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ORIGINAL ARTICLE

Bioactive compounds of *Eugenia punicifolia* fruits: a rich source of lycopene

Compostos bioativos de frutos de Eugenia punicifolia*: uma rica fonte de licopeno*

Elaine Cristina de Oliveira Braga[1], Sidney Pacheco[2], Manuela Cristina Pessanha de Araujo Santiago[2], Ronoel Luiz de Oliveira Godoy[2], Monalisa Santana Coelho de Jesus[2]*, Víctor de Carvalho Martins[3], Marcelo da Costa Souza[4], Alexandre Porte[1], Renata Galhardo Borguini[2]

[1]Universidade Federal do Estado do Rio de Janeiro, Departamento Ciência de Alimentos, Rio de Janeiro/RJ - Brasil
[2]Embrapa Agroindústria de Alimentos, Laboratório de Cromatografia Líquida, Rio de Janeiro/RJ - Brasil
[3]Universidade Federal Rural do Rio de Janeiro, Instituto de Tecnologia, Departamento de Ciência e Tecnologia dos Alimentos, Seropédica/RJ - Brasil
[4]Universidade Federal Rural do Rio de Janeiro, Instituto de Biologia e Ciências da Saúde, Departamento de Botânica, Seropédica/RJ - Brasil

***Corresponding Author:** Monalisa Santana Coelho de Jesus, Embrapa Agroindústria de Alimentos, Laboratório de Cromatografia Líquida, Avenida das Américas, 29.501, Guaratiba, CEP: 23020-470, Rio de Janeiro/RJ - Brasil, e-mail: monalisa.coelho@embrapa.br

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Abstract

This work aimed to characterize some of the bioactive compounds of *Eugenia punicifolia* (Kunth) DC. fruit to enhance the knowledge of its functional potential. Ripe fruits were collected from the restinga of Maricá, in the state of Rio de Janeiro (RJ), Brazil. Bioactive compounds were analyzed by High Performance Liquid Chromatography (HPLC). Ascorbic acid (74.14 mg 100^{-1} g^{-1}), lycopene (504 µg g^{-1}) and total carotenoids (632 µg g^{-1}) contents were superior to other fruits rich in these compounds. In fact, *E. punicifolia* fruits are an excellent source of carotenoids and can be considered a good source of ascorbic acid (vitamin C). Furthermore, its chemical composition has presented phenolic compounds like gallic acid and anthocyanins. Thus, this underutilized Brazilian fruit stands out as a source of bioactive compounds, presenting a good potential as a functional food, especially due to the high content of lycopene.

Keywords: Myrtaceae; Carotenoid; Phenolic acid; Ascorbic acid; Lycopene; HPLC-PDA; Pigments.

Resumo

Este trabalho teve como objetivo caracterizar alguns dos compostos bioativos do fruto da *Eugenia punicifolia* (Kunth) DC. para aprofundar o conhecimento do seu potencial funcional. Frutos maduros foram coletados da restinga de Maricá-RJ, Brasil. Os compostos bioativos foram analisados por cromatografia líquida de alta eficiência. Os teores de ácido ascórbico (74,14 mg 100^{-1} g^{-1}), licopeno (504 µg g^{-1}) e carotenoides totais (632 µg g^{-1}) foram superiores aos de outras frutas ricas nesses compostos. Os frutos de *E. punicifolia* são uma excelente fonte de carotenoides e podem ser considerados uma boa fonte de ácido ascórbico (vitamina C). Além disso, sua composição química apresentou compostos fenólicos, como ácido gálico e antocianinas. Assim, essa fruta brasileira subutilizada

se destaca como fonte de compostos bioativos, apresentando um bom potencial como alimento funcional, principalmente devido ao alto teor de licopeno.

Palavras-chave: Myrtaceae; Carotenoide; Ácido fenólico; Ácido ascórbico; Licopeno; CLAE-DAD; Pigmentos.

