C190-E286A

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Technical Report

Polyphenolic compounds profiling in guabiroba fruits (Campomanesia xanthocarpa Berg.) by comprehensive two-dimensional liquid chromatography

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Abstract:

This study aimed to evaluate the polyphenolic composition of guabiroba (*Campomanesia xanthocarpa* Berg.) fruits using comprehensive two-dimensional liquid chromatography (LC×LC). A simplex centroid design comprising three solvents (methanol, 2% acetic acid and acetonitrile) was used to optimize the extraction mixture for polyphenols from ripe and unripe guabiroba fruits. An LC×LC platform was proposed to characterize the guabiroba extracts using a RP-Amide column and a C18 column in the first and second dimensions, respectively. Total phenolics compounds were more efficiently extracted with 2% acetic acid solution and acetonitrile (50:50, v/v). A total of 37 different compounds were identified and quantified using the proposed LC×LC method. Significant differences were observed between ripe and unripe guabiroba fruits, especially for the compounds geraldone and methyl galangin isomer.

Keywords: *Campomanesia xanthocarpa,* polyphenols, antioxidant activity, LC×LC, simplex-centroid design

1. Introduction

Among the Brazilian flora, the fruit of guabiroba (*Campomanesia xanthocarpa* Berg.), belonging to Myrtaceae family, has a great potential for commercial cultivation, due to its desirable agronomic characteristics, high yield, abundant and succulent pulp, besides the characteristic flavor and aroma. It also possess interesting nutritional properties, such as high content of vitamin C, mineral salts and phenolic compounds, and thus can be considered a functional food [1].

The characterization of the polyphenolic content of *Campomanesia xanthocarpa* Berg. is somewhat limited since so far only a few works on this fruit were carried out by HPLC-PDA focusing on a few compounds. Considering the potential complexity of the sample, in this work comprehensive two-dimensional LC (LC×LC), was employed. The sample was initially analysed in the first dimension (^{1}D) and afterwards the ^{1}D effluent was transferred into the second dimension (^{2}D) through a modulator or interface, equipped with identical sample loops thus achieving a "comprehensive" separation.

A thorough insight into the composition of such a fruit is quite important in order to expand the possibilities of using this fruit that is highly perishable. In addition, native fruits such as guabiroba lack studies about their beneficial health potential considering the few data present in the literature.

2. Experimental

2-1 Chemicals

LC-MS grade water, methanol, acetonitrile and acetic acid were attained from Merck Life Science (Merck KGaA, Darmstadt, Germany). Gallic acid, catechin, hesperidin, epicatechin, sinapic acid, isoquercetin, isorhamnetin-3-O-glucoside, naringenin, quercetin, apigenin and kaempferol were obtained from Merck Life Science (Merck KGaA, Darmstadt, Germany). Stock solutions for each standard were prepared at a concentration of 1000 mg/ L by dissolving 10 mg in 10 mL of methanol.

2-2 Samples

Guabiroba fruits (*Campomanesia xanthocarpa* Berg) were collected in populations of plants native from the West of Santa Catarina, Brazil (latitude 27°09'46"S, longitude 51°22'58"W). The collections were made after obtaining the required authorizations (Sisgen A4D350D). The fruits were harvested manually and randomly in various positions and orientations of the plants. The unripe fruits were harvested with green colored epidermis, with an average total acidity of 0.68% citric acid and 1.1 °Brix. The ripe fruits had orange skin, average total acidity of 0.47% citric acid and 16.2 °Brix. The guabirobas were selected by the uniformity of color and absence of injuries, were cleaned with tap water and freeze-dried (LIOTOP, L101, São Carlos, Brazil) at -46 °C, with pressure from 15 to 30 μHg.

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2-3 Extraction of polyphenolic compounds and experimental design

Polyphenols were extracted from freeze-dried ripe guabiroba fruits using a simplex-centroid design comprising three pure solvents (x1=methanol; x2=2% acetic acid solution; x3=acetonitrile), three binary mixtures, six ternary mixture, with three replicates from the central point. The response function was expressed as concentration of polyphenolic compounds (mg GAE/100 g). Twelve experiments were generated and executed in random order.

