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Optimization of the Extraction of Antioxidant Compounds from Roselle Hibiscus Calyxes (*Hibiscus sabdariffa*), as a Source of Nutraceutical Beverages

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Optimization of the Extraction of Antioxidant Compounds from Roselle Hibiscus Calyxes (*Hibiscus sabdariffa*), as a Source of Nutraceutical Beverages

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Abstract: Secondary metabolites from *Hibiscus sabdariffa* have been used to prevent different diseases. Roselle Hibiscus is known for being rich in phenolic bioactive compounds. The extraction conditions are directly related to the chemical composition and then to the overall bioactivity of the extract. In this study, a Box Behnken experimental design has been used to optimize the antioxidant activity, considering four variables: the ethanol:water ratio, the temperature, the extraction time, and the solvent:solid ratio. The experiment comprises 27 experiments and 3 repetitions at the central point. The results are described by surface response analysis and a second-degree polynomial equation. The model explains 87% of the variation in the response. The maximum antioxidant activity is yielded when 1% solids are extracted in 35.5% ethanol at 60°C for 33 min. Finally, a nutritional functional supplement of 495 µmol Trolox Equivalent (TE) antioxidant capacity was prepared with the optimized extract.

Keywords: *Hibiscus sabdariffa*; antioxidants; nutraceuticals; phenolic compounds; functional foods

1. Introduction

Oxidative stress of cells and tissues causes aging in human beings. Several oxidizing agents are produced by endogenous and exogenous processes. Those agents are called Reactive Oxygen Species (ROS). Oxidizing stress and aging are related to several diseases, such as diabetes, cancer, cardiovascular illnesses, and others [1]. The antioxidant compounds eliminate the free radicals of ROS, through hydrogen atom transfer (HAT), single electron transfer (SET), or chelation using transition metals [2]. Those compounds can be sorted in two classes: radicals and non-radicals.

Phenolic compounds, carotenoids, and vitamins are some metabolites from fruits and vegetables responsible for antioxidant capacity. [3]. Roselle Hibiscus is a tropical plant also known as rozelle, sorrel, red sorrel, Jamaican sorrel, Indian sorrel, Guinea sorrel, sour-sour, queensland jelly plant, jelly okra, lemon bush, and Florida cranberry [4]. Water-based roselle calyxes extracts are used worldwide to prepare beverages of good taste and antioxidant properties [5]. The antioxidant properties are related to the content of organic acids, flavonoids, and phenolic acids [6–8]. Roselle extracts show functional properties and are interesting for nutraceutical product development. Those bioactive products can reduce the risk for some illnesses and improve some organ functions and overall health. Roselle is cytocompatible and it can even replace dyes for histological staining [9].

Previous studies have evaluated different solvents [10], including water [11]. Also, different solid/solvent ratios are reported from 1-10 [12,13] to 100 or greater [14]. Most previous articles had focused on extraction time and stability of molecules [15]anthocyanin yield [16], or some specific bioactivities such as enzymatic inhibition [17] In this work we are optimizing the antioxidant

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capacity. A Box-Behnken Design is used for such a purpose. This information is useful to reduce cost and time, and to obtain more functional foods. This work aims to optimize the antioxidant activity of extracts from *H. sabdariffa* calyxes in order to produce nutraceutical products.

2. Results and discussion

Solid-liquid extraction is a separation process used for transferring solutes from a solid matrix to a solvent. This technique is used to obtain bioactive compounds from plants. The efficiency of solid-liquid extraction is related to many factors such as temperature, solvent composition, stirring speed, solid-liquid rate, time, particle size, pH, and others. Four variables shown in *Table 1* were selected based on a bibliographic review [18–22].

Table 1. Data matrix of Box-Behnken Design for extraction of antioxidants from hibiscus calyxes.

