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Multi-response Optimization of a Solvent System for the Extraction of Antioxidants Polyphenols from Jambolan Fruit (*Syzygium cumini* (L.) Skeels)

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Abstract

The identification of bio-polyphenols from jambolan fruit is of great interest and requires extraction conditions that preserve their bioactivities. This study aimed to optimize a solvent system for the extraction of polyphenols from jambolan fruit pulp using a simplex-centroid design with axial points. We evaluated various proportions of water, ethanol, and acetone to quantify the total polyphenol content and determine antioxidant activity. Polyphenols in the optimized extracts were identified using high-efficiency liquid chromatography and mass spectrometry. The optimal yield was obtained with a binary mixture of water and acetone (0.5:0.5, v/v), which produced more than 90% of the maximum multi-response values. The identification of bio-polyphenols showed that gallic, quinic, and protocatechuic acids; glycosylated anthocyanins; myricetin isomers; and proanthocyanidins as epigallocatechin trimers were the main compounds responsible for the antioxidant activity of jambolan. In conclusion, a binary mixture of water and acetone in equal proportions was effective in extracting polyphenols with antioxidant activity, thus establishing jambolan as a potential source of bioactive compounds.

Keywords *Syzygium cumini* (L.) Skeels · Mixture design · Phenolic compounds · Anthocyanins · Antioxidant capacity

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Introduction

Scientific evidence has shown that the consumption of food sources rich in bioactive compounds reduces oxidative stress and significantly decreases the incidence of non-communicable chronic diseases. In this context, jambolan (*Syzygium cumini* (L.) Skeels) is an important source of polyphenols, mainly anthocyanins, which are responsible for its antioxidant and other pharmacological properties (Tavares et al. 2017).

Various solvents, such as absolute organic solvents or their aqueous mixtures, have been employed for the extraction of polyphenols from jambolan. However, to maximize yield, the solvent and extraction method must allow total separation of the compounds of interest from a complex matrix without changing their chemical structures. Furthermore, at this stage, several factors that determine the type and efficiency of their biological activity, as well as the medium and cell structure in which these compounds can act, must be considered (Mussi et al. 2015).

Despite the efforts employed by researchers worldwide, the Association of Official Analytical Chemists (AOAC) has not been successful in establishing an official method/solvent to extract the bioactive compounds. This is due to the large number and structural complexity of these compounds. Thus, the need for the development, validation, and optimization of new polyphenol extraction methods is important as it is a step prior to their characterization in an accurate and reliable manner (Azmir et al. 2013).

Several studies have assessed different extraction processes, with emphases on mass-solvent and solvent–solvent ratios, extraction time, temperature, and pressure, as well as ways to optimize the extraction of phenols from jambolan pulp (Maran et al. 2014; Migliato et al. 2011). However, they were not able to establish a suitable solvent system for the concomitant extraction of several polyphenol classes from the fruit.

Thus, multivariate statistical techniques for the optimization of mixtures, such as simplex-centroid design (SCD) with axial points, have been employed to analyze different proportions with a reduced number of experiments, and examine possible synergistic or antagonistic interactions between variables (Bochi et al. 2014). This tool can provide the appropriate solvent system for extracting and characterizing polyphenols from the jambolan fruit.

The aim of this study was to optimize the solvent system for extracting bio-polyphenols from jambolan pulp and to obtain the maximum response efficiency of their antioxidant activities using a multivariate simplex-centroid experimental design. We emphasize that, to our knowledge, this is the first scientific study to optimize the extraction of various polyphenolic classes from this fruit using this statistical model.

Material and Methods

Sampling and Sample Preparation

Ripe jambolan fruits (15.20 Brix) were collected in November 2014 in the city of Teresina, northeast of Brazil (5° 03′ 17.2″ S; 42° 47′ 28.0″ W). The plant material was deposited at the Graziela Barroso Herbarium of the Federal University of Piauí, under voucher 30.573. The fruits were sanitized with 100-ppm sodium hypochlorite for 10 min. The edible parts (flesh and skin) were separated manually from the seed while still frozen (− 20 °C) to minimize enzymatic degradation and loss of juice. The pulps were lyophilized (30×10^{-3} mmHg; − 50 °C; 72 h), crushed in an ultra-food processor, sieved with a 50 Tyler mesh for 10 min, wrapped in laminated plastic packaging, sealed, and stored at − 20 °C until sample preparation for extracting bioactive compounds by simplex-centroid design. This experimental design provided analysis of ten samples in duplicate, with three repetitions each.

Reagents and Standards

The chemical compounds (S)-(-)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric chloride, Folin-Ciocalteu reagent, gallic acid, potassium persulfate, catechin, cyanidin chloride, p-dimethylaminocinnamaldehyde (DMACA), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), 3-O-β-D-glucoside, cyanidin-3-O-glucoside, peonidin-3-O-glucoside, and malvidin-3-O-glucoside chlorides were purchased from Sigma-Aldrich® (St. Louis, MO, USA), and HPLC grade reagents were purchased from Merck® (Darmstadt, Germany).

