for 1 hour at 25°C. Subsequently, the blend was centrifuged at 15,000 G for 15 minutes at 4°C. The supernatant was collected and the volume completed to 50 mL.

2.3 Experimental design

The choice of solvent was the first step in the extraction of antioxidants. The most commonly used solvents are methanol, acetone, and mixtures thereof with water. So, initially, pretests were performed with the water and solvent blends: ethanol: water (60:40), methanol: water (60:40), and acetone: water (60:40). These experiments were carried out in order to verify the mixture with greater capacity for extraction of antioxidant compounds and help to decide which solvent should be used to optimize the extraction process.

In this pilot test, acetone in water (60%) provided the best extracting of antioxidants from murici (27.94 ± 0.28 mg polyphenols/100 mg extract and $IC50_{DPPH}$ 169.39 ± 10.62 μg/mL) and, thus, it was chosen as solvent in this study, while distilled water was the most inefficient solvent in the extraction of these compounds in murici (8.79 ± 0.09 mg polyphenols/100 mg extract and $IC50_{DPPH}$ 496.27 ± 8.53 μg/mL).

Response surface methodology (RSM) was employed to determine the optimum levels of the four in dependent variables (X1, solid-liquid ratio; X2, extraction time; X3, extraction temperature; and X4, solvent concentration), and five levels were used to evaluate the optimum combinations for antioxidant extraction. For the optimization of extraction, parameter designs consisting of 27 experiments, including three replicates in a central point, were used by the authors of [20]. The dependent variables studied were total phenolic content (TPC) and DPPH radical scavenging activity. The experimental runs for RSM were shown in **Table 1**. The variation ranges for the independent variables were established based on data from the literature and previous tests.

Independent variable	**Factor levels**				
	−2	**−1**	**0**	**+1**	**+2**
Solid-liquid ratio (mg/mL)	—	20	60	100	140
Time (minutes)	0	40	80	120	160
Temperature (°C)	10	25	40	55	70
Independent variable	**Factor levels**				
	−1.33	**−1**	**0**	**+1**	**1.66**
Solvent % (acetone in water)	10	20	50	80	100

Table 1.
Experimental domain of RSM.

2.4 Determination of total phenolic content (TPC)

The total phenolic compound (TPC) measurements in extracts were adapted from Singleton and Rossi [21] using gallic acid as a standard. Absorbance was measured at 750 nm. Results were expressed as mg equivalent gallic acid (GAE) per 100 mg of murici's dry weight (DW) (mg GAE/100 mg DW). All experiments were performed in three replicates.

2.5 DPPH radical scavenging activity

The extract (20 μL) was incubated with 150 μmol/L DPPH (200 μL) in 95% ethanol. Then, it was mixed and kept at room temperature in the dark for 30 min. Finally, the absorbance was measured at 515 nm using a microplate reader. The blank of each concentration was performed with ethanol instead of DPPH solution. The standard curve was a logarithm between 2 and 25 mg/mL gallic acid [22].

The % inhibition was calculated as described by the equation below. The antioxidant activity was expressed as IC50 (required extract concentration to reduce 50% of DPPH).

$$\%\ Inhibition = \frac{Absorbance\ Control - Absorbance\ Sample}{Absorbance\ Control} \times 100 \qquad (1)$$

2.6 Statistical analysis

Statistical analysis was performed using the Statistica Software v.11 and fitted to a second-order polynomial regression model. An analysis of variance (ANOVA) with 95% confidence level was carried. The coefficient of determination (R^2) was used to estimate the fitness of the polynomial equation to the responses. The experimental and predicted values were compared in order to determine the validity of the model.

3. Results and discussion

The selection of solvent is as crucial stage when antioxidants extractions are concerned [23]. In order to evaluate the better solvent for the extraction of murici antioxidants, in terms of TPC and antioxidant activity (DPPH), a preliminary experiment was performed using ethanol, acetone, methanol, and water.

With the polarity change of the solvent, the amount of dissolved antioxidant compound varied. Solvents with low viscosity have low density and high diffusivity, which allows them to easily diffuse into the pores of vegetable materials, and consequently, extract the bioactive compounds [24]. As the result of that, the antioxidant activity of the extract also tends to increase, as observed in this study with the acetone. Hence, this solvent was deemed the better option in this research.