Experiment number	X_1	X_2	X_3	X_4	Antioxidant capacity (µmol TE/ g DM ±SD)	Adjust
1	-1	-1	0	0	68.24±2.46	71.97
2	1	-1	0	0	20.05±1.72	19.37
3	-1	1	0	0	72.45±1.73	75.34
4	1	1	0	0	53.22±3.55	52.13
5	0	0	-1	-1	95.08±13.37	97.17
6	0	0	1	-1	102.46±1.58	97.17
7	0	0	-1	1	65.14±0.17	65.91
8	0	0	1	1	65.60±2.03	65.91
9	0	0	0	0	106.35±25.55	103.61
10	-1	0	0	-1	114.10±19.46	99.49
11	1	0	0	-1	66.33±1.36	60.76
12	-1	0	0	1	58.47±2.46	68.23
13	1	0	0	1	27.00±3.68	29.50
14	0	-1	-1	0	73.72±0.88	76.51
15	0	1	-1	0	99.23±4.84	95.34
16	0	-1	1	0	75.61±6.94	76.51
17	0	1	1	0	96.10±3.98	95.34
18	0	0	0	0	101.81±10.57	110.69
19	0	-1	0	-1	81.44±3.66	76.45
20	0	1	0	-1	31.96±2.72	37.72
21	0	-1	0	1	73.23±5.17	76.45
22	0	1	0	1	38.62±0.51	37.72
23	-1	0	-1	0	74.69±2.91	80.24
24	1	0	-1	0	93.61±2.41	99.08
25	-1	0	1	0	61.27±2.46	48.98
26	1	0	1	0	70.43±1.72	67.82
27	0	0	0	0	100.23±1.73	101,04

BBD was utilized to find the best extraction conditions for hibiscus roselle, in order to optimize the antioxidant capacity. The experimental values obtained in this work were used to obtain the second-order empirical coefficients for each variable. Only significant coefficients (P-value <0.05) for both the variables and the interactions were included in the model. Eq 1 describes the overall polynomial model explaining the antioxidant capacity in terms of ethanol:water, temperature (°C), time (min), and Solid/Solvent ratio, described as X_1, X_2, X_3, and X_4, respectively.

$$\mu\text{mol TE/gDM} = 94.72 - 18.95\,X_1 + 9.03\,X_2 - 15.63\,X_4 - 35.16\,X_1X_1 - 15.43\,X_2X_2 - 10.00\,X_3X_3 + 7.35\,X_1X_2 \quad \text{(Eq1)}$$

Results of ANOVA test can be found in *Table 2* P-value, and F-value for the regression (Eq. 1) were < 0,0001 and 36,190, respectively.

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Table 2. Estimated regression coefficients, model adequacy checking, and ANOVA analysis of the model.

Source	DF	Sc Adjust	MC Adjust	F-value	P-value
Model	9	15361.9	1706.9	36.2	<0.0001
X_1	1	4291.9	4291.9	91.0	<0.0001
X_2	1	975.3	975.3	20.7	<0.0001
X_4	1	3719.4	3719.4	78.9	<0.0001
X_1X_1	1	7174.4	7174.4	152.1	<0.0001
X_2X_2	1	1381.3	1381.3	29.3	<0.0001
X_3X_3	1	574.6	574.5	12.2	0.003
X_1X_2	1	216.7	216.7	4.6	0.047
Lack of fit	16	1400.0	87.5	8.7	0.108
		R-Squared	0.9504		
		Adjusted R-Squared	0.9241		
		Predicted R-Squared	0.8747		

DF: degrees of freedom.

Analysis of variance was realized for the adjusted model for the antioxidant capacity of Hibiscus extracts. The determination coefficient (R^2) of the model is 0.940. The R^2 value confirms the regression model explains well the actual behavior of the system [23]. The adjusted R^2 is 0.9241. Although it is smaller than the regular determination coefficient, both values are very close to each other. It means the values predicted by the model are a good representation of the experimental results [24].

A lack-of-fit test showed a P-value of 0.108. It is higher than 0.05, and it means there is no evidence of a lack of fit. The model is an appropriate representation of the relationship between the experimental factors and the response variable [23].

The regression model predicts the effect of the four variables on the antioxidant capacity after the extraction process. The relationship between dependent and independent variables is illustrated through the surface 3D graphs generated from the model (*Figure 1*). The optimal points from the 3D graphs are the highest antioxidant capacity from the subset of conditions considered within the graph.