Highlights

- *E. punicifolia* fruits are a richer source of carotenoids than tomatoes and their products
- *E. punicifolia* is an underutilized species with the potential for use as a functional ingredient in natural and healthy products
- A small fruit of the Brazilian hotspots of the Restinga ecosystem with great contents of colorful bioactive compounds was chemically characterized in order to promote the knowledge, use and preservation of the species

1 Introduction

Brazil is one of the countries with the highest biodiversity on the planet. This abundant variety of life, which is more than 20% of the total number of species on Earth, puts Brazil at the top of the list of 17 nations with mega-biodiversity (Brasil, 2015). Among the species found in the Brazilian flora, those belonging to the Myrtaceae family are important for the production of pharmaceutical substances (Leitão et al., 2014).

The Myrtaceae family has significant economic potential. Several of its species contain edible fruits much appreciated by humans and wildlife, such as guava (*Psidium guajava* L.), jabuticaba (*Plinia cauliflora* (DC.) Kausel), and pitanga (*Eugenia uniflora* L.) (Lorenzi et al., 2015). However, these species represent only a small portion of the potential of this family that includes other Myrtaceae species (Landrum & Kawasaki, 1997), such as *E. punicifolia* (Kunth) DC and its edible fruit (Figure 1).

Figure 1. *Eugenia punicifolia* (Kunth) DC fruits.

Eugenia punicifolia is a neotropical specie with high occurrence in Brazil, popularly known as beach cherry or field cherry. The *E. punicifolia* tree can grow up to 8 meters high (Silva & Pinheiro, 2007; Lorenzi et al., 2015). Its flowers are white, with a flowering period from March to June, and fruiting occurs from October to February. It produces a small and protruding berry-like fruit, which has an intense red color when ripe (Souza & Morim, 2008; Lorenzi et al., 2015), and has aroused interest, since red is a characteristic color of bioactive compounds, especially carotenoids.

The majority of studies concerning *E. punicifolia* species have been related to the botanical characteristics and the characterization and therapeutic application of the essential oils. There is a lack of scientific data concerning the chemical characterization of the fruits. Therefore, this work aimed to characterize the bioactive compounds of *E. punicifolia* fruits, using High Performance Liquid Chromatography (HPLC) to determine its carotenoids, ascorbic acid, anthocyanins, and phenolic acid profile.

2 Material and methods

2.1 Plant material

In fact, *E. punicifolia* fruits were collected in the restinga of Maricá (22°56'57.4"S 42°53'14.2"W), in the state of Rio de Janeiro, Brazil. The fruits were botanically identified using the voucher specimen (RB554365), filed in Rio de Janeiro Botanical Garden Herbarium, RJ, Brazil. The study was conducted with a sample of approximately 500 g of ripe fruits collected from eight (8) plants. The fruits were selected according to their stage of maturity (red ripe fruits), sanitized, and manually separated into parts (pulp and seed), and stored at -18 °C until analysis. Ascorbic acid (vitamin C) was extracted and analyzed on the same day due to its chemical instability. All analyses were performed in triplicate.

2.2 Chemicals and standards

All solvents were HPLC grade. Methanol, acetonitrile, acetone, petroleum ether, and methyl *tert*-butyl ether were purchased from Tedia® (Brazil). Sulfuric acid (98%), hydrochloric acid (37%), and phosphoric acid (85%) were purchased from Tedia® (Brazil). Standards of ascorbic acid (99%) and phenolic acids (95%) were purchased from Sigma Aldrich® (EUA). Carotenoid and anthocyanin standards were obtained through isolation from natural sources by HPLC (purity grade > 95%) at the Embrapa Food Technology Laboratory (Rio de Janeiro, Brazil), according to Kimura & Rodriguez-Amaya (2002) and Gouvêa et al. (2012), respectively. Ultrapure water (0.054 µS cm^{-1}) was obtained using a Milli-Q system (Millipore®, Milford, MA, USA). Mass spectrometry grade solvents (acetonitrile and formic acid) from Merck® (Darmstradt, Germany) were used for Mass Spectrometry (MS) analysis.