Prior to the extraction of total polyphenolic compounds, the freeze-dried ripe fruits were ground in an electric grinder. Approximately 1.5 g of each sample was used with 15 mL of solvent according to the experimental design (Table S1). The mixtures were then shaken on an orbital shaker (Solab, SL 180/D, Piracicaba, SP, Brazil) for 15 min at 100 rpm. Thereafter, the mixtures were placed in an ultrasonic bath (Cristófoli, Campo Mourão , PR, Brazil) at 25 ºC for 5 min at a frequency of 42 kHz, centrifugated (Centribio, 80-2B, São Paulo, SP, Brazil) for 10 min at 4400 rpm. The organic phase was separated and evaporated in a rotatory evaporator (Quimis, Q344B, Diadema, Sp, Brazil) with controlled temperature at 28 ºC. The extract was resuspended in methanol and filtered through a 0.45-µm Acrodisc nylon membrane (Merck Life Science, Merck KGaA, Darmstadt, Germany).

2-4 Instrumentation and Analytical Conditions

LC×LC analyses were carried out on a Nexera-e liquid chromatograph, equipped with a CBM-20A controller, a LC-Mikros binary pump, two LC-30AD dual-plunger parallel-flow pumps, a DGU-20A5R degassing unit, a CTO-20AC column oven, a SIL-30AC autosampler, an SPD-M30A photodiode array (PDA) detector (1.0 µL detector flow cell volume). Two high speed/high pressure two-position, six-ports switching valves with micro-electric actuator (model FCV-32 AH, 1.034 bar; Shimadzu, Kyoto, Japan), placed inside the column oven and equipped with two 10 µL stainless steel loops, were employed for connecting the two dimensions. For peak identification, the Nexera-e liquid chromatograph was connected to an LC-MS-8050 triple quadruple mass spectrometer, equipped with an ESI source (Shimadzu, Kyoto, Japan).

The LC×LC-LCMS-8050 system and the switching valves were controlled by the Shimadzu LabSolutions software (version 5.93). LC×LC-Assist software (version 2.00) was used for setting up the ^{2}D gradient analyses. The LC×LC data were visualized and elaborated using Chromsquare version 2.3 software. Table 1 details the analytical conditions used in this analysis.

For quantification, eleven standards representatives of the chemical classes under study were selected. Standards calibration curves were prepared in a concentration range from 1.0 to 100 mg/L. The calibration curves with the external standards were obtained using concentrations (mg/L) with respect to the area obtained from the integration of the PDA peaks at a wavelength of 280 nm for benzoic acid-like and flavan-3-ol-like compounds, 320 nm for cinnamic acid-like compounds, 336 nm for flavone-like compounds, 346 nm for flavanone-like compounds and 360 nm for flavonol-like compounds. The compounds for which there was no standard available were semi-quantified

Table 1: Analytical Conditions

[Separation Conditions]

^{1}D Column	: Amide Type (250 mmL.×1.0 mm I.D., 5 µm)
Gradient	: 0 min, 2% B; 5 min, 2% B; 40 min, 40% B; 50 min, 60% B; 60 min, 100% B; 90 min, 100% B
Flow Rate	: 10 µL/min
^{2}D Column	: C18 column (50×4.6 mm I.D., 2.7 µm)
^{2}D Gradient	: I) 10 to 40 min (cycle: 0.01-0.80 min, 10-16% B; 0.81-1.0 min, 10% B) II) 40 to 60 min (shifted from 16%B to 26%B; gradient height 6%) III) 60 to 70 min (cycle: 0.01-0.80 min, 30-50% B; 0.81-1.0 min, 30% B) IV) 70 to 105 min (cycle: 0.01-0.80 min, 50-90% B; 0.81-1.0 min, 50% B)
Gradient Elution	: Segmented-In-Fraction
Flow Rate	: 2.5 mL/min
Modulation	: 1 minute
Loop Volume	: 10 µL
Mobile Phase A	: 0.1% formic acid in H_2O (pH 3)
Mobile Phase B	: 0.1% formic acid in Acetonitrile
Column Temp.	: 30 ˚C
Injection Volume	: 5 µL

[Detection]

Photo Diode Array Detector	
Wavelength Range	: 200-450 nm
Sampling Rate	: 40 Hz
Time Constant	: 0.025 sec

