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RESEARCH ARTICLE

Study of Acidified Aqueous Extraction of Phenolic Compounds from *Hibiscus sabdariffa L.* calyces

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Abstract:

Introduction:

Hibiscus calyces are important sources from anthocyanins and pigments. The recovery of these bioactive compounds using non-organic solvents becomes very attractive for the food industry.

Methods:

For this reason, the separation of phenolic compounds by acidified aqueous extraction from hibiscus calyces was studied. The experiments were conducted by a fractional factorial design.

Result and Conclusion:

Four factors were evaluated: temperature, time, stirring speed and enzyme concentration. The extracts produced were subjected to analysis of color (*L**, *a**, *b** and *Chroma*), total monomeric anthocyanins, antioxidant capacity by ABTS and fourteen phenolic compounds were quantified. The results showed that the best condition to obtain hibiscus calyces extract was using an enzyme concentration of 50 µL/1000 g hibiscus extract, 400 rpm of stirring speed at 55 ºC by 4 hours of extraction, that corresponded to concentrations of 17595, 7516, 2568 µg/g, expressed on a dry basis, for total phenolic compounds, delphinidin 3-sambubioside and cyanidin 3-sambubioside, respectively, and antioxidant capacity measured by ABTS of 7.8 µmol of Trolox equivalent per gram.

Keywords: Extraction, Hibiscus calyces, Phenolic compounds, Antioxidant activity, HPLC-DAD-MS/MS, Anthocyanins, Antioxidant capacity.

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1. INTRODUCTION

Hibiscus is a native plant from Africa and Asia, belonging to *Malvaceae* family. Depicted as a bushy and branched plant, with a height of up to 2.5 m, of the purplish stem with green-purple leaves, whose solitary flowers consisting of 5 valves that have a shape of calyx with an intense red hue [1]; growing in tropical and subtropical areas of both hemispheres of the world [2].

Hibiscus calyx is widely used to make infusions or teas, as well as in the preparation of jellies and/or as a natural dye source [3]. In traditional medicine, the hibiscus is used in the form of tea to treat several disorders such as constipation, cancer, heart disease, urinary tract infections, diabetes, high blood pressure, and hepatic disorders [4 - 6].

Nowadays, natural antioxidants from plant sources have had special importance, especially compounds, such as polyphenols and flavonoids (anthocyanins) [1]. Several studies have reported on the antioxidant capacity of hibiscus [7], as well as activity anti-inflammatory [8], antimicrobial [9], anticholesterol [10], hepatoprotective [11], anticancer [12], antihypertensive [13], cardioprotective [14, 5], anti-adipogenic [15], immunomodulatory [8], and diuretic activity [14]. The compounds responsible for these effects are the molecules of phenolic and anthocyanins present in hibiscus [6, 16].

The extraction of bioactive compounds from natural sources has become important due to their use as phytochemicals in the preparation of functional food ingredients, food supplements, and additives, as well as pharmaceutical and cosmetic products [17]. However, the extraction of bioactive compounds from the fruits and vegetable sources depends on

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several factors, such as the solvent type, stirring speed, solid and solvent ratio, extraction time and temperature [18]. In addition, several works have reported the use of mixed of enzymes such as pectinases, cellulases, and proteases, in order to increase the yield of the extraction of bioactive compounds in foods [19, 20].

The anthocyanins are commonly used in the food industry due to their coloring properties, which can provide several hues and chromas in food [21]. Many edible plants, including the hibiscus calyces, are sources of anthocyanins, representing the largest group of water-soluble pigments in the plant [2, 22]. According to Da-Costa-Rocha *et al*. [23], aqueous extracts from the calyces of hibiscus contain two main anthocyanins: cyanidin 3-sambubioside and delphinidin 3-sambubioside, besides other phenolic compounds as phenolic acids.

In this context, the aim of this work was the extraction and quantification of the major phenolic compounds from hibiscus calyces, obtained by different conditions, using acidulated water as a solvent.

2. MATERIAL AND METHODS

2.1. Raw Material

The hibiscus was supplied for Association Lomba do Pinheiro, Brazil. Once received in the laboratory, the calyces were separated from the seeds. Then, calyces were cleaned, dried and packed in polyethylene bags, sealed and storage at -18 °C.

2.2. Chemicals

Enzyme complex of cellulose, hemicellulase and pectinase (Novozym 33095) was donated by Novozymes (Spain). HPLC-grade methanol was supplied by J.T. Baker (Trinidad and Tobago), formic acid and acetonitrile from Panreac (Darmstadt, Germany). Millipore (Massachusetts, USA) membranes (0.22 μm and 0.45 μm) were used before the HPLC analyses. The ABTS (2,2'azino-bis-3-ethylbenzo-thiazoline-6-sulfonic acid), trolox (6-hydroxy-2,5,7,8-tetramethylch-romane-2-carboxylic acid), standards caffeic acid, coumaric acid, ferulic acid, quercetin and cyanidin were purchased from Sigma-Aldrich (St. Louis, USA). All reagents were analytical grade.