2.3 Carotenoid analysis

The extraction was performed according to Rodriguez-Amaya (2001). Approximately 1 g of pulp and seed samples was macerated with celite and acetone. The extraction procedure was repeated until the sample no longer exhibited the characteristic color of carotenoids. Subsequently, the acetone extract was partitioned with petroleum ether. The ether fraction was filtered through anhydrous sodium sulfate and transferred to a volumetric flask.

The ether extract was saponified with potassium hydroxide (KOH) solution 10% in methanol (10:90, v/v) at room temperature and a reaction time of 16 h. Later, the level of total carotenoids in the saponified sample extract was determined by spectrophotometry at 450 nm. Carotenoid profiles were determined by HPLC as described by Pacheco et al. (2014) using a Waters HPLC system, C30 YCM S-3 carotenoid column (4.6 mm × 250 mm), gradient elution of methanol and methyl *terc*-butyl ether with photodiode array (PDA) detector. The flow rate was 0.8 mL min^{-1}, and the run time was 28 min. The carotenoids were identified by comparing their retention times and UV/Vis absorption spectra with those of the carotenoid standards. The quantification was performed by external standardization with analytical standards.

2.4 Ascorbic acid analysis

Ascorbic acid (vitamin C) was determined by the method described by Rosa et al. (2007). Pulp and seed samples were weighed, and 10 mL of an aqueous solution of H_2SO_4 0.05 Mol L^{-1} was added. The extraction proceeded in an ultrasonic bath for 10 min. The samples were filtered and the extracts were subjected to chromatographic analysis using HPLC Alliance Waters model 2690/5, ion exchange HPX 87 H BIO RAD (7.8 x 300 mm) column, isocratic elution with an aqueous solution of H_2SO_4 0.05 Mol L^{-1} and PDA Waters model 2996. The flow rate of 0.7 mL min^{-1} and run time of 10 min were employed. Ascorbic acid was quantified by external standardization.

2.5 Phenolic acid analysis

Phenolic acids were extracted using the method described by Mattila & Kumpulainen (2002). The analysis of free phenolic acids was performed by extracting pulp and seed in an ultrasonic bath for 30 min, with 10 mL of a methanol solution containing butylhydroxytoluene (BHT) (2 g L^{-1}): 10% acetic acid in water (85:15 v/v), obtaining the free phenolic acid fraction. Then, 17 mL of a 3 Mol L^{-1} NaOH aqueous solution was added to the extract, followed by mechanical stirring for 16 hours. The pH was adjusted to 2.0 with a 6 Mol L^{-1} HCl aqueous solution for subsequent partitioning with 15 mL of ethyl ether: ethyl acetate (1:1 v/v) solution in a separating funnel, resulting in the basic hydrolysis fraction. To obtain the acid hydrolysis fraction, 2.5 mL of concentrated HCl was added to the aqueous phase, followed by heating in an oven at 85 °C for 35 min. The pH was adjusted to 2.0 with a 3 Mol L^{-1} NaOH aqueous solution to a new partition with ethyl ether: ethyl acetate (1:1 v/v), resulting in the acid fraction. Three fractions were analyzed in a HPLC Alliance Waters model 2690/5, Thermo HYPERSIL BDS C_{18} column (100 mm x 4.6 mm x 2.4 um), elution gradient, using an aqueous solution of 0.0015% phosphoric acid and acetonitrile with photodiode array detector model 2996. Phenolic acids were identified by comparing their retention times and the UV/Vis absorption spectra with those of commercial standards. The quantification of phenolic acids was performed by external standardization.

2.6 Anthocyanin analysis

The anthocyanins were extracted in an ultrasonic bath using 1 g of sample and 10 mL of methanol: formic acid solution (90:10 v/v). This step was repeated until the absence of characteristic color in the extraction solution. Subsequently, an aliquot of the extract was dried and solubilized in 5% formic acid solution in water: methanol (90:10 v/v) (Brito et al., 2007). Analyses were carried out on a Waters Alliance 2695 system, Thermo Scientific C^{18} BDS (100 mm × 4.6 mm; 2.4 µm) column, using a gradient elution method with formic acid and acetonitrile with a PDA. All chromatographic conditions were adopted according to Gouvêa et al. (2015). The main anthocyanins were identified by comparing their retention times and UV/Vis absorption spectra with those of the standards. This identification was confirmed through isolation and direct injection of each anthocyanin into a High-Resolution Mass Spectrometer (HRMS). The quantification was performed by external standardization.