Mass Spectrometry	
MS Ionization Mode	: ESI negative mode
Mass Range	: 100-1200 *m/z*
Event Time	: 0.2 sec
Heat Block Temp.	: 400 ˚C
DL Temp.	: 250 ˚C
Nebulizing Gas Flow	: 3 L/min
Drying Gas Flow	: 15 L/min
Heat Gas Flow	: 10 L/min
Interface Voltage	: 3.5 kV
Interface Temp.	: 300 ˚C
Detector Voltage	: 1.8 kV

3. Results and Discussion

3-1 Effects of the solvent system on phenolic compounds extraction

The quadratic model fitted to the experimental data had determination coefficients (R^2) of 92%. Regarding the phenolic compounds extraction from freeze-dried ripe guabiroba fruits, the binary interaction coefficients (methanol/2% acetic acid solution, methanol/acetonitrile and 2% acetic acid solution/acetonitrile) and ternary (methanol/2% acetic acid solution/acetonitrile) were not significant ($p \leq 0.05$). However, the factors methanol (x1; regression coefficient = 156.05), 2% acetic acid solution (x2; regression coefficient = 239.29) and acetonitrile (x3; regression coefficient = 251.70) were significant ($p \leq 0.05$) for the total phenolic compound's concentration response function (Figure 1A). The combination of solvent used to obtain the highest value for total phenolic compounds (274.49 mg/GAE 100 g) was 0:0.5:0.5 (v/v/v, methanol:2% acetic acid solution : acetonitrile), as seen in the response surface plot (Figure 1B), contour plot (Figure 1C) and profiles for the predicted values and overall desirability as a function of the solvent system (Figure 1D).

The extraction of polyphenolic compounds from different matrices was influenced by the polarity of the solvents and the solubility of the molecules [2]. The polarity index of water was 9.0, while methanol was 6.6 and acetonitrile was 6.2. By using aqueous solvent mixtures like methanol and acetonitrile, the solvent system is able to extract phenolic compounds from high and medium range of polarity [3]. The solvent system (v/v, 0.5:0.5, 2% acetic acid:acetonitrile) was used do validate the model. The model was verified to be significant and appropriate for estimative finalities because the experimentally observed value (276.68 mg GAE/100 g) was not significantly different ($p \leq 0.05$) from the estimated value of the model (274.49 mg GAE/100 g).