2.3. Aqueous Extraction

The calyces were submitted to steam blanching for 4 min by autoclave at 100 ºC and right away, cooled in an ice bath for 3 min. The acidified water (2% of citric acid, w/v) [24] was added to the calyces in a proportion of 1:5 (w/w), and the mixture was mixed and triturated in a blender (Britânia).

During the extraction procedure, the agitation was realized in a mechanical stirrer (RW20, IKA) with a rod stirrer (R1342). The temperature was controlled by a water bath (Laborota

Table 1. Treatments defined by fractional factorial experimental design with range of coded and real values to aqueous extraction of hibiscus calyces.

Treatment	X_1 Enzyme (μL/1000 g)	X_2 Temperature (ºC)	X_3 Stirring Speed (rpm)	X_4 Time of Extraction (h)
T1	-1 (0)	-1 (35)	-1 (200)	-1 (3)
T2	+1 (50)	-1 (35)	-1 (200)	+1 (5)
T3	-1 (0)	+1 (55)	-1 (200)	+1 (5)
T4	+1 (50)	+1 (55)	-1 (200)	-1 (3)
T5	-1 (0)	-1 (35)	+1 (400)	+1 (5)
T6	+1 (50)	-1 (35)	+1 (400)	-1 (3)
T7	-1 (0)	+1 (55)	+1 (400)	-1 (3)
T8	+1 (50)	+1 (55)	+1 (400)	+1 (5)

Table 2. Color parameters of the hibiscus calyces extracts prepared following the treatments defined by fractional factorial experimental design showed in (Table 1).

Treatments	*L**	*a**	*b**	*Chroma**
T1	35.03 ± 0.68^{a}	33.40 ± 0.69^{a}	19.88 ± 0.64^{a}	38.87 ± 0.92^{a}
T2	32.61 ± 0.67^{ab}	32.67 ± 0.78^{ab}	17.79 ± 0.81^{ab}	37.20 ± 1.07^{ab}
T3	31.14 ± 0.69^{bc}	29.82 ± 0.59^{cde}	15.24 ± 0.14^{cd}	33.49 ± 0.59^{cde}
T4	30.66 ± 0.21^{bc}	30.56 ± 0.16^{bcd}	16.64 ± 0.19^{bc}	34.80 ± 0.05^{bcd}
T5	31.22 ± 0.09^{bc}	31.09 ± 0.09^{abc}	16.26 ± 0.15^{bc}	35.08 ± 0.15^{bc}
T6	31.16 ± 0.92^{bc}	32.63 ± 0.95^{ab}	17.61 ± 0.99^{abc}	37.08 ± 1.30^{ab}
T7	29.59 ± 0.79^{c}	27.44 ± 0.74^{e}	12.91 ± 0.73^{d}	30.32 ± 0.98^{e}
T8	28.81 ± 0.99^{c}	28.24 ± 1.03^{de}	13.49 ± 0.52^{d}	31.30 ± 1.16^{de}

Means followed by the same letters in the same columns do not differ by Tukey test at 5% probability.
Measures were performed in triplicate.

4000, Heidoplph). After the extraction process, the extracts were centrifuged at 6.000 rpm (CR21GIII, Hitachi Koki) to separate the water-insoluble solids.

2.4. Colorimetric Analysis

The measured color of the extracts was made using a colorimeter (CR400/410, Minolta), according to the CIELAB (L^*, a^*, b^*) system. Before measurement, the instrument was calibrated using a white ceramic plate. *Chroma* ($C^* = [a^{*2} + b^{*2}]^{1/2}$) was calculated, which indicates color's purity or saturation.

2.5. Total Monomeric Anthocyanins (TMA)

The total anthocyanin content was determined by the method of differential pH [25]. The extracts were mixed with buffer solutions pH 1.0 and 4.5. The absorbance was measured in a spectrophotometer (Genesys S10, Thermo Scientific) at 520 and 700 nm. The concentrations were expressed in mg of delphinidin 3-sambubioside per g extract on a dry basis, using molecular weight 577 g/mol, and molar absorptivity 26000 L/mol/cm [26].

2.6. Antioxidant Capacity (ABTS)

The radical scavenging ability of the extracts was measured by the ABTS (2,2'azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) antioxidant capacity assay following the methodology recommended by Re *et al*. [27]. The results were calculated based on a calibration curve of Trolox (1.5 – 20 μM) (R^2 = 0.9995), and Trolox-equivalent antioxidant capacity (TEAC) values were expressed as μmol of Trolox per g extract on a dry basis.