These results match with the results previously described where the mixture water:acetone is the more efficient at the extraction of phenolic antioxidants of oat grains [25] and *Etlingera elatior* Jack flowers [24], respectively. In addition, this mixture is also recommend as the best system for the extraction of polyphenols [26, 27].

In this study, RSM was employed to determine the optimum conditions for the extraction of antioxidant from fruits of *Byrsonima crassifolia* (L) Kunth (murici). In order to optimize the extraction parameters (extraction time, temperature, acetone concentration, and solid-liquid ratio), a five-level, four-factor experimental design

was performed. The experimental conditions and results of 27 runs are presented in **Table 2**.

Original variables					TPC	IC_{50}
Test*	S/S	T (°C)	Time (min)	Acet. (%)		
1	20	25	40	20	24.57	146.12
2	100	25	40	20	24.38	144.06
3	20	55	40	20	23.90	162.26
4	100	55	40	20	23.55	165.41
5	20	25	120	20	23.15	152.07
6	100	25	120	20	23.27	157.85
7	20	55	120	20	20.79	174.72
8	100	55	120	20	20.86	179.86
9	20	25	40	80	21.39	160.05
10	100	25	40	80	21.73	162.49
11	20	55	40	80	17.50	188.85
12	100	55	40	80	18.06	184.94
13	20	25	120	80	19.84	163.47
14	100	25	120	80	20.07	169.16
15	20	55	120	80	12.83	205.09
16	100	55	120	80	13.21	203.07
17	60	40	80	50	27.03	132.73
18	60	40	80	50	27.52	128.68
19	60	40	80	50	26.58	137.29
20	20	40	80	50	27.80	129.90
21	140	40	80	50	24.51	142.48
22	60	10	80	50	23.73	167.38
23	60	70	80	50	20.81	192.18
24	60	40	0	50	24.95	144.93
25	60	40	160	50	20.29	186.91
26	60	40	80	10	16.58	201.93
27	60	40	80	100	13.73	218.58

**Does not correspond to order of processing. TPC—total phenolic content (mg GAE/100 mg), DPPH radical scavenging activity (IC_{50}—μg/mL), S/S—solid-solvent ratio (mg/mL), T—temperature, and Acet.—acetone in water.*

Table 2.
Response sheet for RSM experimental design with process variables and experimental results for the preparation of fruits of Byrsonima crassifolia *(L.) Kunth.*

The values of the TPC ranged from 13.21 to 27.80 mg GAE/100 mg, while the values of the IC 50 ranged from 128.68 to 218.58 μg/mL. The significance and model adequacy were tested by ANOVA, and it revealed that the model was highly significant ($p < 0.05$) for TPC and DPPH. The lack of fit of each model was not significant ($p > 0.05$), indicating that the developed model adequately explains the relationship between the independent variables and responses (**Tables 3** and **4**).

The linear and quadratic effects of the acetone concentration, temperature, and time extraction demonstrated significant effects on TPC ($p < 0.01$), as well as the

Source	Sum of squares	Degrees of freedom	F_{value}	R^2
Regression	385.75	6	18.42	0.8466
Residues	69.9	20		
Lack of fit	69.46	18	17.54	
Pure error	0.44	2		
Total	455.65	26		

Table 3.
ANOVA for the regression of the second-degree polynomial for the TPC response.

Source	Sum of squares	Degrees of freedom	F_{value}	R^2
Regression	12693.05	5	15.85	0.7906
Residues	3362.56	21		
Lack of fit	3325.45	19	9.43	
Pure error	37.11	2		
Total	16055.61	26		

Table 4.
ANOVA for the regression of the second-degree polynomial for the DPPH response.

interactions' temperature/time and time/acetone % ($p < 0.05$). In the case of DPPH, the linear and quadratic effects of temperature, time, and acetone % were significant ($p < 0.05$). The solid-liquid, in the studied range, did not interfere in the two responses ($p > 0.05$). Conversely, in the study of [28], the solid-to-solvent ratio (10–30 g/mL) showed a significant effect on the antioxidant compound extraction from germinated chickpea. Probably, an increase in the solvent to sample ratio helps the solute dissolve in solvent and hence the phenolic compounds extraction rate increased with decreasing of solid-to-liquid ratio [29]. However, the extraction rate trend stabilizes when the extraction rate reached a certain value [30]. In this study, we tested low solid-to-solvent ratios (20–140 mg/mL), which seems to have already provided maximum dissolution of the antioxidant compounds.