2.7 Mass spectrometry anthocyanin analysis

The main anthocyanins present in *E. punicifolia* fruits were individually collected from the HPLC system. Then, each anthocyanin was directly injected into a Synapt Waters ESI-qTOF HRMS. The electrospray ionization (ESI) source was operated in the following conditions: positive mode, capillary voltage at 3.0 kV, sampling cone energy at 25.0 V, extraction cone energy at 4.0 V, temperature of 120 °C, and N_2 as the desolvation gas delivered at 12.5 L min^{-1} at 500 °C. Accurate mass and fragmentation patterns by MS-MS were employed to confirm the structural formula of each anthocyanin.

2.8 Data analysis

Quantification analyses were carried out with three replicates. All results were expressed as mean ± standard error of the mean (SEM). Results were reported on a wet basis.

3 Results and discussion

3.1 Carotenoid content

The pulp of the *E. punicifolia* fruit presented high content of carotenoids (632 ± 4.20 μg g^{-1}), with lycopene as the main carotenoid, corresponding to 85% (503 ± 3.60 μg g^{-1}) of the total carotenoids, followed by β-cryptoxanthin (32 ± 4.50 μg g^{-1}), β-carotene (5.70 ± 0.56 μg g^{-1}), zeaxanthin (4.45 ± 4.30 μg g^{-1}), and lutein (3.20 ± 1.76 μg g^{-1}). Figure 2 shows the chromatogram of the carotenoid profile of the saponified extracts. This reaction is important for the release of xanthophylls (hydroxylated carotenoids), such as β-cryptoxanthin, which present pro-vitamin A activity (Rodriguez-Amaya, 2010), lutein and zeaxanthin, which are considered the main carotenoids associated with a risk reduction of cataracts and macular degeneration (Delcourt et al., 2006; Trieschmann et al., 2007). Carotenoids were not found in the seed.

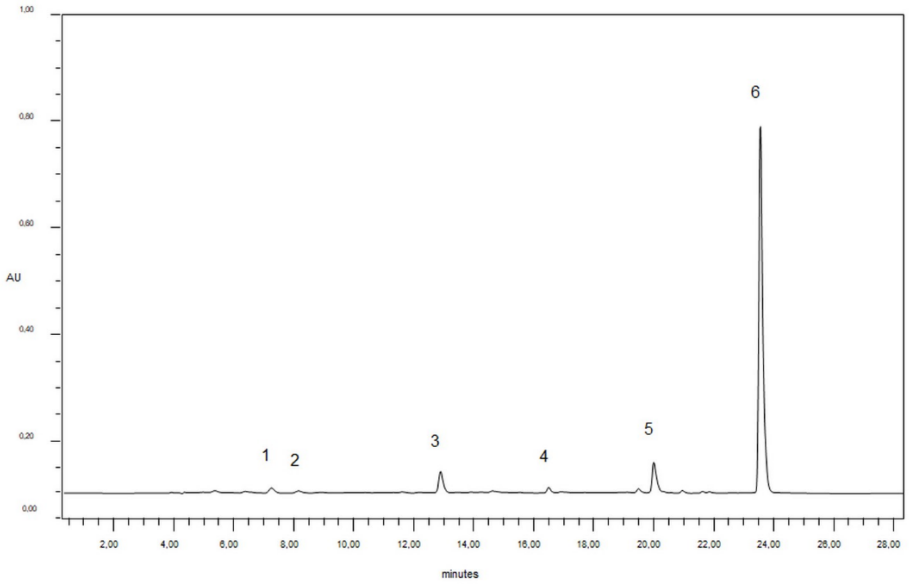

Figure 2. Chromatogram of carotenoids obtained from the *E. punicifolia* pulp extracts at 450 nm after saponification. Peak identification: (1) lutein, (2) zeaxanthin, (3) β-cryptoxanthin, (4) β-carotene, (5) *cis*-lycopene, and (6) lycopene.