2.7. Quantification of Phenolic Compounds

Determination of the phenolic compounds was realized according to Rodrigues *et al*. [28]. The liquid chromatographic analysis was carried out in a Shimadzu HPLC system with a diode array detector, using an Atlantis T3-RP C18 column (250 × 4.6 mm, 5 μm). In order to improve the peak of separation a Synergi Hydro-RP C18 column (250× 4.6 mm, 4 μm) was also used. Mobile phase consisting of two solvents (A) water acidified with formic acid (0.5%, v/v) and (B) acetonitrile acidified with formic acid (0.5%, v/v), and the following gradient was used: A/B from 99:1 to 50:50 (0 - 50 min), from 50:50 to 1:99 (50 - 55 min) and finally held for 5 min. Column conditions were of 29 °C, 0.7 mL/min and 20 μL for temperature, the flow rate of mobile phase and injection volume, respectively. The UV-vis spectra were monitored from 200 to 800 nm and the chromatograms were obtained at 260, 320, 360 and 520 nm [29].

HPLC was connected in series to a diode array detector and a mass spectrometer with a Q-TOF analyzer and electrospray ionization source (micrOTOF-QIII, Bruker Daltonics). Electrospray ionization and MS analysis conditions were: capillary voltage 2.0kV (positive mode) and 3.0kV (negative mode), nitrogen was used as both the nebulizing gas (flow rate of 8 mL/min) and dry gas (pressure of 2 bar), probe temperature of 310 °C and the mass spectra scan from 100 to 700 m/z. MS2 was set in automatic mode applying fragmentation energy of 34 V.

The phenolic compounds were identified based on retention time and elution order in the reversed phase column, UV−Vis and MS spectrum characteristic compared to standards analyzed on similar conditions and data of the literature [30].

The phenolic compounds were quantified by HPLC-DAD, using analytical curves with nine-point of caffeoylquinic acid, ferulic acid, quercetin and cyanidin. Data analysis was very well fixed to linear models ($r^2 > 0.99$).

2.8. Experimental Design and Statistical Analysis

For this study, a 2^{4-1} fractional factorial design was utilized (Table **1**), resulting in eight treatments. Four independent factors at two levels were studied: enzyme (x_1) (0; 50 μL/1000 g extract), extraction temperature (x_2) (35; 55 °C), stirring speed (x_3) (200; 400 rpm) and extraction time (x_4) (3; 5 h).

The variables x_i were coded as X_i, according to the equation:

$$X_i = (x_i - \bar{x}_i)/\Delta x_i \quad \textbf{(1)}$$

Where $\bar{x}_i$ is the mean value of each independent variable, and Δx_i is the step change value. The levels of the independent variables in coded (-1, +1) and real values are shown in Table **1**.

The following model was fitted to the data (Equation 1) through regression analysis:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j \quad \textbf{(2)}$$

Where Y is the response variable, β, β_i, and β_{ij} are coefficients of the term independent, the linear and interaction effects, respectively, and X_i and X_j the coded level of variables x_i and x_j.

The analysis of *Chroma, TMA* (total monomeric anthocyanins), ABTS and phenolic compounds were the responses variables. The resulting models were used to plot response surfaces. Data were subjected to ANOVA and Pearson correlation, and the treatments to Tukey's multiple comparison tests, using the software SAS 9.3. The experiments were performed in triplicates and a 95% confidence level was used.

3. RESULTS AND DISCUSSION

3.1. Colorimetric Analysis

Table **2** shows the color parameters for different extraction conditions. L^* values were significantly lower (darker color) when either higher levels of temperature or stirring speed were used. This might be due to the formation of dark compounds during the extraction. The higher values of a^*, b^* and *Chroma* were obtained at 35 ºC. The results also showed that they are located in the first quadrant of the hue circle, indicating a tendency to yellowness and redness. The best extracts with red color may be related to the extraction of anthocyanins [31].

3.2. Total Monomeric Anthocyanin (TMA)

The mean values of *TMA* varied from 3.07 to 3.82 mg/g extract on a dry basis for the hibiscus calyces extracts Fig. (**1A**), where the increase in the stirring speed and the temperature increased the TMA concentrations. These values are in agreement with Christian and Jackson [32] that reached mean values from 1.8 to 3.5 mg/g extract on a dry basis for TMA using organic solvents in the extraction. Thus, when used to ideal conditions in the hibiscus aqueous extraction process, it is possible to achieve similar levels of total monomeric anthocyanins compared with extraction with organic solvents. The highest anthocyanins extractions were obtained for T8 and T7 and they did not show a significant difference Fig. (**1A**).