2.1. Effect of the ethanol content on the antioxidant capacity

(*Figures 1(A), 1(B), &12(C)*) show the effect of increasing the ethanol content of the solvent on the extraction, respecting the solid:solvent ratio, the temperature, and the time, respectively. For either (*Figure 1*(A), 1(B), or 1(C)), the extraction efficiency of antioxidants starts growing when the ethanol concentration in the solvent increases from 0 to 34.5% ethanol. However, the antioxidant extraction decreases its efficiency when the ethanol concentration increases by more than 34.5% in the extraction solvent. When the ethanol:water ratio is fixed at 34.5:65.5, the maximum antioxidant capacity is reached at a low solid solvent ratio, intermediate values of temperature, and intermediate values of time.

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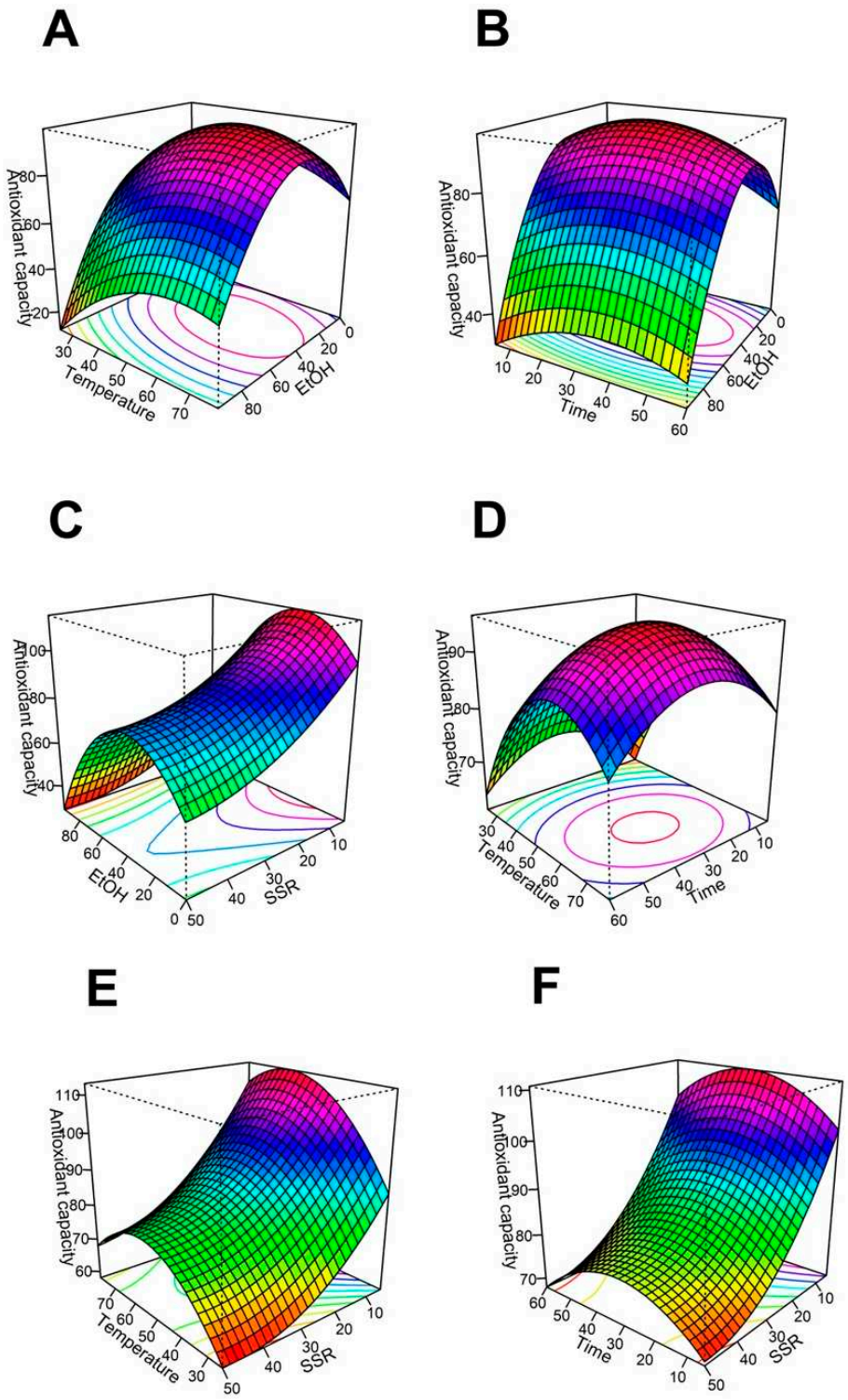