Simplex-Centroid Design for Extraction of Bioactive Compounds

A simplex-centroid experimental design was applied to discover the optimal conditions for the extraction of bioactive compounds from jambolan, maintaining their bioactivities. In this experimental design, the extracting solvents acetone, water, and ethanol, as well as their mixtures, were evaluated. Thus, this experimental design involved three pure solvents (x_1 = water, x_2 = acetone, and x_3 = ethanol) on the vertex, three binary mixtures (1:1) at the center of the edges, one ternary mixture (1:1:1) on the overall center point, and three axial points (2/3 of each solvent and 1/6 of the other) on the triangular face, providing ten treatments

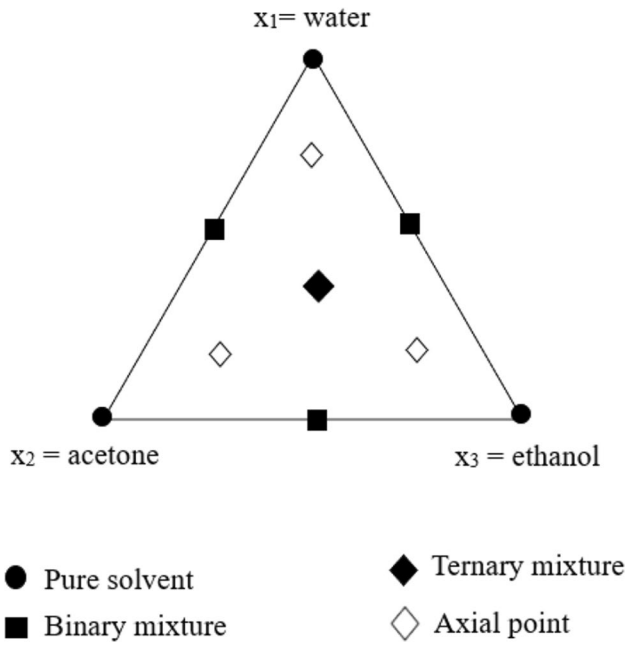

Fig. 1 Simplex-centroid mixture design for the extractor solvent selection of bioactives compounds in jambolan

(samples), as shown in Fig. 1 (Calado and Montgomery 2003). This experimental design was carried out in duplicate, with three repetitions each.

The extraction of bioactive compounds from jambolan was based on the protocol developed by Bochi et al. (2014), with some modifications. The lyophilized powder of the fruit pulp (0.5 g) was mixed with the extraction solvent (25 mL) determined by the experimental design, homogenized with a magnetic stirrer (25 °C, 60 min), sonicated (80 kHz/20 W, 20 min), centrifuged (1200 × *g*, 15 min, room temperature), vacuum-filtered, and re-extracted under the same conditions. The supernatants were collected using the same extraction solvent to a final volume of 50 mL, reaching a proportion of 1:100 ($m{\cdot}v^{-1}$). Then, the extracts obtained were subjected to laboratory analysis.

The responses measured were the total content of polyphenols (Y_1), flavonoids (Y_2), flavanols (Y_3), monomeric anthocyanins (Y_4), and proanthocyanidins (Y_5), as well as the results of the DPPH (Y_6), ABTS (Y_7), and FRAP (Y_8) assays. Based on these results, an optimized solvent system was validated. The optimized extract was concentrated using a rotary evaporator (40 °C, 25 min), lyophilized (30×10^{-3} mmHg, − 50 °C, 24 h), and subjected to identification of the compounds via HPLC–MS.

Analysis of Bioactive Compounds

Total Polyphenol Content

Total polyphenol content was analyzed using the Folin-Ciocalteu method in alkaline medium, measured at 720 nm in a UV–VIS spectrophotometer, and expressed as milligrams of gallic acid/gram of sample (Swain and Hills 1959). The total phenolic content was calculated from the linear regression equation of a standard curve of gallic acid ($y = 0.0047x - 0.004$; $r = 0.9998$), which was prepared at concentrations of 5–180 $\mu g{\cdot}mL^{-1}$ as a function of the optical density for these concentrations.

Total Flavonoid Content

Total flavonoid content was quantified using the aluminum trichloride ($AlCl_3$) method, measured at 510 nm in a UV–VIS spectrophotometer, and expressed as milligrams of sample/gram of catechin (Zhishen et al. 1999). The total content of flavonoids was calculated from the linear regression equation of a standard catechin curve ($y = 3.575x - 0.0079$; $r = 0.9983$), prepared at concentrations of 0.02–0.12 $mg{\cdot}mL^{-1}$ as a function of the optical density for these concentrations.

Total Content of Flavanols

Total flavanols were analyzed using 0.1% DMACA in 1 N HCl in methanol, measured at 640 nm in a UV–VIS spectrophotometer, and expressed as milligrams of catechin equivalent/gram of sample (Arnous et al. 2002). The total flavanol content was calculated from the linear regression equation of a standard catechin curve ($y = 0.0731x + 0.0708$; $r = 0.999$) prepared at concentrations of 2–12 $\mu g{\cdot}mL^{-1}$, as a function of the optical density at these concentrations.

Total Content of Monomeric Anthocyanins

The monomeric anthocyanins were quantified using the pH difference method and measured at 510 nm and 700 nm on a UV–VIS spectrophotometer in buffers with pH 1.0 and 4.5 (Giusti and Wrolstad 2001). The total absorbance of the dilutions was calculated using Eq. 1.

$$A = \left(A_{510} - A_{700}\right)_{pH\ 1.0} - \left(A_{510} - A_{700}\right)_{pH\ 4.5} \quad (1)$$

The concentrations of monomeric anthocyanins were expressed as milligrams of cyanidin-3-glucoside per gram of the sample. The molecular weight (MW) and molar absorptivity (ε) of cyanidin-3-glucoside used were 449.2

and 26,900, respectively (Eq. 2). For greater reliability of the result, the appropriate dilution factor of each sample (DF) was used in the determination total content of monomeric anthocyanins (Giusti and Wrolstad 2001).

$$\text{Monomeric anthocyanins}(\text{mg.L}^{-1}) = (\text{A} \times \text{MW} \times \text{DF} \times 1000)(\varepsilon \times 1)^{-1} \quad (2)$$

Total Content of Proanthocyanidins

The total proanthocyanidin content in the acid-butanol assay was measured at 550 nm using a UV–VIS spectrophotometer and expressed as milligrams of cyanidin chloride per gram of sample (Porter et al. 1986). The total proanthocyanidin content was calculated from the linear regression equation of a standard cyanidin chloride curve ($y = 0.0139x + 0.0262$; $r = 0.9999$) prepared at concentrations of 5–50 μg·mL^{-1}, as a function of the optical density at these concentrations.