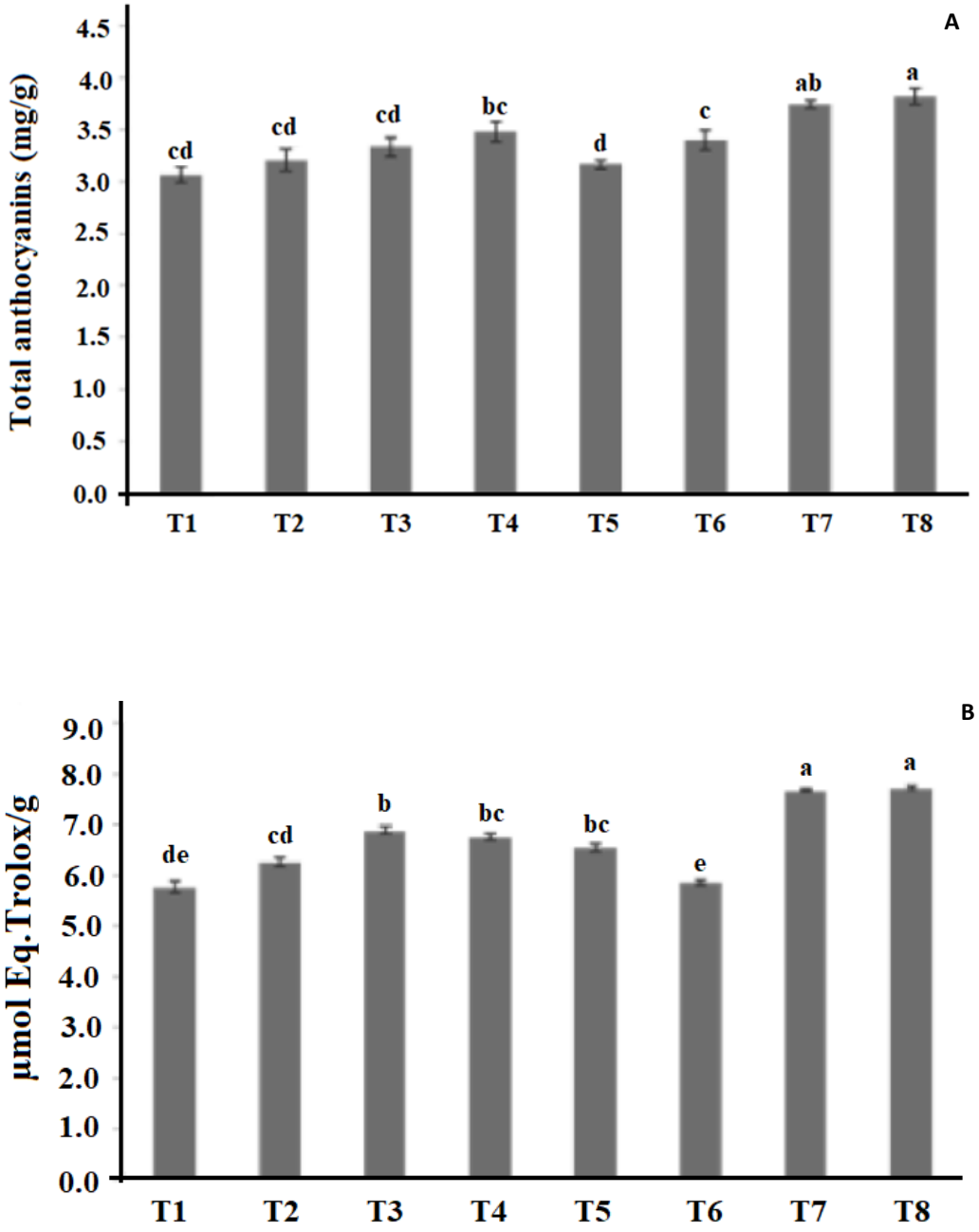

Fig. (1). Determination of Total Monomeric Anthocyanins (*TMA*) **(A)** and ABTS **(B)** for the hibiscus calyces extracts prepared following the fractional factorial experimental design showed in Table **1**.

Table 3. Phenolic compounds concentration in the hibiscus calyces extracts prepared following the fractional factorial experimental design showed in (Table 1).

Phenolic Compounds	Concentration (μg/g extract on dry basis) [1]							
	T1	T2	T3	T4	T5	T6	T7	T8
3-caffeoylquinic acid[1]	2088.86	2560.28	2979.78	3211.35	2632.31	2775.98	3486.63	3568.43
Delphinidin 3-sambubioside[2]	4035.49	5304.83	5945.75	6602.71	5129.33	5409.26	6937.87	7515.74
3-p-Coumaroylquinic acid[1]	162.90	202.45	233.85	271.31	201.02	212.47	295.41	310.36
Cyanidin 3-sambubioside[2]	1420.70	1786.52	2012.17	2185.27	1745.77	1950.48	2457.71	2568.34
5-caffeoylquinic acid[1]	1726.27	2047.86	2537.48	2609.59	2096.17	2203.34	2678.89	2750.79
4-caffeoylquinic acid[1]	63.21	74.14	84.47	95.92	80.31	80.83	96.96	107.08
Ferulic acid derived[3]	12.28	19.62	25.06	21.52	10.08	11.69	21.06	17.78
Myricetin 3-sambubioside [4]	51.39	63.73	82.83	72.34.	50.68	59.92	65.93	87.12
5-p-Coumaroylquinic acid[1]	72.31	88.70	100.97	107.09	83.75	93.49	126.55	126.88
5-O-Caffeoylshikimic acid[3]	39.70	40.82	58.95	46.67	43.05	42.17	46.36	47.96
Quercetin 3-sambubioside[4]	152.61	179.82	219.04	230.75	184.14	205.37	235.55	262.83
Quercetin 3-rutinoside[4]	110.23	125.71	151.83	160.42	123.88	129.34	164.11	183.36
Quercetin 3-glucoside[4]	13.06	13.63	16.58	14.34	14.42	13.64	14.03	17.83
Kaempferol 3-O-rutinoside[4]	21.94	25.44	27.51	27.55	24.51	24.77	29.32	30.33
Total phenolic acids[5]	4165±89^{g}	5033±69^{f}	6020±36^{d}	6363±134^{c}	5146±33^{f}	5419±5^{e}	6751±13^{b}	6929±114^{a}
Total anthocyanins[6]	5456±65^{h}	7091±86^{f}	7957±110 ^{d}	8787±101^{c}	6875±63^{g}	7359±141^{e}	9395±48^{b}	10084±93^{a}
Total flavonoids[7]	349±5^{d}	408±27^{c}	518±13^{b}	505±26^{b}	397±11^{c}	433±7^{c}	509±19^{b}	581±23^{a}
Total phenolic compounds[8]	9970±160^{g}	12533±183^{f}	14476±159^{d}	15657±262^{c}	12419±108^{f}	13213±143^{e}	16656±81^{b}	17595±231^{a}
Yield of anthocyanins	28.36	36.86	41.36	45.68	35.74	38.25	48.84	52.42
Yield of total phenolic content	29.38	36.93	42.66	46.13	36.59	38.93	49.08	51.84