Figure 1. Surface-response analysis of the effect of EtOH (%), Temperature (°C), Time (min), and SSR (g/500 mL) on the antioxidant capacity (µmol TE/gDM). (A) SSR vs EtOH, (B) EtOH vs temperature, (C) EtOH vs Time, (D) Time vs SSR, (E) Temperature vs SSR, & (F) Temperature vs Time. Abbreviations: TE Trolox equivalents, DM dry mass, SSR: solid solvent ratio, EtOH (mass % ethanol in solvent, the remaining percent corresponds to water).

Low antioxidant capacity of the extracts is obtained when the ethanol concentration is high in the extractant phase [25]. These results may confirm the high efficiency of water:ethanol as a solvent to evaluate the antioxidant capacity of *H. Sabdariffa*. Antioxidants found in literature for hibiscus aqueous-ethanolic extracts are: organic acids, phenolic acids, flavonoids, and anthocyanins [14].

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Those compounds are soluble in hydroalcoholic mixtures containing equal amounts of ethanol and water, but their solubility decreases when ethanol concentration is near the azeotrope. In our results, the best antioxidant capacity is obtained when the solvent is composed of a mixture of water and organic solvent (*Table 1*). The combination of those solvents dissolves a wide range of phenolic compounds [26]. The dipoles from phenolic compounds (such as delphinidin-3-O-sambubioside, and cyanidine-3-O-sambubioside) interact with the dipoles from ethanol and water, yielding a higher extraction rate [27].

The optimal ethanol concentration in the solvent is 34.5%. There is no additional improvement in antioxidant capacity when the ethanol concentration increases above 34.5%. This behavior is due to the average affinity of the antioxidant compounds. Nonetheless, a higher water concentration could promote the degradation of anthocyanidins [28] because Flavylium cation stability. This ion is the predominant form of the anthocyanins in acidic medium. Flavylium ion is susceptible to nucleophilic attack of water, and after reacting, it generates a pseudo hemiketal with reduced antioxidant capacity [29].

2.2. Effect of extraction temperature on the antioxidant capacity

Temperature is an important parameter in the extraction of Hibiscus. (*Figure 1* B, E, and F) contain the effects of the temperature, solid-solvent ratio, and time on the antioxidant capacity of the extract. The highest antioxidant capacity is reached at 60°C, combined with low solid/solvent ratios and medium values for % ethanol in solvent and time. Similar behavior was reported [30]. They found that the total phenolics and the antioxidant capacity of the extract decays when the temperature was increased to 90°C. The high temperatures can decompose or modify the thermosensitive bioactive compounds, such as polyphenols and other antioxidants [31]. Also, very high temperatures can accelerate the co-extraction of other non-active components such as sugars and fiber. Then, the antioxidant concentration in the extract would decrease [32].

A low concentration is also inconvenient in order to obtain an antioxidant-rich extract. The cell wall from hibiscus is weakened when the temperature is increased. Then, the cellular components, and the chemical compounds have more interaction with the solvents. [33]. Results from (*Figures* 1 B, E, & F) show that the best temperature for extraction of antioxidants from Hibiscus is 60°C. Commonly, Hibiscus calyxes are boiled to obtain infusions. Although our results suggest Hibiscus should not be boiled because that process decreases its quality.

2.3. Effect of extraction time on the antioxidant capacity

(*Figure 1* C, D, and F) show the effect of the time on the antioxidant capacity, respecting the % of ethanol in the solvent (*Figure 1*C), the solid/solvent ratio (*Figure 1*D), and the temperature (*Figure 1*F). There are no significant differences in the antioxidant activity when the time changes between the limits included in this study. However, prolonged extraction times at high temperatures can decompose and oxidize phenolic compounds, and consequently, the antioxidant capacity is reduced [34].