Evaluation of the Antioxidant Activity

DPPH· Radical Scavenger Activity

The study of antioxidant capacity through the sequestration of DPPH radicals was carried out according to Kim et al. (2002). Antioxidant capacity was measured at 517 nm using a UV–VIS spectrophotometer, 30 min after the beginning of the reaction, and calculated from the linear regression equation of a standard Trolox curve ($y = 0.0007x - 0.0031$; $r = 0.9999$), prepared at concentrations of 40–800 μmol·L^{-1}, as a function of the optical density for these concentrations. The results were expressed in TEAC as micromole Trolox/gram.

ABTS$^{\bullet+}$ Radical Scavenger Activity

Antioxidant activity was also determined through the sequestration of ABTS$^{\bullet+}$ radicals according to method by Re et al. (1999). The antioxidant activity was measured at 734 nm using a UV–VIS spectrophotometer 6 min after beginning the reaction. The antioxidant activity was calculated from the linear regression equation of a standard Trolox curve (y = 0.0009x—0.0722; r = 0.9985), prepared at concentrations of 25–700 μmol·L^{-1}, as a function of the optical density at these concentrations. The results were expressed in TEAC as micromole Trolox/gram.

Fe^{3+} Reduction Potential (FRAP)

The iron-reducing capacity was evaluated according to the method proposed by Arnous et al. (2002). Samples were reacted for 30 min with an iron (III) chloride solution (3 mM) in a water bath at 37 °C. The acid solution TPTZ was added, and the optical density was subsequently measured at 620 nm in a UV–VIS spectrophotometer after 10 min. The reduction potential was calculated from the linear regression equation of a standard Trolox curve ($y = 0.0023x + 0.1699$; $r = 0.9999$), prepared at concentrations of 30–625 μmol·L^{-1} as a function of the optical density for these concentrations. The results were expressed in TEAC as micromole Trolox/gram of the sample.

Determination of Polyphenols by High-Performance Liquid Chromatography Coupled to Mass Spectrometry (HPLC–MS)

The separation of polyphenols using high-performance liquid chromatography (HPLC) was performed according to the procedures described by De-Melo et al. (2018) and Martínez-Villalba et al. (2013), with modifications. A reverse phase LiChroCART C18 column (250 × 4 mm, 4.5 μm; Merck, Darmstadt, Germany) maintained at 25 °C was used. A Shimadzu Prominence chromatograph (model LC-20ADX, Kyoto, Japan) (Software LabSolutions), equipped with two high-pressure pumps and a diode detector (model SPD-M20A) and thermostat (model CTO-20A) coupled to the mass spectrometer, was also used. The mobile phase was composed of water (solvent A) and methanol (solvent B) that were both acidified with 0.1% formic acid, filtered using a 0.45-μm nylon filter (Whatman, Maidstone, UK), and degassed in a sonicator for 10 min. The mobile phase flow rate was 1 mL.min^{-1}, and the elution in the gradient mode occurred as follows: 0 min, 15% B in A; 20 min, 30% B in A; 40 min, 45% B in A; 45 min, 50% B in A; 50 min, 55% B in A; 65 min, 70% B in A; and 75–80 min, 100% B. Then, the system returned to the initial conditions to stabilize the column.

Mass spectrometry (MS) was used to identify the compounds. We used a Bruker Amazon Speed model mass spectrometer (Billerica, MA, USA) with an ion trap analyzer and electrospray ionization source (ESI) in negative and positive mode, controlled by HyStar software, according to the following conditions: the temperature and voltage of the ionization capillary were set at 230 °C and 3.5 kV, respectively, and the desolvation gas flow (N_2) was 360 L·h^{-1}. The spectra were captured in the range of 100–1000 mass/charge (m/z) ratio. The samples were injected in triplicate, and the phenolic compounds were identified by comparing their molecular ions (m/z) and major fragments with commercial standards or published data in the literature.

Statistical Analysis

All experiments were performed in a random order and replicated to minimize systematic errors. The simplex-centroid experimental design allows linear (β_1, β_2, and β_3), quadratic (β_{12}, β_{13}, and β_{23}), and special cubic (β_{123}) assessments (Eq. 3) for each response under this study (Calado and Montgomery 2003).

$$Y = \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3 \quad (3)$$

The mathematical models were subjected to analysis of variance (ANOVA) and regression, using Statistica software 13.5 (StatSoft, Tulsa, OK, USA). The simultaneous optimization of the response variables was based on the maximum desired response for all variables. The models were validated using Student's t test ($\alpha = 0.05$; 95% confidence interval), in which the average of the experimental values ($n = 3$) was compared with the estimated responses ($n = 3$) of the model.

Results and Discussion

Effects of the System Solvent on the Total Content of Bioactive Compounds and Their Antioxidant Activity

The results obtained from the experiments on solvent system optimization by SCD are shown in Table 1. The models displayed a coefficient of determination (R^2) from 53 to 99% and an adjusted model R^2 from 77 to 98% (Online Resource 1).

As shown in Table 1, low yields of bioactive compounds were obtained using pure solvents or binary mixtures without water. Extraction with water was more efficient than with pure ethanol and acetone, a result attributed to the formation of hydrogen bonds with the polyphenols and the presence of sugars in the chemical structure of these compounds, which improve their solubility (Maran et al. 2014). Pure acetone, a polar aprotic solvent, was not able to form hydrogen bonds, which reduced the solubility of polyphenols, and, therefore, was the least efficient solvent to extract these compounds (Bhebhe et al. 2016). However, the extraction of polyphenols and flavanols with acetone and water in a 0.5:0.5 (v/v) binary mixture produced highest efficiency. In contrast, in the total content of flavonoids, monomeric anthocyanins, and proanthocyanidins, the ternary mixture of water–acetone-ethanol (0.33:0.33:0.33, v/v/v) yielded the best result for these polyphenolic classes. These findings were contrary to those obtained by Chanda and Kaneria (2012).