Different letters in the same line indicate significant differences ($p < 0.05$). The peaks were quantified as equivalent of 5-caffeoylquinic acid[1], cyanidin[2], ferulic acid[3] and quercetin[4]. [5]Total phenolic acids include 3-caffeoylquinic acid, 3-p-Coumaroylquinic acid, 5-caffeoylquinic acid, 4-caffeoylquinic acid, Ferulic acid derived, 5-p-Coumaroylquinic acid and 5-O-Caffeoylshikimic acid. [6]Total anthocyanins include Delphinidin 3-sambubioside and Cyanidin 3-sambubioside. [7]Total flavonoids include Myricetin 3-sambubioside, Quercetin 3-sambubioside, Quercetin 3-rutinoside, Quercetin 3-glucoside and Kaempferol 3-O-rutinoside. [8]Total phenolic compounds include all compounds.

When acid citric is added to extraction water, recovery of anthocyanins increase due to low pH of the solution denatures cell membranes permitting higher solubilization of the anthocyanins, and the free hydrogen ions allow stabilizing the red color produced by flavylium cation form of the anthocyanins [33]. In addition, at low pH, the flavylium is highly soluble in water [34].

3.3. Phenolic Compounds

Fourteen phenolic compounds, namely, 3-caffeoylquinic acid, delphinidin 3-sambubioside, 3-p-coumaroylquinic acid, cyanidin 3-sambubioside, 5-caffeoylquinic acid, 4-caffeoylquinic acid, ferulic acid derived, myricetin 3-sambubioside, 5-p-coumaroylquinic acid, 5-O-caffeoylshikimic acid, quercetin 3-sambubioside, quercetin 3-rutinoside, quercetin 3-glucoside and kaempferol 3-O-rutinoside were identified [30] and quantified. The concentrations on a dry basis of the phenolic compounds obtained by different extraction assays are shown in Table **3**.

The total phenolic acids varied from 4165 to 6929 μg/g extract on dry basis, being the 3-caffeoylquinic acid the main phenolic acid of the hibiscus extract (2089 to 3568 μg/g extract on dry basis), followed, in descending order, by 5-caffeoylquinic acid (1726 to 2751 μg/g extract on dry basis), 3-*p*-Coumaroylquinic acid (163 to 310 μg/g extract on dry basis), 5-p-coumaroylquinic acid (72 to 127 μg/g extract on dry basis), 4-caffeoylquinic acid (63 to 107 μg/g extract on dry basis), 5-O-caffeoylshikimic acid (40 to 59 μg/g extract on dry basis) and ferulic acid derived (10 to 25 μg/g extract on dry basis). The T8 and T7 extractions provided the highest values of total phenolic acid (6929 and 6751 μg/g extract on dry basis, respectively), in these treatments can be observed that the use of both the higher levels of temperature and stirring speed, showing the importance of these variables for the extraction of phenolic acids. Several studies reported the importance of phenolic acid in antioxidant activity [35, 5].

The content of total anthocyanins quantified by HPLC ranged from 5456 to 10084 μg/g extract on dry basis. The highest content for total anthocyanins was also obtained by T8 and T7 treatments, 10084 and 9395 μg/g extract on dry basis, respectively. These results were much higher than those found by Salazar-González *et al*. [36] who reported 2147 μg/g of total anthocyanins in hibiscus aqueous extract. Extraction efficiency depends on several variables, such as the technique of separation, the raw material composition, and the type of solvent [37]. Thus, phenolic compounds profile contained in raw materials, for having different polarities, may affect the extraction process [38].