The extract with the best antioxidant capacity was reached when the extraction temperature was kept at 60°C for 33 min. According to Ramírez et al. [20], the concentration of polyphenolic compounds increases with the extraction time, at the appropriate temperature. The compounds need time to migrate to the solvent [34].

2.4. Effect of the solid/solvent ratio on the antioxidant capacity

The solid/solvent ratio is an important parameter during the extraction process. The solid is the mass of powdered calyxes from H. sabdariffa, and the solvent is the hydroalcoholic mixture. (*Figures 1* A, D, & E) show the surface response graph for 500 mL total volume and variable solid/solvent ratio. For example, 5g of hibiscus extracted with 500 mL of solvent represents a 1/100 ratio. The maximum antioxidant capacity was reached at this condition (1/100 solid/solvent). The antioxidant activity increases with the decrease in the solid/solvent ratio until it reaches the optimal value. Similar

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results were seen by Tan et al. [35]. A greater concentration gradient increases the diffusion rate when the solvent amount increases, according to the mass transfer principle. This is consistent with the principle of mass transfer [36]. The trend can be reversed at some point by the dilution rate. Although this behavior is not observed within the conditions included in this study.

2.5. Contribution of total polyphenolic compounds (TPC) and anthocyanins (ACs) to antioxidant activity

The two main ACs (delphinidin -3-O-sambubioside and cyanidine-3-O-sambubioside), and the TPC were determined for each extract. The ACs selected were found as main components of Hibiscus aqueous extracts by Segura et al. [37].

According to Ramírez et al. [20], ACs handle 51% of antioxidant capacity. (*Figure 2)* shows TPC and ACs for the 26 experiments included in the BBD. TPC values are higher than ACs as expected. Although both TPC and ACs follow the same trend, both are related to antioxidant capacity. Also, the experiment runs showing lower content of both TPC and ACs were extracted with 95% ethanol. It means that ethanol is not a good solvent for extracting phenolic compounds.