Based on these results, it was evident that the efficient extraction of a high content of bioactive compounds results from the increase in the polarity index and dielectric constant of the organic solvents, with a subsequent reduction of water parameters. The greater polarity of the solvent system increases the dissolution of polyphenols, including those of medium polarity, such as flavonoids bound to methyl and

Table 1 Proportions of solvent extractors of polyphenols of jambolan (*S. cumini* (L.) Skeels) in accordance with SCD and responses observed

Races	Independent variables*			Response							
	×1	×2	×3	TP	TF	TFl	MA	TPr	DPPH	ABTS	FRAP
1	1.00	0.00	0.00	6.01 ± 0.05	0.76 ± 0.01	0.00 ± 0.00	2.40 ± 0.04	0.89 ± 0.01	48.56 ± 0.07	27.78 ± 0.16	10.39 ± 0.15
2	0.00	1.00	0.00	3.31 ± 0.02	0.90 ± 0.01	0.00 ± 0.00	0.39 ± 0.02	0.38 ± 0.01	31.41 ± 0.28	14.76 ± 0.11	2.54 ± 0.13
3	0.00	0.00	1.00	4.86 ± 0.02	1.28 ± 0.04	0.00 ± 0.00	1.85 ± 0.00	1.13 ± 0.01	41.96 ± 0.14	23.31 ± 0.22	0.61 ± 0.04
4	0.50	0.50	0.00	11.65 ± 0.02	1.61 ± 0.03	0.29 ± 0.02	2.82 ± 0.03	1.92 ± 0.00	80.28 ± 0.07	53.97 ± 0.61	22.32 ± 0.06
5	0.50	0.00	0.50	8.78 ± 0.03	1.33 ± 0.08	0.09 ± 0.00	2.70 ± 0.01	1.72 ± 0.00	64.23 ± 0.35	42.81 ± 0.17	4.36 ± 0.17
6	0.00	0.50	0.50	5.95 ± 0.01	1.37 ± 0.07	0.00 ± 0.00	2.12 ± 0.02	1.38 ± 0.00	48.56 ± 0.35	25.80 ± 0.16	1.51 ± 0.13
7	0.33	0.33	0.33	7.84 ± 0.01	1.82 ± 0.03	0.23 ± 0.02	3.11 ± 0.03	2.04 ± 0.01	76.58 ± 0.07	48.02 ± 0.16	10.18 ± 0.32
8	0.67	0.17	0.17	9.47 ± 0.04	1.50 ± 0.04	0.16 ± 0.01	2.82 ± 0.04	1.10 ± 0.01	70.09 ± 0.28	48.04 ± 0.44	13.46 ± 0.26
9	0.17	0.67	0.17	9.77 ± 0.01	1.60 ± 0.03	0.17 ± 0.01	2.86 ± 0.01	1.53 ± 0.02	70.06 ± 0.14	42.78 ± 2.48	9.28 ± 0.84
10	0.17	0.17	0.67	7.70 ± 0.02	1.46 ± 0.03	0.06 ± 0.00	2.78 ± 0.03	1.74 ± 0.01	59.27 ± 0.21	36.99 ± 0.38	1.55 ± 0.04

*x_1 water, x_2 acetone, x_3 ethanol

Means ± standard ($n = 2$; three repetitions each). Results were expressed in milligrams of gallic acid equivalent.g^{-1} of the lyophilized sample for total polyphenols (TP); milligrams of catechin equivalent.g^{-1} of the lyophilized sample for total flavonoids (TF); milligrams of catechin equivalent.g^{-1} of the lyophilized sample to total flavanols (TFl); milligrams equivalent of cianidina-3-glucoside.g^{-1} of the lyophilized sample for monomeric anthocyanins (MA); milligrams equivalent of cyanidin chloride.g^{-1} of the lyophilized sample for proanthocyanidins (TPr); micromole of Trolox equivalent.g^{-1} of the lyophilized sample for DPPH, ABTS, and FRAP

acetyl radicals. For the same reason, aqueous acetone solutions have a higher extraction yield than hydroethanolic solutions (Bravo et al. 2012).

Regarding the antioxidant action of the extracting solvents, the study showed that the aqueous acetone solution (0.5:0.5, v/v) also provided the best results. The antioxidant action measured by the DPPH and ABTS assays is attributed to polyphenols with a high degree of hydroxylation, such as flavonoids, flavanols, anthocyanins, and proanthocyanidins, which by donating hydrogen atoms can stabilize the free radicals formed. All these compounds showed a strong positive correlation in both tests (Online Resource 2). In the FRAP assay, the compounds that showed highest possible correlation were flavanols ($r_{\text{flavanols}} = 0.81$; $p < 0.05$). Anthocyanins, proanthocyanidins, and other flavonoids showed a low correlation ($r_{\text{anthocyanins}} = 0.48$; $p < 0.05$; $r_{\text{proanthocyanidins}} = 0.33$; $p > 0.05$; $r_{\text{flavonoids}} = 0.33$; $p > 0.05$), suggesting that the reduction of ferric ion (Fe^{3+}) is independent of the concentration of these compounds. These results agree with the results obtained by Slatnar et al. (2012), who attributed the higher antioxidant activity to flavanols due to their molecular structures because of the presence of the O-dihydroxy structure in the B ring, unsaturation in the C ring, and groups 3-OH and 5-OH with 4-oxo function in rings A and C, which are required for the maximum elimination of radicals/ions.