Analysis of the model clearly shows that temperature and acetone were the most significant factors. The acetone and temperature had a negative effect (coefficient = −4.98 and −3.46, respectively), indicating that a reduction in the temperature and acetone favored the recovery of TPC in the extract. A decrease in the acetone concentration enhanced the solvent polarity, which helps TPC dissolve in solvent [29]. Likewise, the acetone and temperature had a positive effect on DPPH-IC50 (coefficient = 19.35 and 26.12, respectively). This means that the extraction of antioxidants was less favorable at high temperature and acetone because a higher IC50 value corresponds to weaker antioxidant activity of tested extract. These results are similar to those found for [31] for mulberry (*Morus nigra*) pulp. On the other hand, high ethanol concentration and high temperature were the most effective factors for increasing TPC and antioxidative activities in mango peels [30].

As shown in **Figure 1**, the TPC increases rapidly as a result of acetone concentration, time and the temperature increase in the range of 10 and 45%, 40–50 min and 10 and 30°C, respectively. However, beyond acetone concentration of 50%, 55 min, and 35°C, the TPC decreases. Generally, temperature ranging from 60 to 70°C might increase the TPC yield due to higher solubility and diffusion in the solvent [30]. However, these temperatures are not recommended for polyphenol extraction from murici. It might be possible that higher temperatures for extended extraction caused the degradation of the some thermosensitive phenolic compounds