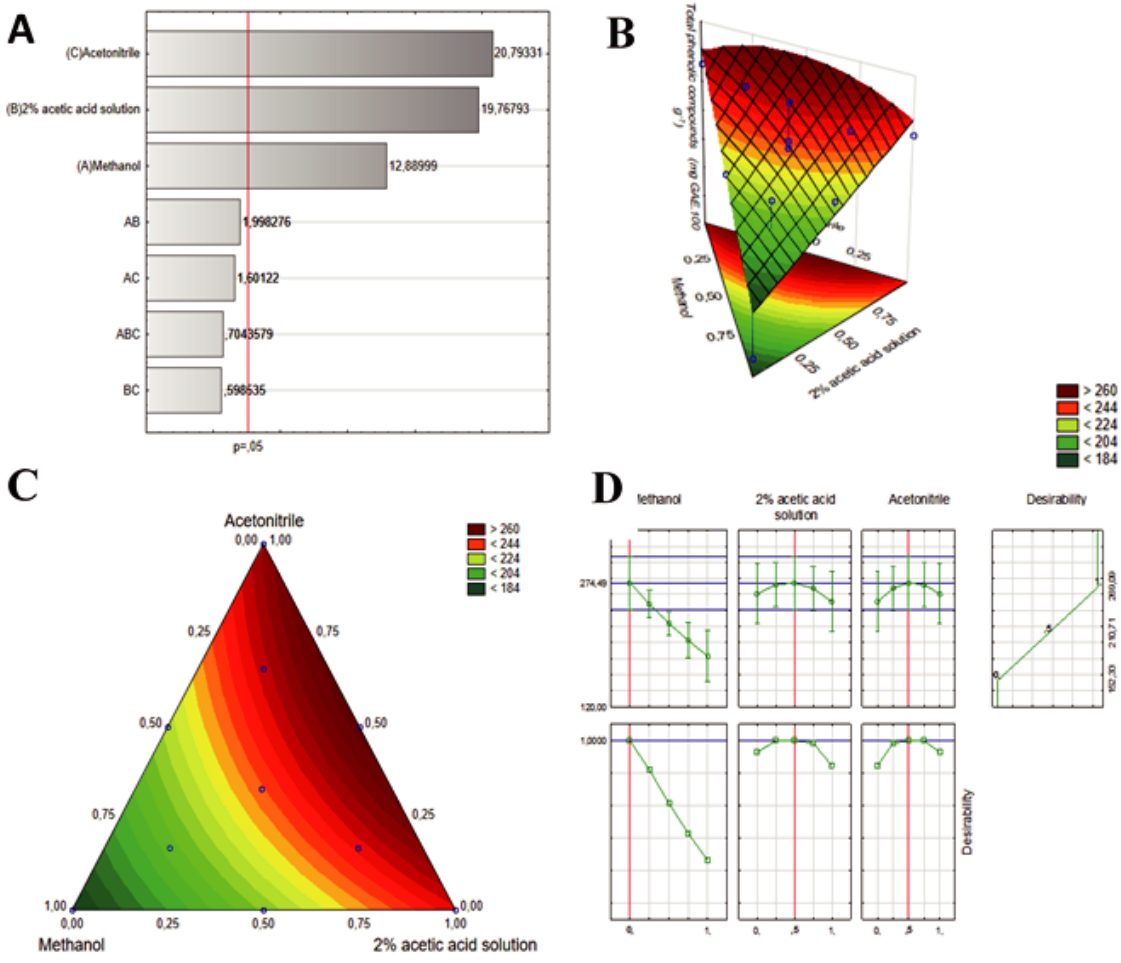

Figure 1: Pareto chart (A), response surface plot (B), contour plot (C) and profiles for the predicted values and overall desirability as a function of the solvent system (D) for extraction of polyphenolic compounds from freeze-dried guabiroba fruits.

3-2 LC×LC method optimization

The first step in determining the polyphenolic compounds occurring in guabiroba fruits was the optimization of the LC×LC methodology. The first approach consisted of the careful selection of several stationary phases to test the potential for the ^{1}D separation. Unripe guabiroba fruits were selected as a model matrix for the development of these experiments, using the extraction method previously optimized in this study. Different conditions have been tested independently, firstly looking at the performance achievable by three different stationary phases in the ^{1}D and then, studying their potential when combined with a C18 column in the ^{2}D Microbore Amide column (250 mmL.×1.0 mm I.D., 5 µm), Phenyl column (250 mmL.×1.0 mm I.D., 5 µm) and Amide type column (250 mmL.×1.0 mm I.D., 5 µm) stationary phases were studied, using the appropriated mobile phases. Among them, the Amide type column provided more separated peaks and better peak shape and thus it was therefore selected for definitive optimization. Once the column for the ^{1}D was selected, the separation conditions were optimized adjusting the gradient to provide wide peaks that could be appropriately sampled into the ^{2}D.

For the ^{2}D optimization, the extract obtained from the matrix model was injected directly into the C18 column (50 mmL.× 4.6 mm I.D., 2.7 µm). In this case, an RP separation mode was selected because it has been shown as the most suitable separation mechanism to provide fast (< 2 min) and efficient separations with shorter column equilibrium times. A target total time cycle of 1 min was established, corresponding to the allotted time for each modulation and it included besides the separation gradient, the column re-equilibration (20 s). Considering the apparent similarity of the separation mechanisms in both dimensions, a tailored ^{2}D elution gradient was developed to improving the diffusion of the solutes in the ^{2}D separation space [4].

A ^{2}D multi-segmented gradient approach, gradually increasing the concentration of organic solvent was investigated. Due to the polarity range of polyphenolic compounds, four gradients were used: the first segment used a gradient slope of 6% per modulation cycle, starting with 10% ACN in the first 40 min and increasing to 16% in 40 min; the second segment used a slope of 10% per modulation cycle, starting with 16% ACN and increasing to 26% ACN in 60 min; the third segment used a slope of 20% per modulation cycle, starting with 30% ACN and increasing to 50% ACN in 70 min; the fourth segment used a slope of 40% per modulation cycle, starting with 50% and increasing to 90% ACN in the last 105 min. Afterwards, unripe and ripe guabiroba samples were analyzed using the previously optimized conditions and the two-dimensional plots (280 nm) of the samples analyzed using this method are shown in Figure 2.