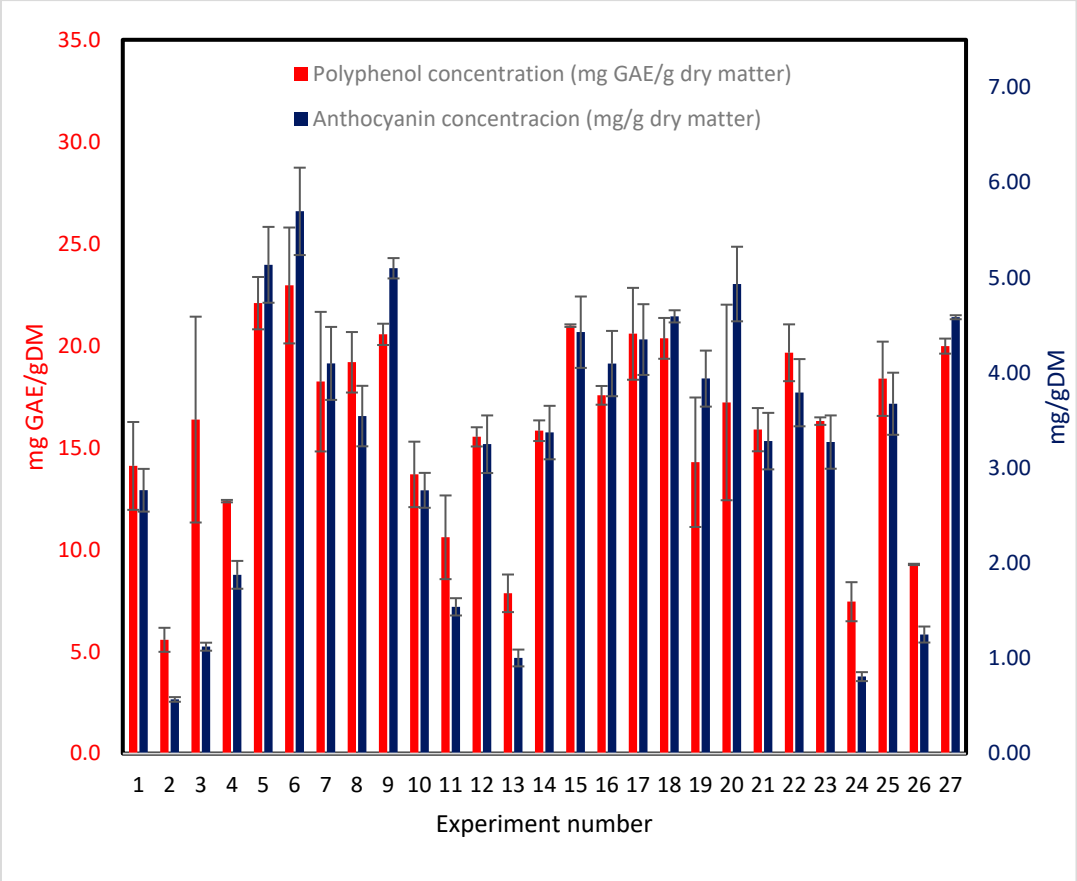

Figure 2. Total polyphenolic compounds and anthocyanins for individual extraction experiments from H. sabdariffa. Error bars represent standard deviation. Tuckey test results for the 27 experiments is shown in Supplementary Tables S1 (TPC) and S2 (PAC).

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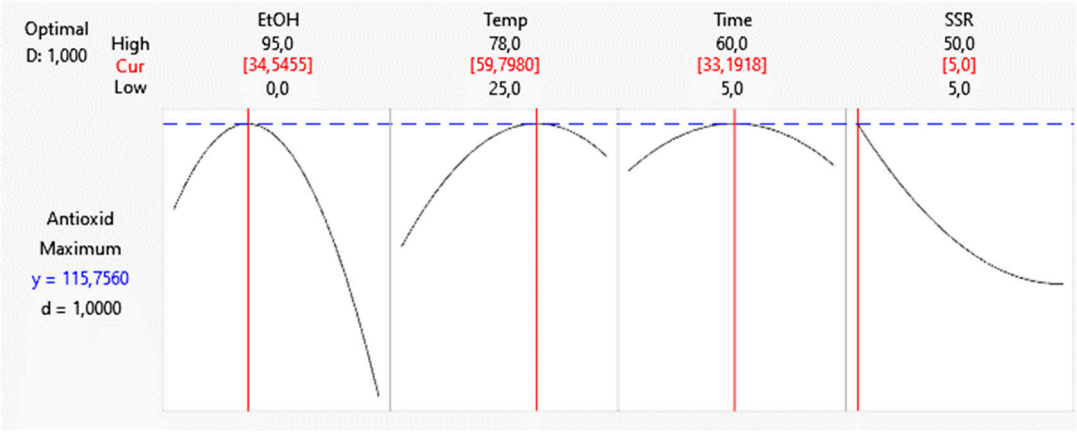

Figure 3. Optimized variables of the extraction of antioxidant compounds from *Hibiscus sabdariffa* calyxes.

2.6. Study of a formula prepared using the optimized Hibiscus extract as a base component

Nutraceutical products can prevent or treat several diseases. Nutraceutical intake is recommended as complementary or alternative treatments [38]. Hibiscus is commercially utilized to prepare hot beverages, mainly. For this reason, in this work, a bioactive nutraceutical product was formulated using Hibiscus extract. When the extract is obtained using the optimized conditions, as shown in *Figure 3*, it showed an antioxidant capacity of 103,36 μmol TE / g DM, with a prediction error of 10.71 % when compared to the prediction value obtained in the statistical analysis whose result 115,76 μmol TE / g DM is shown in *Figure 3*. The hibiscus dried extract was dried by atomization and blended with a non-caloric sweetener and polydextrose. This procedure helps to preserve the stability of the bioactive compounds and requires less time than freeze-drying or vacuum-drying [39]. The drying process have some advantages: the powder is lighter, its volume is decreased, then it is easier to handle and transport. The spray-drying technique produces a microbiological and oxidative resistant powder. The method is simple, easily automatable, and fast. However, the atomization drying yield is 30% lower than freeze dryer, but the process still can be improved by optimizing some parameters such as the inlet temperature, the feeding flux, and the outlet temperature. The proximate analysis of the resulting powder shows 1.14 μg/g of total sugars, 3.81 Kcal/g, and 0.258 mg/g of sodium. There is not a standard daily consumption of antioxidants, however, the United States Department of Agriculture (USDA) recommends ingestion of 3000-5000 μmol TE [40].