For the successful extraction of polyphenols from different plant matrices, the effect of solvent polarity was qualitatively considered. However, it is important to note that the extraction procedure is also affected by solute/solvent and solvent/solvent interactions. The study of the interactions between organic solvents and the studied responses demonstrated that the extraction solvents that showed a significant synergistic effect in all interactions were water and acetone, showing no need in using more than two solvents in extracting polyphenols from the fruit (Online Resource 3). This evidence agrees with the results presented in this study.

Based on these results, joint optimization of the phenolic compound content and antioxidant activity of the lyophilized jambolan pulp was performed (Fig. 2). This optimization indicated that the maximum response could be estimated using the water–acetone-ethanol (0.5:0.5:0, v/v/v) solution. The choice of solvent system arose from the results obtained, which exceeded 90% of the maximum values of the multiresponse. Thus, we adopted this model to validate the tested methods and characterize their phenolic profiles. This model was considered satisfactory, as it presented experimental values that did not differ significantly ($p > 0.05$) from the estimated values, as shown in Table 2.

The interactions between the extraction solvents and the fruit matrix are comprehensive and complex. This demonstrates that the selection of the most suitable solvent for the extraction of bioactive compounds from the jambolan depends on the edaphoclimatic conditions (climate, relief, lithology, temperature, air humidity, radiation, soil type, wind, atmospheric composition, and rainfall) and the cultural management of the plant, as well as on the interactive chemical components, such as the functional group and length of the pigment chain, in addition to the nutritional composition (Marcheafave et al. 2019). Therefore, no precise recommendation can be made for the use of a specific solvent or mixture of solvents for a particular sample, without a systematic optimization study.

Polyphenolic Profile

The chromatographic and spectrometric characteristics of the polyphenols obtained from the optimized jambolan extract (acetone–water, 0.5:0.5, v/v) are presented in Tables 3 and 4. Detection via ESI–MS in both negative and positive ionization modes was conducted in a complementary manner. A total of 41 compounds were found in this extract, comprising 24 non-anthocyanin polyphenols and 17 anthocyanins. Among the non-anthocyanin compounds, isomers of dihydromyricetin and its methylated forms were identified, which accounted for 37.25% of the compounds present in the fruit, together with caffeic acid (m/z 179) and its derivatives, which represented 34.27% of the compounds (Table 3). This study also demonstrated a strong prevalence of hydroxycinnamic acids and flavonols in jambolan fruit, confirming the results obtained by Tavares et al. (2016) and Lestario et al. (2017). The chromatograms obtained for the compounds by negative ionization are shown in Fig. 3.

Hydroxycinnamic acid has a C6-C3 structure with a double bond in the side chain in *cis* or *trans* configuration, causing the loss of the CO_2 entity [M-H-44] (Heras et al. 2017). However, this was not observed in the present study because the hydroxycinnamic acids identified were characterized by a typical loss of one H_2O molecule (18 u). Caffeic acid and quinic acid (m/z 191) released a fragment with m/z ratios of 161 and 173, respectively. The same activities were observed on the derivatives of these acids due to the presence of water in the extraction solvent, which enabled the formation of inter- and intramolecular hydrogen bonds, facilitating the release of these molecules.

The hydroxybenzoic acids found in the optimized jambolan extract were adducts of protocatechuic acid hexoside with H_2O (m/z = 333), gallic acid 4-O-hexoside (m/z 331), and gallic acid (m/z 169). These acids, while presenting a different chemical structure (C6-C1) from hydroxycinnamic acids, also presented a similar loss $[M\text{-}H\text{-}44]^-$ in MS_2 (Quifer-Rada et al. 2015). However, for monoglycosylated gallic acid, this loss occurred only in MS_3, where m/z 125 ions were present. This change in the fragmentation stage was attributed to the initial release of the glycosidic group in MS_2, generating m/z 169 and postponing