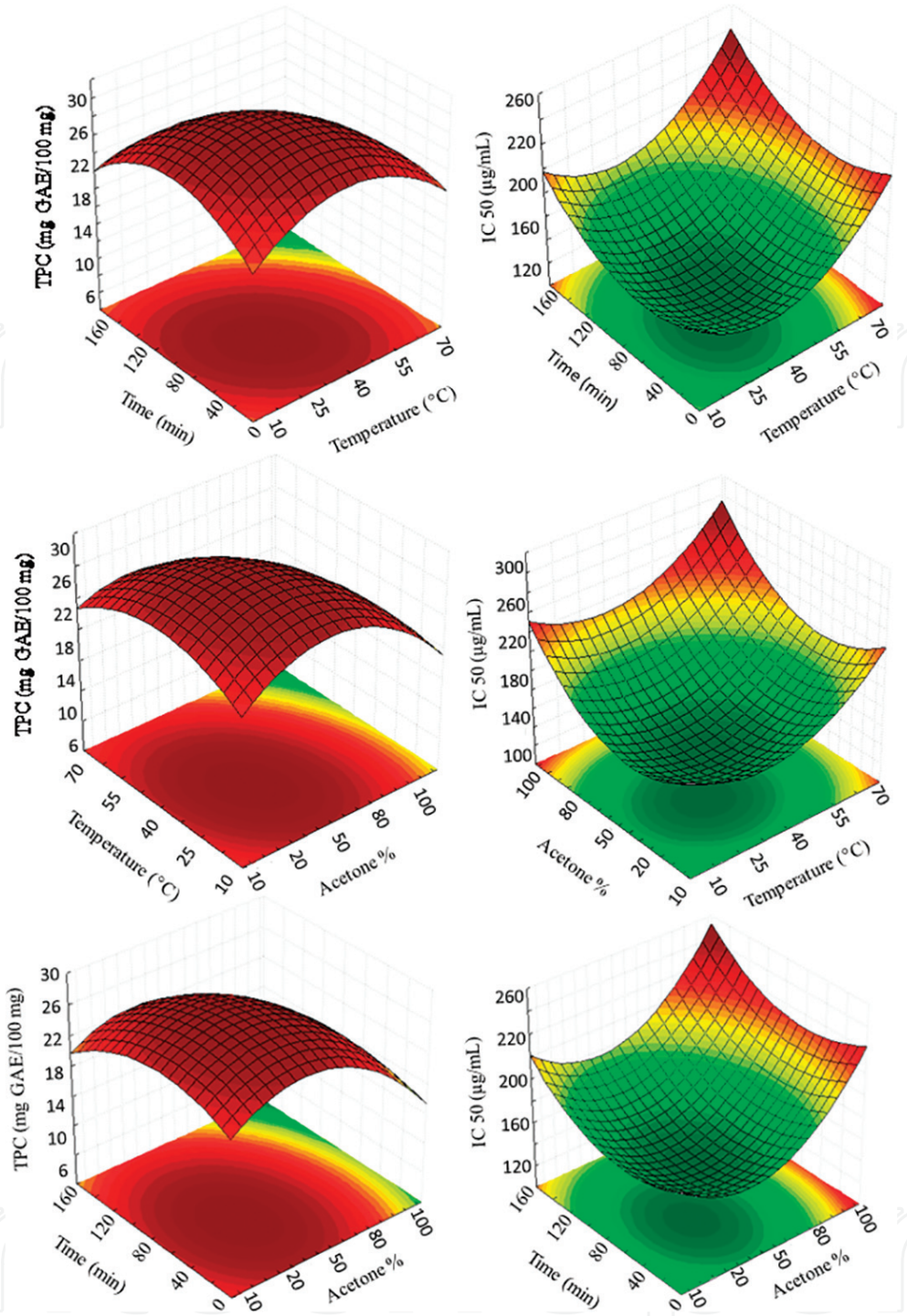

Figure 1.
Response surface plots for the effect of independent variables (stepping time, temperature, and acetone %) on total phenolic content (TPC) and total DPPH radical scavenging activity (IC50 - µg/mL) of fruits from Byrsonima crassifolia *(L.) Kunth.*

presented in murici [31, 15]. Similarly, the rise in acetone concentration, time, and the temperature decreased the antioxidant activity of murici after DPPH scavenging measurements. This result is in agreement with the findings to *Lycium ruthenicum* Murr. fruit [29] where lower solvent concentration was found to be conducive to total antioxidant activity.

The determined coefficient for TPC showed a good regression value (R^2 = 0.84), and the relationship between the TPC and the extraction parameters of the acetone concentration, the temperature, and the extraction time. By applying multiple regression analysis on the experimental data, the model for the response variable could be expressed by the following quadratic polynomial equation in the form of

	TPC (mg GAE/100 mg)	DPPH (IC_{50} - μg/mL)
Predicted value	27.61	137.65
Experimental value	28.04 ± 1.14*	139.00 ± 9.89*

Mean ± standard deviation (n = 3 replicates).

Table 5.
Predicted and experimental values under optimum conditions based on the multiple responses of TPC and DPPH.

coded values: $-\hat{y} = 26.42–1.40X_2–0.95X_2^2–1.27X_3–0.86X_3^2–1.87X_4–4.23X_4^2$, where X_2 = temperature, X_3 = time, and X_4 = acetone %.

The determined coefficient for DPPH also showed a reasonable regression value ($R^2 = 0.84$). The model for the response variable could be expressed by the following quadratic polynomial equation in the form of coded values:
$-\hat{y} = 141.9 + 10.77X_2 + 7.88X_2^2 + 7.29X_3 + 7.32X_4 + 23.35X_4^2$, where X_2 = temperature, X_3 = time, and X_4 = acetone %.

Based on the experimental results and statistical analysis, numerical optimizations were performed in order to determine the optimum level of independent variables. In our work, the optimum conditions for TPC compounds were as follows: extraction solvent using 44% acetone in water, an extraction temperature of 29°C, and an extraction time of 51 minutes. The optimum conditions for antioxidant activity (DPPH) were as follows: extraction solvent with 45% acetone in water, an extraction temperature of 40°C, and an extraction time of 53 minutes. These results suggest that the optimum parameters of extraction of murici's antioxidants occur with moderate percentages of acetone in water. The optimum temperature for antioxidants extraction evaluated by DPPH was in the middle range, and it was slightly higher for total phenolic compounds.

In order to verify the suitability of the response surface methodology model for quantitative predictions, experiments on estimated optimal conditions were performed. **Table 5** shows the predicted and experimental results for the variables selected. The predicted results matched well with the experimental results obtained at optimal extraction conditions proving the validity of the model to describe the process. These findings justified the selection of the RSM design, which was demonstrated to be accurate and reliable for predicting the TPC and antioxidant capacity of extracts from murici.

4. Conclusion

In this study, the RSM was successfully used to determine optimal levels of experimental parameters for the extraction of murici antioxidants. It is possible to verify that temperature, time, and acetone concentration negatively interfere in the extraction of polyphenols and in the antioxidant activity of fruits of *Byrsonima crassifolia* (L.) Kunth. The efficiency of the extraction can significantly be improved by using the novel extraction techniques, which may contribute to large-scale industrial applications and future pharmacological research.

Conflict of interest

All other authors report no potential conflict of interests.

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