A mass of 5 g of the previously prepared formula can be dissolved in 100 mL of water. The drink showed an antioxidant capacity of 495 μmol TE. Our antioxidant activities are close to the shown by other reference herbal infusions such as the green tea [41]. Although, most ready-to-drink formulas are supplemented with ascorbic acid and other antioxidant compounds [42]. The formula proposed in this study is just supplemented with stevia such as sweetener and polydextrose for flavor stabilization. The only source of antioxidants in the formulation proposed is the herbal extract.

Some of the advantages of a dry powder formula are the antioxidant activity is not affected by the preparation conditions [42], the humidity content is very low, the product is long time stable [43], and the phytochemical characteristics are preserved.

3. Materials and Methods

3.1. Plant material preparation and maceration

Dry whole roselle calyxes were purchased from Doña Rosa Food Products (San Isidro, Heredia, Costa Rica). The material was freeze-dried in a Freezone 2.5 Plus (from Labconco Corp., Kansas City,

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MO), and ground to 2 mm in a cutting mill SM100 (from Restsch GmbH, Germany). Samples were stored at room temperature.

A double jacket laboratory reactor model LR-2.ST (from IKA WERKE, Germany) was equipped with a circulating thermostat Ecoline E306 (from LAUDA, Germany) was used for the extraction procedure. The reaction flask was equipped with a glass condenser and deflectors. Mechanical agitation was kept at 100 rpm. Different ratios of the previously grounded material and solvent were tested. The reactor was filled with 500 mL of the selected solvent and the corresponding previously-ground roselle calyxes. Specific extraction times, temperature, solvent composition, and solid/solvent ratio are explained in the experimental design section. The extracts were directly used for further analyses.

3.2. Antioxidant activity determination

The antioxidant activity was measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) antiradical test. We followed the 96-well microplate protocol [44] 30 µL of each extracted sample was mixed with 270 µL of a 0,04 mg/mL DPPH solution in 80% methanol. After 20 min of incubation at room temperature, absorbance was measured at 515 nm in a microplate reader Synergy HT Multi-Mode (from BioTek Instruments, EUA). A standard curve ranging from 0 to 250 µmol/mL 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used. Sample antioxidant activity was reported as Trolox Equivalents per gram of Dry Mass (µmol TE/DM).

The ORAC procedure used an automated plate reader Synergy HT Multi-Mode (from BioTek Instruments, EUA) [45] Analyses were conducted in phosphate buffer pH 7,4. Peroxyl radical was generated using 2,2-azobis(2-amidino-propane) dihydrochloride which was prepared fresh for each run. The standard curve was linear between 0 and 125 µmol/L Trolox. Fluorescein was used as the substrate. Fluorescence conditions were as follows excitation at 485 nm at emission at 520 nm. Sample antioxidant activity was reported as Trolox Equivalents per gram of Dry Mass (µmol TE/DM).

3.3. Box-Behnken Design (BBD)

Box-Behnken design was used. It comprises 27 experiments with 3 central points, and 4 variables at three levels. Variables were the solvent composition (ethanol:water ratio), the temperature, the time, and the solid:solvent ratio (ground roselle mass and volume of ethanol/water). Table 1 explains the levels for each variable selected. The response variable is the Antioxidant Activity determined by the DPPH method.

Table 3. Independent variables and levels for Box Behnken Experimental Design.