Regarding the anthocyanins of hibiscus extract, two anthocyanins, highlighting the delphinidin 3-sambubioside (up to 7516 μg/g extract on dry basis) as the main anthocyanin of the hibiscus were identified and quantified, followed by

cyanidin-3-sambubioside (up to 2568 μg/g extract on dry basis), which is found to be in agreement with the results obtained by Ramirez-Rodrigues *et al*. [31] and Christian *et al*. [39], who reported delphinidin and cyanidin sambubiosides as the anthocyanins in hibiscus aqueous extracts.

Anthocyanins are responsible for the blue, red, purple and their complementary colors of most fruits, vegetables, and flowers [40]. They constitute a large percentage of phenolic compounds from hibiscus, representing the most important compounds of the phenolic profile in this flower. These molecules have polar characteristic and are very reactive and water soluble [41], and for this reason, the water might be used as a solvent for extraction. Moreover, add a small amount of acid would allow rising the stability of them [41]. Besides, they are unstable at different factors, such as temperature, light, oxygen, pH, water activity and attendance of enzymes and copigments [42, 43].

For total flavonoids found in the hibiscus extracts, the values ranged from 349 to 598 μg/g extract on dry basis. The highest concentrations were achieved by T3 and T8 (518 and 581 μg/g extract on dry basis, respectively) while the lowest concentrations were obtained by T1 (349 μg/g extract on dry basis). Among the main flavonoids identified and quantified on the hibiscus calyces extracts, it can be highlighted the derivatives of quercetin, kaempferol, and myricetin, in accordance with the results reported by Borrás-Linares *et al*. [44].

In general, T8 provided the highest extraction of phenolic compounds (17595 μg/g extract on dry basis), followed by T7 (16656 μg/g extract on dry basis), showing a positive effect mainly of the temperature and stirring speed in the extraction of phenolic compounds. According to Pinelo *et al*. [45], the phenolic compounds can be linked or entangled in the polysaccharides of the cell walls, the cell vacuoles, or associated with cell nuclei.

Thus, it can be concluded that treatments in which high temperatures combined with high stirring speed provided greater degradation of hibiscus cells, increasing the release of intracellular components and of phenolic compounds bound to the cell wall polysaccharides. These results are in accordance with other studies that also emphasized the importance among temperature, time and stirring on the extraction of phenolic compounds [18, 45].

The extraction yields are shown in Table **3**. The yields ranged from 28.36 to 52.42 and from 29.38 to 51.84 for anthocyanins and total phenolic compounds, respectively. The highest yield for these two compounds was obtained by T8 treatment.

3.4. Antioxidant Capacity ABTS

The highest antioxidant capacities measured by ABTS were obtained by T7 and T8 Fig. (**1B**). These results are also in agreement with Wong *et al*. [46], who observed that the antioxidant capacity values increased with the increase of temperature, due to the higher mass transfer and the higher separation of antioxidant phenolic compounds.

Thus, the highest antioxidant capacities were obtained by treatments that provided the highest extraction of phenolic compounds. It is important to highlight that several studies have correlated the antioxidant activity with the phenolic content [16, 47].

Table **4** shows the Pearson correlation coefficients between the color parameters, phenolic acids, total phenolics, anthocyanins, total monomeric, flavonoids and ABTS in several extractions. The highest correlation coefficients were observed between ABTS (0.90) with phenolic acids, total phenolics, and anthocyanins. On the other hand, both parameters a^* or b^* were also highly correlated, but showed the negative relationship (ranged between -0.98 and -0.85 and -0.95 and 0.84, respectively) with phenolic acids, total phenolics, anthocyanins total monomeric, flavonoids and ABTS.

3.5. Effect of the Variables on the Responses

The equations that describe the behavior in the extraction of the *Chroma* (Y_1), TMA (Y_2), ABTS (Y_3) and phenolic compounds (Y_4) in relation to the responses variables are presented in the equations below.

$$Y_1 = 34.77 - 2.29X_2 - 1.32X_3 \quad \textbf{(3)}$$

$$Y_2 = 3.40 + 0.074X_1 + 0.19X_2 + 0.13X_3 + 0.06X_2X_3 \quad \textbf{(4)}$$

Table 4. Coefficients of Pearson correlation between the color parameters (*L, *a**, *b**, *Chroma*), phenolic acids (Phen acids), anthocyanins (Antho), flavonoides (Flavo), total phenolics (T phen), Total Monomeric Anthocyanins (*TMA*) and ABTS.**