In term of orthogonality [5], the degree was 65% for unripe guabiroba and 52% for ripe guabiroba. Consequently, these values can be considered satisfactory considering the same selection mechanism in the two dimensions. Values of theoretical peak capacity as high as 2826 and 2331 were attained for unripe and ripe samples, respectively. However, to provide more realistic values, the "effective" peak capacity, was also calculated considering the effects of undersamplig which can cause the remix of already separated compounds in the first dimension during the collection of the ^{1}D effluent in the sample loops [6]. Using this approach, values of 803 and 808 for unripe and ripe guabiroba, respectively, were obtained. Finally, the "corrected" peak capacity, considering both orthogonality and undersampling was determined and a maximum limit of 522 peaks for unripe guabiroba and 418 peaks for ripe guabiroba, were attained.

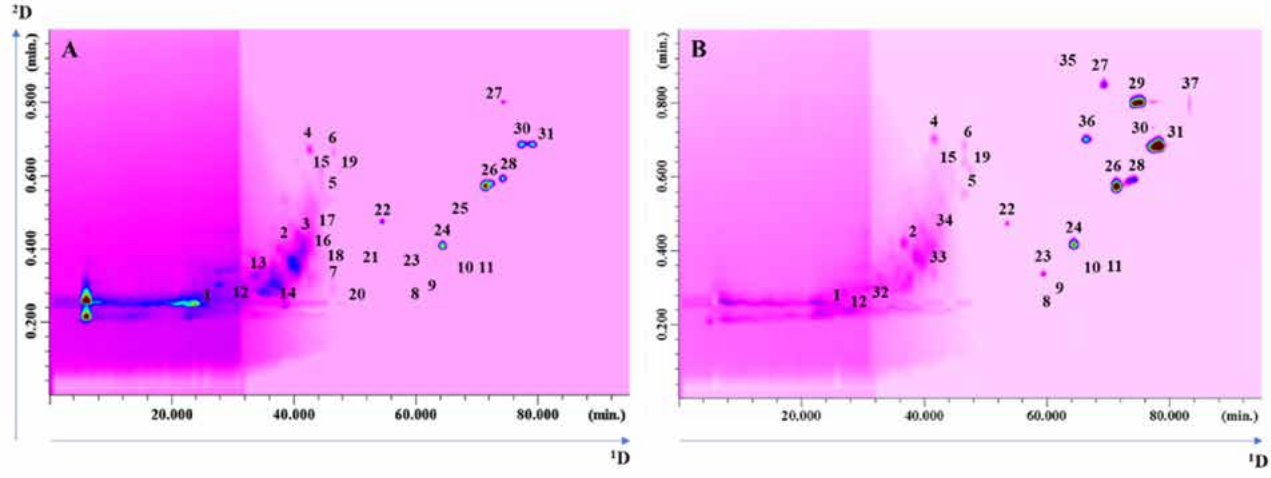

Figure 2: Contour plots (280 nm) obtained using the optimized LC×LC methodology for the analysis of unripe (A) and ripe (B) guabiroba extracts.

In terms of quantification, an evident discrepancy between the ripe and unripe fruit. 37 compounds, through LC×LC method, were positively identified (Table 2) and quantified by combining the information obtained with PDA and MS detection and literature data. Eleven compounds were quantified by authentic standard; on the other hand, when the reference standards were not available, each compound was quantified with the commercial standard belonging to the same chemical family. The compounds occurring in the samples can be divided in different chemical classes, 10 out of them to flavonols, 9 to flavones, 6 to phenolic acids, 5 to flavan-3-ols, 4 to phenones, 2 to flavanones and 1 to another class. Methyl galangin isomers (no.29,30) were the most abundant in ripe samples (151.66 mg/100 g and 142.24 mg/100 g). Galangin and methyl galangin were previously reported in fruits of the Myrateceae family, such as Eugenia catharinensis [7] and Eugenia brasiliensis [8].