The fruit is shown to be a significant source of lycopene since it has a concentration approximately six times higher than that found in pitanga (*E. uniflora* L.), with 76 μg g^{-1} and guava (*P. guajava* L.), with 70 μg g^{-1} (wet basis), which are fruits belonging to the same family and, in the case of the pitanga, the same genus as *E. punicifolia*. It also showed a concentration about 15 times higher when compared to other fruits considered good sources of lycopene and commonly consumed in Brazil, such as watermelon (*Citrillus lanatus* (Thunb.) Matsum. & Nakai), with 36 μg g^{-1} and tomato (*Lycopersicum esculentum* L.), with 35 μg g^{-1} (wet basis) (Rodríguez-Amaya et al., 2008). The lycopene content found in the *E. punicifolia* fruit (503 μg g^{-1}) is much higher than that found in products considered an excellent source of lycopene, such as tomato pulp (93 μg g^{-1}), tomato sauce (100 μg g^{-1}), and ketchup (155 μg g^{-1}) (wet basis) (Rodríguez-Amaya et al., 2008; Oliveira et al., 2011).

Therefore, the *E. punicifolia* fruit can be considered an excellent source of lycopene and has high potential as a functional food since the consumption of lycopene is associated with a lower risk of chronic diseases like some cancers, especially prostate cancer (Cheung et al., 2003; Clinton, 1998; Giovannucci, 2005; Chen et al., 2013).

3.2 Ascorbic acid (vitamin C)

The pulp of the *E. punicifolia* fruit showed 74.2 ± 3.6 mg 100^{-1} g^{-1} of ascorbic acid, presenting a similar content compared to guava fruit (80.6 mg 100^{-1} g^{-1}), considered a good source of vitamin C, and higher than jabuticaba (*Myrciaria cauliflora* O Berg) (16.2 mg 100^{-1} g^{-1}) (Tabela Brasileira de Composição de Alimentos, 2011), both fruits belonging to the Myrtaceae family. This substance was not detected in the seeds.

According to the Recommended Daily Intake (RDI) for vitamin C (45 mg for an adult) (Food and Agriculture Organization, 1998; U.S. Department of Agriculture, 2000; Brasil, 2005), *E. punicifolia* fruit may be considered as a source of this nutrient.

Vitamin C acts as a preservative agent in food and participates in many metabolic processes in the body, such as collagen formation and synthesis of bile acids and acting as an enzyme cofactor, therefore, extremely important for health (Du et al., 2012).

3.3 Phenolic acid content and profile

The phenolic compounds are commonly observed as soluble and insoluble conjugated through covalent bonds with sugars or some cell structural components (Cheung et al., 2003; Acosta-Estrada et al., 2014). Thus, the analysis of phenolic acids was carried out in three steps. The first was the extraction of free phenolic acids, which releases the acids that are not linked to other substances, followed by basic hydrolysis providing most of the linked phenolic acids, and then acid hydrolysis, to release the remaining acids that were not released in the previous steps.

No phenolic acids were identified in the free fraction. Five phenolic acids were identified in the fraction obtained by basic hydrolysis of the pulp extract: gallic, syringic, *p*-coumaric, ferulic, and ellagic acids (Figure 3a). The fraction obtained using acid hydrolysis of the pulp extract presented only gallic and ellagic acids (Figure 3b). While for seed, the fraction obtained by acid hydrolysis had protocatechuic acid in addition to the acids mentioned in the pulp, making six acids identified in the two fractions (Figure 4a-4b). Ramos et al. (2019) also detected the presence of gallic and ellagic acids in the extract of *E. punicioflia* fruits, besides myricetin 3-rhamnoside and quercitrin.