-	*L**	*a**	*b**	*Chroma*	Phen acids	Antho	Flavo	T phen	*TMA*	ABTS
L*	1.00	-	-	-	-	-	-	-	-	-
a*	0.85	1.00	-	-	-	-	-	-	-	-
b*	0.91	0.98	1.00	-	-	-	-	-	-	-
Chroma	0.88	0.99	0.99	1.00	-	-	-	-	-	-
Phen acids	-0.94	-0.91	-0.91	-0.91	1.00	-	-	-	-	-
Antho	-0.93	-0.89	-0.89	-0.89	0.99	1.00	-	-	-	-
Flavo	-0.87	-0.85	-0.84	-0.85	0.96	0.95	1.00	-	-	-
T phen	-0.94	-0.89	-0.89	-0.90	0.99	0.99	0.96	1.00	-	-
TMA	-0.88	-0.86	-0.85	-0.86	0.93	0.95	0.88	0.95	1.00	-
ABTS	-0.84	-0.98	-0.95	-0.98	0.90	0.90	0.86	0.90	0.85	1.00

All coefficient were significant ($p < 0.05$); statistical significance of 0.05.

$$Y_3 = 6.70 + 0.57X_2 + 0.26X_3 + 0.18X_4 - 0.14X_1X_3 + 0.18X_2X_3 \quad \textbf{(5)}$$

$$Y_4 = 14.08 + 0.69X_1 + 2.03X_2 + 0.91X_3 \quad \textbf{(6)}$$

Where, X_1, X_2, X_3, and X_4 are the coded independent variables for enzyme concentration, temperature, stirring speed and time, respectively. The significance of each coefficient and the coefficients of determination, the linear and interaction effects of the variables are shown in Table **5**. For better understanding, contour lines plots for *Chroma*, *TMA*, ABTS and phenolic compounds were generated in accordance with Equations 3 to 6.

For *Chroma* only the linear effects of the temperature and stirring speed were significant (Equation 3 and Fig. **2A**). For phenolic compounds, the linear effects of the enzyme, temperature and stirring speed were significant (Equation 6 and Fig. **2B**). Three linear effects were significant for *TMA* (X_1, X_2 and X_3) and ABTS (X_2, X_3 and X_4). Furthermore, the interaction effect of X_2X_3 was significant for *TMA*, while X_1X_3 and X_2X_3 were significant for ABTS (Equations 4 and 5, Figs. (**3A** and **3B**), respectively).

Chroma values increased when temperature and stirring speed decreased Fig. (**2A**). On the other hand, in (Fig. **2B**), it was observed that phenolic content increased with stirring speed and temperature. In (Figs. **3A** and **3B**), for anthocyanins (*TMA*) and ABTS can be observed a typical behavior of the interaction between factors, where the better extraction conditions were achieved when for these two variables (stirring speed and temperature) were used in their higher levels.

It was also observed that the increase in the values of concentration of enzymes will increase response variables TMA and phenolic content. The use of commercial enzymes with pectinase, cellulase and protease activities help in the degradation of cell walls, and this way may facilitate the extraction of phenolic and anthocyanin compounds [20, 29].

All dependent variables were temperature-dependents. . The temperature had a significant effect on *Chroma* (Y_1), TMA (Y_2), ABTS (Y_3), and phenolic compounds (Y_4). In addition, for TMA (Y_2), temperature also caused interaction with stirring speed. Temperature is a variable of great importance in the extraction of phenolic compounds, due to its influence on the viscosity, solubility, rates of mass transfer, and mass diffusion coefficient.

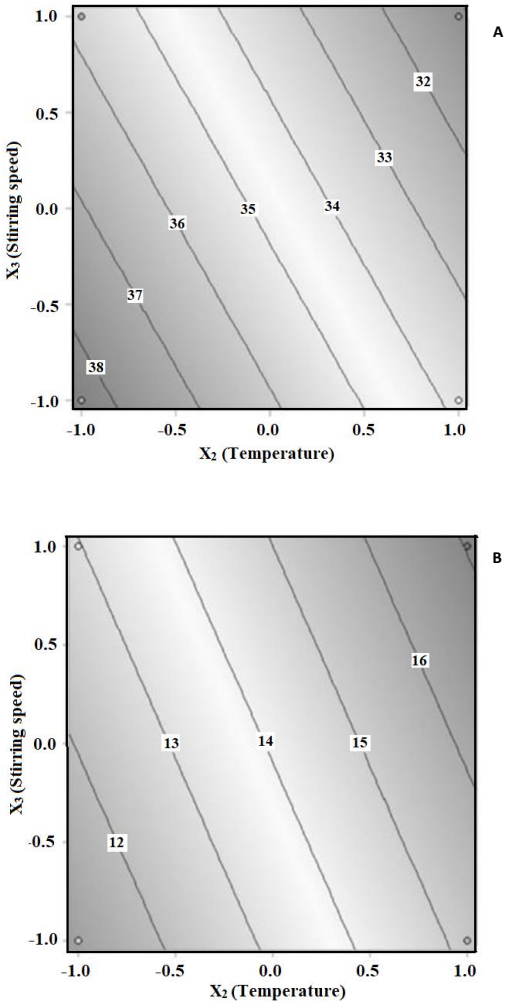

Fig. (2). Contour plots for stirring speed versus temperature in the hibiscus calyces extraction for *Chroma* (Y_1) **(A)**; and phenolic compounds (Y_4) **(B)**, where the variable X_1 (enzyme) was fixed in 0.5.