They are formed by the phenylpropanoid pathway, thus they are derived from the amino acid phenylalanine. Phenylpropanoids are some of the most important sets of secondary metabolites in plants, especially in fruit ripening, which explains the considerable increase in the methyl galangin concentration in ripe fruits. The production of this flavonol has as its precursor cinnamic acid, which is converted to cinnamoyl-CoA by 4-coumaroyl-CoA ligase (4CL), forming pinocembrin, which is converted to methyl galangine by the action of flavanol synthase (FLS) [9]. According to Lee et al [10], galangins may be promising therapeutic agents against inflammation. The other most abundant compounds are represented by geraldone (no. 26) (133.36 mg/100 g and 50.31 mg/100 g in unripe fruit and unripe fruit) and cirsimaritin (no. 36) (49.48 mg/100 g in unripe fruit).

Table 2: Polyphenolic compounds identified in the Campomanesia xanthocarpa Berg. fruits investigated by LC×LC-PDA-MS

Peak numbering	Compound	$[M-H]^-$	^{1}tR (min)	^{2}tR (min)	Guabiroba unripe	Guabiroba ripened
1	Gallic acid [a]	169	26	0.256	X	X
2	Catechin [a]	289	37	0.428	X	X
3	Hesperidin [a]	609	42	0.443	X	–
4	Epicatechin [a]	289	42	0.703	X	X
5	Quercetin 3-O-glucoside [a]	463	47	0.556	X	X
6	Isorhamnetin 3-O-glucoside [a]	477	47	0.685	X	X
7	Sinapic acid [a]	223	48	0.289	X	–
8	Naringenin [a]	271	60	0.337	X	X
9	Quercetin [a]	301	66	0.383	X	X
10	Kaempferol [a]	285	67	0.384	X	X
11	Apigenin [a]	269	69	0.377	X	X
12	Galloyl-bis-HHDP-hexoside [b] (casuarinin)	935	28	0.237	X	X
13	5-p-coumaroylquinic acid [b]	337	34	0.340	X	–
14	Quercetin 3-O-arabinopyranoside (guajaverin) [b]	433	39	0.252	X	–
15	Myrciaphenone B [b]	481	43	0.648	X	X
16	Epicatechin gallate [b]	441	44	0.413	X	–
17	Quercetin 3-O-xyloside (reynoutrin) [b]	433	44	0.455	X	–
18	Myricetin 3-O-rhamnoside [b]	463	47	0.376	X	–
19	Kaempferol 3-O-glucoside [b]	447	48	0.627	X	X
20	Ellagic acid-rhamnoside [b]	447	49	0.251	X	–
21	Isorhamnetin 3-O-rhamnoside [b]	461	52	0.336	X	–
22	Guavinoside B [b]	571	54	0.476	X	X
23	7,3',4'-trihydroxyflavone [b]	269	60	0.337	X	X
24	Biochanin A [b]	283	65	0.419	X	X
25	Arbutin [b]	271	67	0.495	X	–
26	Geraldone [b]	283	72	0.580	X	X
27	Galangin [b]	269	74	0.805	X	X
28	Hispidulin [b]	299	75	0.593	X	X
29	Methyl galangin isomer [b]	283	76	0.801	–	X
30	Methyl galangin isomer [b]	283	77	0.681	X	X
31	Sativol [b]	297	79	0.684	X	X
32	p-Coumaric acid ethyl ester [b]	191	29	0.276	–	X
33	Epigallocatechin [b]	305	40	0.361	–	X
34	Epigallocatechin 3-O-gallate [b]	457	42	0.436	–	X
35	Hesperitin [b]	301	62	0.919	–	X
36	Cirsimaritin [b]	313	67	0.705	–	X
37	[6]-gingerol [b]	299	84	0.805	–	X

[a] identified with commercial standards
[b] identified using PDA, MS and literature data ("tentatively identified")

Conclusions

A simplex-centroid design proved to be an efficient tool for the optimization of the extraction of phenolics from guabiroba fruits. The extraction mixture comprised of aceteonitrile:2% acetic acid (0.5:0.5, v/v) can be used to obtain extracts with high phenolics content from guabiroba. In addition, this is the first time that a quantitative LC×LC method has been implemented to characterize the phenolic fraction of ripe and unripe guabiroba fruits. 37 different compounds separated and detected in the extracts studied were quantified. The polyphenolic composition of ripe and unripe guabiroba was strongly different, especially regarding the concentration of flavonoids. More research is needed to fully assess the real biological activity of polyphenols and other secondary metabolites presents in the fruits to correlate with biological activities.

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