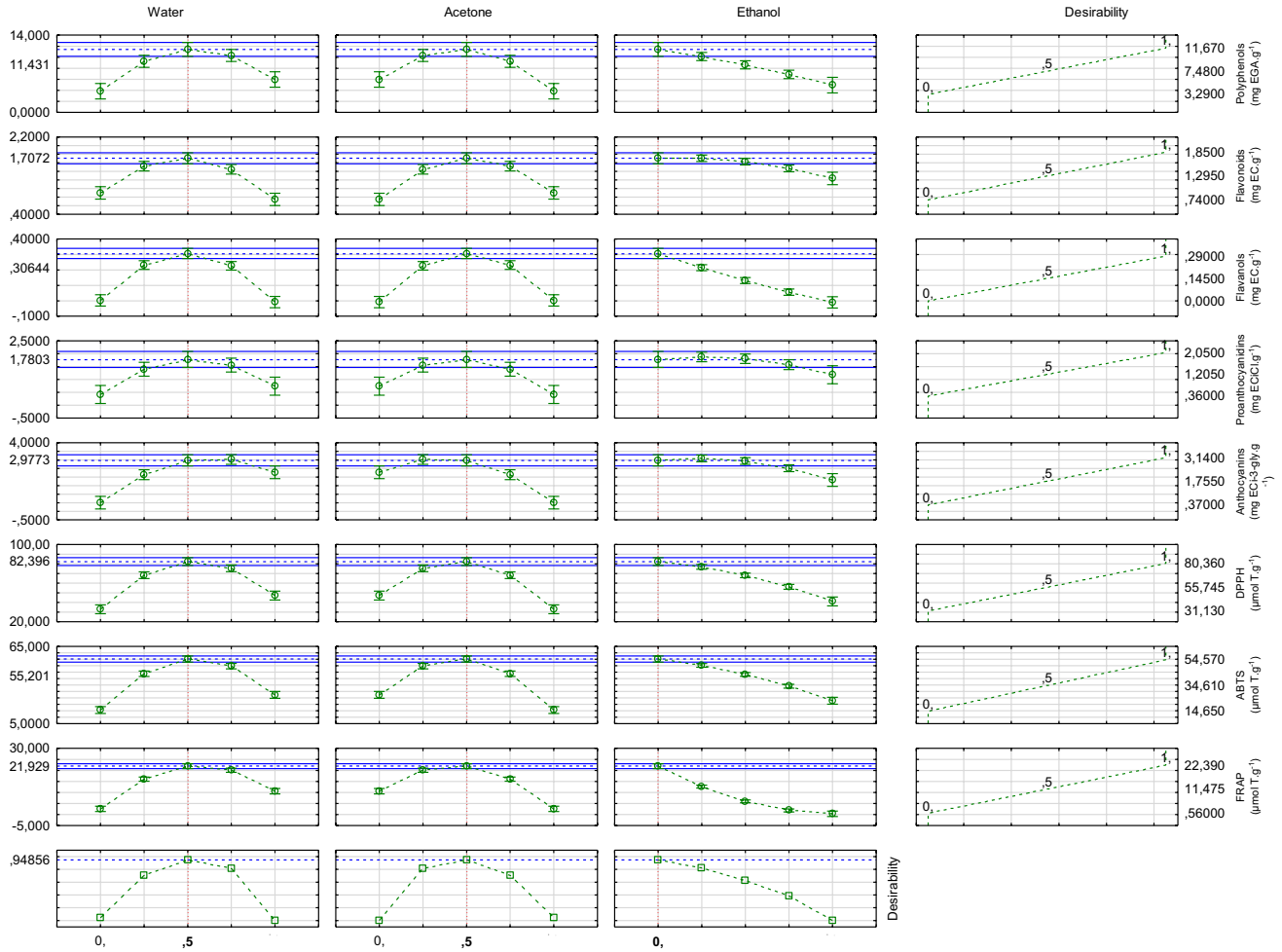

Fig. 2 Profiles of the predicted values of the maximum response as a function of the solvent system for the extraction of bioactive compounds and determination of their antioxidant activity. EGA, equivalent of gallic acid. EC, equivalent of catechin. ECi-3-gli, equivalent of cyanidin-3-glucoside. ECiCl, equivalent of cyanidin chloride equivalent. µmolT = µmol Trolox equivalent

the release of the CO_2 molecule. These findings are similar to those reported by Faria et al. (2011).

In addition to the aforementioned acids, the presence of eight flavonols and isomers of dihydromyricetin was observed. These isomers are characterized by a loss of 162 u, which was attributable to the neutral loss of the dehydrated hexose. The loss of an entire hexose molecule (180 u) is only possible if it was bound to position C3 of the flavanol C ring, resulting in the formation of a double bond between positions C2 and C3 in the ring. In contrast, if the hexose is bound to one of the hydroxylic groups of the A and B rings of flavonol, the hexose loss is possible only by dehydration. Therefore, according to Tavares et al. (2016), the detection of the $[M\text{-hexose-H}]^-$ fragment as the main signal in the MS/MS spectrum suggests that the hexoses are linked to the C5 and C7 positions of the A ring, or to one of the hydroxylic groups of the B ring.

In this study, we further identified the presence of quercetin deoxyhexoside (m/z 447) and epigallocatechin trimer attached to two molecules of H_2O (m/z 949) based

Table 2 Total contents and antioxidant activities of polyphenols, flavonoids, flavanols, monomeric anthocyanins, and proanthocyanidins obtained using the optimal mixture of solvents (50% aqueous acetone)

Response functions	Predicted values	Observed values	*p* value
TP (mg EGA.g^{-1})	11.67	11.88 ± 0.10	0.19
TF (mg EC.g^{-1})	1.85	1.74 ± 0.04	0.14
TFl (mg EC.g^{-1})	0.29	0.33 ± 0.02	0.13
MA (mg ECi-3-gly. g^{-1})	3.14	2.95 ± 0.05	0.06
TPr (mg ECiCl.g^{-1})	2.05	1.97 ± 0.01	0.06
DPPH (µmol T.g^{-1})	80.36	80.76 ± 1.02	0.76
ABTS (µmol T.g^{-1})	54.57	53.63 ± 0.33	0.13
FRAP (µmol T.g^{-1})	22.39	22.73 ± 0.61	0.65

Results expressed as mean ($n = 3$) ± standard deviation. Student's *t* test ($p < 0.05$)

TP total polyphenols; *TF* total flavonoids; *TFl* total flavanols; *MA* monomeric anthocyanins; *TPr* total proanthocyanidins; *DPPH* total antioxidant activity determined by the DPPH test; *ABTS* antioxidant activity determined by testing $ABTS^+$; *FRAP* reducing capacity of the ferric ion Fe^{3+}

Table 3 Identification of polyphenols in jambolan (binary mixture acetone:water, 0.5:0.5, v/v) by HPLC–MS in negative ionization mode