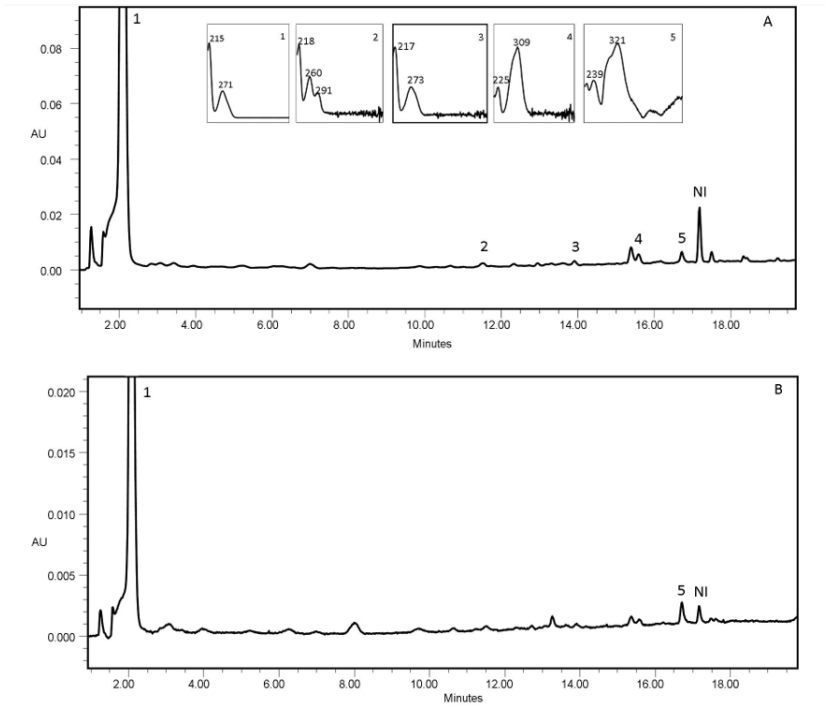

Figure 3. Chromatogram of phenolic acids in the pulp extract of the *E. punicifolia* fruit obtained after basic hydrolysis (A) and acid hydrolysis (B) at 270 nm. Peak identification: (1) gallic acid, (2) syringic acid, (3) *p*-coumaric acid, (4) ferulic acid, (5) ellagic acid, (NI) not identified.