Table 5. *P*-values for the effect of the explanatory variables on the different responses.

Source[a]	Color (*Chroma*)	*TMA* [b]	ABTS	Phenolic Compounds
X_1	n.s.[c]	0.021*	n.s	0.023*
X_2	0.005*	0.001*	0.003*	0.001*
X_3	0.023*	0.004*	0.006*	0.011*
X_4	n.s.	n.s.	0.009*	n.s.
X_1X_2	n.s.	n.s.	n.s.	n.s.
X_1X_3	n.s.	n.s.	0.0118*	n.s.
X_1X_4	n.s.	n.s.	n.s.	n.s.
X_2X_3	n.s.	0.037*	0.009*	n.s.
X_2X_4	n.s.	n.s.	n.s.	n.s.
X_3X_4	n.s.	n.s.	n.s.	n.s.
R square	0.961	0.990	0.992	0.993

[a] X_1: enzyme; X_2: temperature; X_3: stirring speed; X_4: time.
[b] Total Monomeric Anthocyanins (*TMA*).
[c] n.s.: not significant.
*Significant (*p*-value<0.05); statistical significance of 0.05.

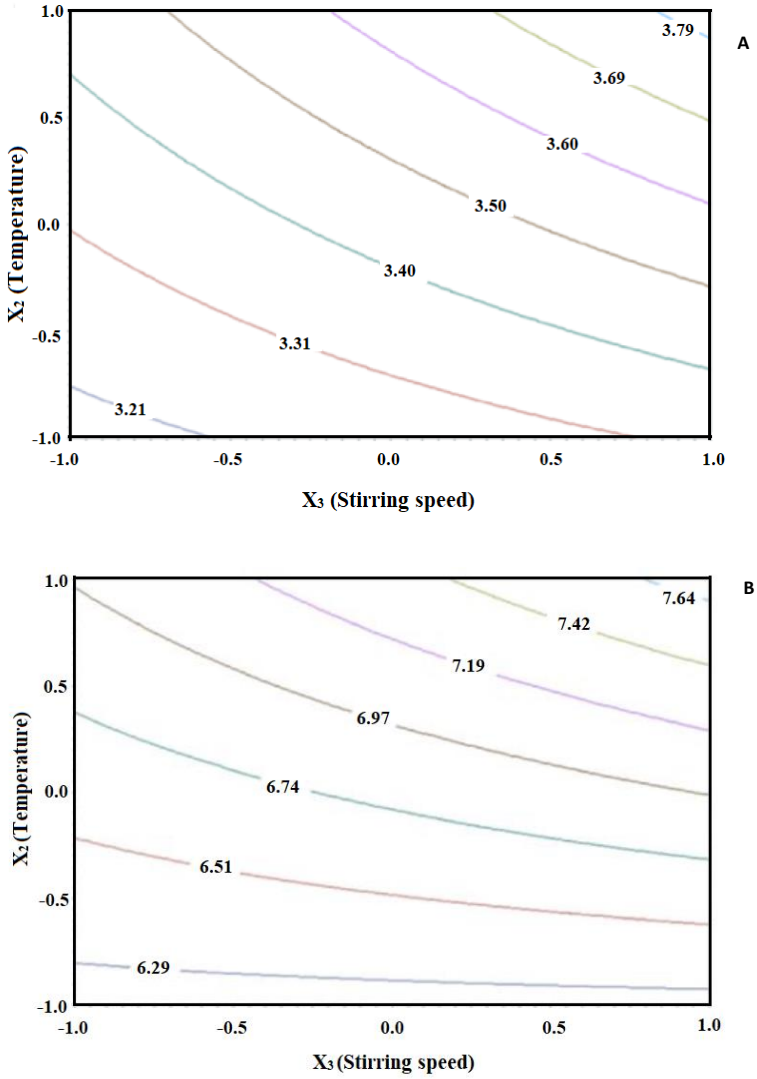

Fig. (3). Contour plots for temperature versus stirring speed in the hibiscus calyces extraction for *TMA* (Y_2) **(A)**, where the variable X_1 was fixed in 0.5; and for ABTS (Y_3) **(B)**, where the variables X_1 and X_4 were fixed in 0.5 (X_1: enzyme concentration; X_2: temperature; X_3: stirring speed; X_4: time).

CONCLUSION

In this work, it was observed that the treatments that used the higher levels of temperature and stirring speed were able to improve the extraction of phenolic compounds in the hibiscus. The best extract was considered the treatment eight, that obtained a aqueous extract with higher concentrations of total phenolic acids (6.93 mg/g extract on dry basis), total anthocyanins (10.08 mg/g extract on dry basis), total flavonoids (0.58 mg/g extract on dry basis) and total phenolic compound (17.59 mg/g extract on dry basis). The use of acidulated water as a solvent has shown promising effects when compared with other studies that use organic solvent extraction used in the food industry, for example, for production of microcapsules or natural colorant.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest financial or otherwise.

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