Peak	tR (min)	MS $[M-H]^-/[M-H-H2O]^-$	MS^2	MS^3	Identification proposal	Reference[1]	Relative abundance (%)[2]
1	2,8	412	366	203, 179, 142	Caffeic acid derivative	Wu et al. 2009	0.94
2	3,2	488	341, 146, 179	179, 161	Coffeoil hexose-deoxyhexoside	Rivera-Pastrana et al. (2010)	0.55
3a	3,3	359	179, 161	161, 119, 142	Caffeic acid dimer	Wu et al. 2009	10.89
3b	3,3	341	179, 161	161, 143, 113, 131	Caffeoil hexoside	Wu et al. 2009	10.89
3c	3,3	179	161, 143	161, 143, 119, 131	Caffeic acid	Standard	10.89
4	3,4	295	179, 133, 161	161, 143, 113, 89	Caffeic acid ester	Falcão et al. 2010	0.07
5a	3,6	393	295	179, 133, 161	Caffeic acid derivative	Wu et al. 2009	0.04
5b	3,6	333	241, 171, 153	153, 223, 97	Adduct of protocatechuic acid hexoside with H_2O	Lambert et al. 2015	0.03
6	3,9	289	271, 133	115	Apigenin adduct with H_2O	Ferreres et al. 2003	0.66
7a	4,6	405	387, 191	111, 173, 129	Quínic acid derivative	Bastos et al. 2007	1.84
7b	4,6	191	111, 173	111	Quínic Acid	Standard	1.84
8	4,8	331	271, 169	125, 211, 169	Gallic acid 4-O-hexoside	Heras et al. 2017	3.37
9	4,9	169	125		Gallic acid	Standard	0.42
10	6,1	643 463, 481, 355, 505		301, 283, 355, 319, 337, 256	Dihydromyricetin-1	Tavares et al. 2016	11.15
11	7,2	643 481, 319, 463, 355, 301, 283		319, 301, 355, 329, 257, 233	Dihydromyricetin-2	Tavares et al. 2016	2.29
12	7,6	657 495, 477, 355		333, 315, 369, 495, 297, 282, 257	Methyl dihydromyricetin-1	Tavares et al. 2016	0.38
13	7,7	657 495, 477, 519, 355		333, 369, 333, 315, 271	Methyl dihydromyricetin-2	Tavares et al. 2016	9.65
14	7,8	657 495, 477, 355, 319		315, 297, 333, 369, 315, 282	Methyl dihydromyricetin-3	Tavares et al. 2016	9.65
15	10,7	671 509		347, 261, 371, 261, 189	Dimethyl dihydromyricetin-1	Tavares et al. 2016	3.27
16	10,8	657 495, 333, 355		333, 369, 167, 193, 271, 125	Methyl dihydromyricetin-4	Tavares et al. 2016	0.65
17	11	671 509		347, 371, 329, 401, 303, 261, 189	Dimethyl dihydromyricetin-2	Tavares et al. 2016	0.21
18	27,3	757 549, 491, 653		491, 329, 531, 387	Malvidin 3,5-diglucoside derivative	Lestario et al. 2017	1.55
19	28,8	479 461, 317		271, 287, 179, 244, 151	Myricetin 3-glucoside	Sun et al. 2007	0.32
20	37,5	447 301		179, 151, 271, 229, 273, 107	Quercetin deoxyhexoside	Gordon et al. 2011	11.30
21	44,4	949 904		338, 451, 564, 677, 790, 885	Epigallocatechin trimer bound to $2H_2O$	Costa et al. 2016	7.13

[1]Phenolic compounds were identified by comparing their molecular ions (m/z) and major fragments with commercial standards or published data in the literature

[2]Calculation performed based on the percentage of the area of the chromatogram

Table 4 Identification of anthocyanins in jambolan (binary mixture acetone:water, 0.5:0.5, v/v) by HPLC–MS in positive ionization mode

Compound	MS [M] +	MS^2	Identification proposal	Reference[1]
1	353	335, 317, 232	Petunidine derivative	Standard
2	413	301	Peonidin derivative	Standard
3	449	287, 278	Cyanidin 3-O-glucoside	Flamini et al. (2015)
4	451	436, 357	NI	
5	463	425, 301	Peonidine 3-O-glucoside	Flamini et al. (2015)
6	465	425, 303	Delphinidin 3-O-glucoside	Flamini et al. (2015)
7	479	419, 317	Petunidine 3-O-glucoside	Flamini et al. (2015)
8	493	331	Malvidin 3-O-glucoside	Flamini et al. (2015)
9	611	355, 285	Cyanidin 3,5-diglucoside	Faria et al. (2011)
10	627	465, 457, 303	Delphinidin 3,5-diglucoside	Faria et al. (2011)
11	639	301	Peonidin derivative	Standard
12	641	597, 479, 317, 301	Petunidine 3,5-diglucoside	Faria et al. (2011)
13	655	491, 339, 331	Malvidin 3,5-diglucoside	Faria et al. (2011)
14	697	535, 360, 338	Cyanidin malonylglucoside derivative	Schutz et al. 2006
15	773	724, 423, 395, 387	NI	
16	787	616, 447	NI	

[1]Anthocynins were identified by comparing their molecular ions (m/z) and major fragments with commercial standards or published data in the literature

NI not identified

on the characteristic fragments of MS_2, which were m/z 301 and 904, respectively. The release of m/z 301 in the MS_2 of quercetin deoxyhexoside can be confused with the release of ellagic acid, but any doubts were clarified with the MS_3 fragmentation results. In MS_3 fragmentation, quercetin releases fragments at m/z 271, 255, 179, and 155, while ellagic acid releases only m/z 257 fragments (Gordon et al. 2011; Costa et al. 2016). Therefore, the characteristic fragments found in this study are related to quercetin. The compounds mentioned are found abundantly in the optimized jambolan extract, and together with the other identified compounds, are possibly responsible for the total antioxidant activity of the fruit.