Factor	Symbol	Level		
		-1	0	1
Ethanol:water	X_1	5:95	50:50	0:100
Temperature (°C)	X_2	25	50	78
Time (min)	X_3	5	30	60
Solid/Solvent ratio	X_4	1/100	1/50	1/10

3.4. Total Phenolic Content (TPC) determination

TPC was determined by employing colorimetry using the Folin-Ciocalteu method. We follow the 96-well microplate procedure developed by Sánchez-Rangel et al. (2013) [47], with minor modifications. 30 µL of the sample was mixed in a well with 200 µL of distilled water, 15 µL of Folin-Ciocalteu reagent, and 50 µL of 20% Na2CO3 solution. Then, the mixture was incubated for 20 min while mixing in a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments) at 40 °C. Finally, absorbance was measured at 755 nm, against a standard curve of 0.000, 0.020, 0.040, 0.060, 0.080, and 0.120 mg/1 mL of gallic acid.

3.5. Identification and quantification of anthocyanins

Two anthocyanins (Delphinidin-3-O-sambubioside and cyanidin-3-O-sambubioside) were quantified using HPLC-DAD Ultimate 3000 (from Term Scientific Instruments, EUA). Those compounds were previously isolated from roselle and characterized by Segura et al. [37]. The separation was performed using a C18 Dionex Acclaim (250 x 4.0 mm, 5 μm) column. A constant solvent flux of 0.8 mL/min was utilized. A binary pump filled with aqueous 0.01% TFA (trifluoroacetic acid) and acetonitrile as mobile phase components were used. Initially, the pump was set to keep acetonitrile constant at 10% from time 0 to 8 min, then it was linearly increased to 50% (10 min) and finally, increased to 95% (13 min). The diluted TFA completes the remaining sambubioside and cyanidin-3-O-sambubioside (from Sigma-Aldrich, MO) as standard, and read at 530 nm. Chromatographs are shown in (*Figure S1*).

3.6. Preparation and characterization of a nutraceutical drink using the optimized roselle extract as raw material

The optimized roselle extract was concentrated in a rotatory evaporator B-490, the concentrated extract was dried out into a Mini Spray Dryer B-290 (both from Büchi Corporation, Flawil, Switzerland). Finally, the powder of the most antioxidant extract was supplemented with stevia (as sweetener) and polydextrose (vehicle), in a mass ratio of 1:1:3 (extract:stevia:polydextrose).

The caloric content of the final product was determined using a bomb calorimeter model C 200 (from IKA WERKE, Germany), according to the protocol DIN 51900-1 (DIN, 2000). Total carbohydrates were determined by the colorimetric phenol-sulfuric acid method [49]. Sodium was determined by atomic absorption following the AOAC 963.09 procedure [50].

3.7. Statistical analysis

All samples were quantified in a duplicate, and the got results were expressed as mean ± standard deviation. Data processing and statistical analysis (mean value) were performed using Microsoft Excel 2019. Response surface design and statistical analysis of the model were performed through an ANOVA. Minitab 19 version was used for those purposes.

4. Conclusions

A viable method for extraction of H. sabdariffa calyxes was developed after optimization of multivariate experimental conditions. The optimal extraction conditions for antioxidant capacity were found after the development of a Box-Behnken experimental design.

The extraction method decreased the extraction time to 33 min, using 5g of sample, and 500 mL of 34.5% ethanol as solvent, at 60 °C. The method employs a reduced concentration of organic solvent and uses a renewable organic solvent. Thus, this method can be considered environmentally friendly.

Ethanol concentration in the solvent is considered the most important variable for the extraction of Hibiscus calyxes. The optimal ethanol concentration was found to be 34.5%. No significant improvement is observed in the extraction when ethanol concentration increases above that value. The reason is the basic principle of extraction, known as affinity.

Nutraceutical beverages can promote a healthy life and prevent diseases. These beverages are functional and low-caloric alternatives.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: Conceptualization, G.R. and VA.; methodology VA and MV; software, MV AND P.J.; validation, M.J, P.J and O.A..; formal analysis, M.V.; investigation, G.R, V.A and P.J..; resources, G.R. and V.A..; data curation, M.J, V.A. and P.J.; writing—original draft preparation, M.J., P.J. and V.A.; writing—review and editing, M.J., P.J. and V.A.; visualization, M.J.; supervision, O.A.; project administration, V.A..; funding acquisition, G.R. and V.A. All authors have read and agreed to the published version of the manuscript.

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Sample Availability: Samples of the extracts or plant material are available from the authors.

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