The characterization of anthocyanins in the optimized jambolan extract is presented in Table 4. The characterization by positive ionization mode found sixteen

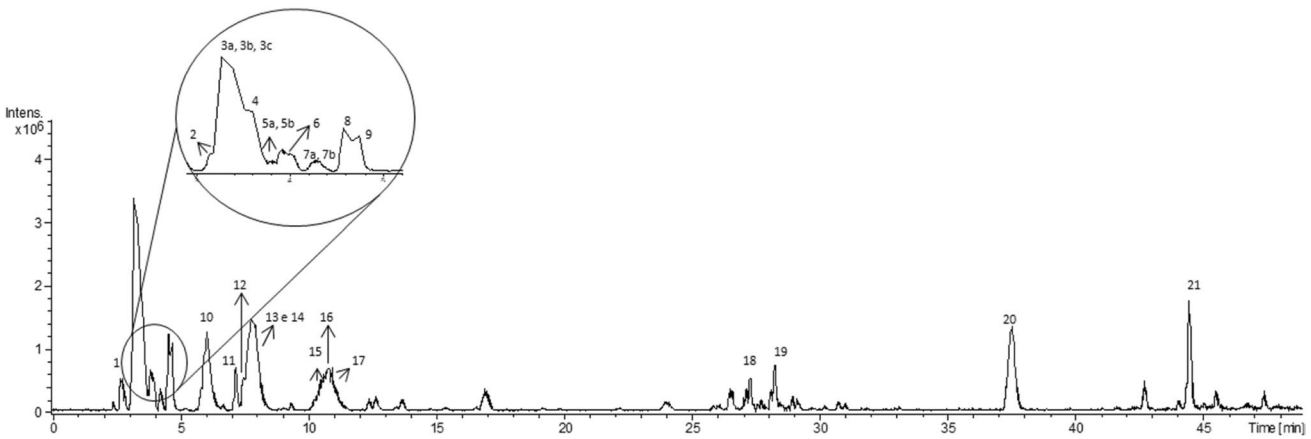

Fig. 3 HPLC–MS chromatogram of polyphenols of acetone:water extract from jambolan (0.5:0.5, v/v) obtained from 200 to 600 nm, negative ionization mode. Peaks: **1**: caffeic acid derivative. **2**: Coffeoil hexose-deoxyhexoside. **3a**: Caffeic acid dimer. **3b**: Caffeoil hexoside. **3c**: Caffeic acid. **4**: Caffeic acid ester. **5a**: Caffeic acid derivative. **5b**: Adduct of protocatechuic acid hexoside with H_2O. **6**: Apigenin adduct with H_2O. **7a**: Quinic acid derivative. **7b**: Quinic acid. **8**: Gallic acid 4-O-hexoside. **9**: Gallic acid. **10**: Dihydromyricetin-1. **11**: Dihydromyricetin-2. **12**: Methyl dihydromyricetin-1. **13**: Methyl dihydromyricetin-2. **14**: Methyl dihydromyricetin-3. **15**: Dimethyl dihydromyricetin-1. **16**: Methyl dihydromyricetin-4. **17**: Dimethyl dihydromyricetin-2. **18**: Malvidin 3,5-diglucoside derivative. **19**: Myricetin 3-glucoside. **20**: Quercetin deoxyhexoside. **21**: Epigallocatechin trimer bound to $2H_2O$

anthocyanins, of which thirteen were identified, including delphinidin (m/z 303), cyanidin (m/z 287), petunidine (m/z 317), peonidine (m/z 301), and malvidin (m/z 331/329) in their mono- or diglycosylated forms and derivatives. Glycosylated anthocyanins have also been identified by Santiago et al. (2016) and Tavares et al. (2017). However, these studies were not able to detect the presence of anthocyanins derived from petunidine (m/z 353), peonidine (m/z 413), and cyanidin (m/z 697) as observed in this study, which were confirmed by co-chromatography with the respective standards.

The extraction of aglycones in their free forms has been previously reported (Santos et al. 2013). However, they were not detected in this study. It is important to note that many bio-polyphenols can be complex with macromolecules present in the cell membrane such as lipids, polysaccharides, and structural proteins (Shahidi and Yeo 2016). To allow the release of the anthocyanin aglycones, the hydrolysis of these glycosides must occur under drastic conditions with high H^+ concentration, heat, and extraction time or through fermentation via gastrointestinal digestion, cleaving covalent bonds, hydrogen bonds, and hydrophobic interactions in the linked phenolics (Maran et al. 2014). These parameters were not used in the extraction of bioactive compounds in this study, due to the possible degradation of other non-anthocyanin polyphenols. This demonstrates that further studies, such as the use of different extraction methods, should be carried out to identify the phenolic and anthocyanin profile of jambolan more accurately.

Conclusions

The maximum yield of bioactive compounds in jambolan and their antioxidant capacity were optimized using a binary mixture of water and acetone in equal proportions. The study also showed that the optimized extract of the fruit includes the main polyphenolic classes with antioxidant action, such as phenolic acids, flavanones, flavonoids, proanthocyanidins, and anthocyanins. Thus, these results establish jambolan fruit as an alternative source of bioactive compounds, especially because of its high content of polyphenols and anthocyanins, which implies its potential commercial application in food coloring products, nutraceuticals, pharmaceuticals, and cosmetics that are beneficial to human health.

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Data and Materials Availability Not applicable.

Code Availability The serial number of Statistica 13.5 software (StatSoft, Tulsa, OK, USA) used in this research was JPZ008H813418FAACD-4.

Declarations

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Informed consent is not applicable to this study.

Conflict of Interest Mariana de Morais Sousa declares that she has no conflict of interest. Alessandro de Lima declares that he has no conflict of interest. Bruno Quirino Araujo declares that he has no conflict of interest. Márcio dos Santos Rocha declares that he has no conflict of interest. Evaldo dos Santos Monção Filho declares that he has no conflict of interest. Renato Pinto de Sousa declares that he has no conflict of interest. Antônia Maria das Graças Lopes Citó declares that she has no conflict of interest. José Augusto Gasparotto Sattler declares that he has no conflict of interest. Lígia Bicudo de Almeida-Muradian declares that she has no conflict of interest. Nadir do Nascimento Nogueira declare that they have no conflict of interest.

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