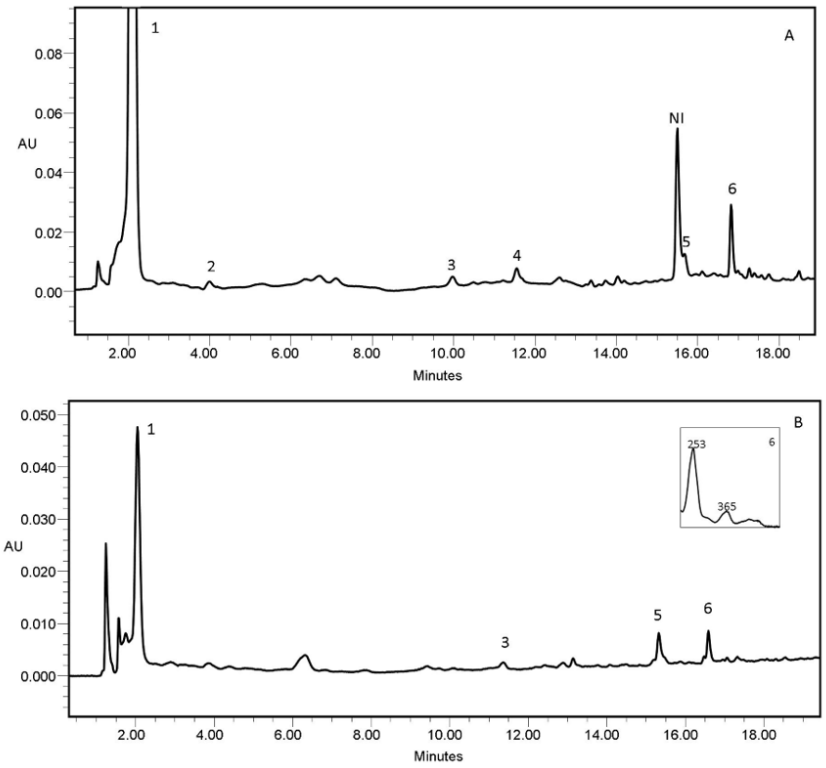

Figure 4. Chromatogram of phenolic acids in the seed extract of the *E. punicifolia* fruit obtained after basic hydrolysis (A) and acid hydrolysis (B) at 270 nm. Peak identification: (1) gallic acid, (2) protocatechuic acid, (3) syringic acid, (4) *p*-coumaric acid, (5) ferulic acid, (6) ellagic acid, (NI) not identified.

Gallic acid is the predominant acid (95%) among the phenolic acids present in the *E. punicifolia* fruit. The other acids accounted for only 2% of the fruit.

The gallic acid content found in the pulp was 1.46 mg g^{-1}, while in the seed, it was 1.54 mg g^{-1}. Compared with the results obtained by Inada et al. (2015) for jabuticaba (1.98 mg g^{-1}), the *E. punicifolia* fruit had similar content.

The gallic acid and the other phenolic acids act as reducing agents, sequestering free radicals from the body and contributing as metal chelators. Fruits, especially with red to purple colors, are the most important sources of these phenolic compounds in food (Mertz et al., 2009; Wang et al., 2014).

3.4 Anthocyanin content and profile

Anthocyanins, which are important components of fruits, are phenolic compounds with beneficial health functions, acting against free radicals that promote chronic diseases and reducing the risk of cancer due to their antioxidant capacity (Sumner et al., 2005).

Two anthocyanins were identified in the *E. punicifolia* fruit pulp, delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside. The first one corresponds to 57% of the total monomeric anthocyanin contents (Figure 5). The pulp had an average total monomeric anthocyanin content of 11.6 mg 100^{-1} g^{-1}.

The identification of these anthocyanins was confirmed through isolated anthocyanin injection by tandem mass spectrometry. A molecular ion of m/z 465 was detected for the first substance, corresponding to the molecular weight of delphinidin-3-*O*-glycoside. Fragmentation of this ion generated an ion of m/z 303, which corresponds to the molecular weight of aglycone delphinidin (Figure 6a). The results for the second anthocyanin present in the fruit were similar, with a molecular ion of m/z 449, referring to cyanidin-3-*O*-glycoside, and a fragment of m/z 287 relating to cyanidin aglycone (Figure 6b).

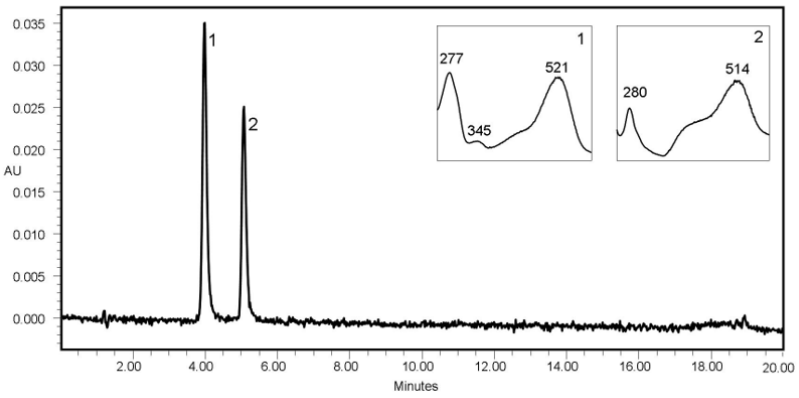

Figure 5. Chromatogram of anthocyanins extract from *E. punicifolia* pulp at 520 nm. Peak and spectra identification: (1) delphinidin-3-*O*-glucoside, (2) cyanidin-3-*O*-glycoside.

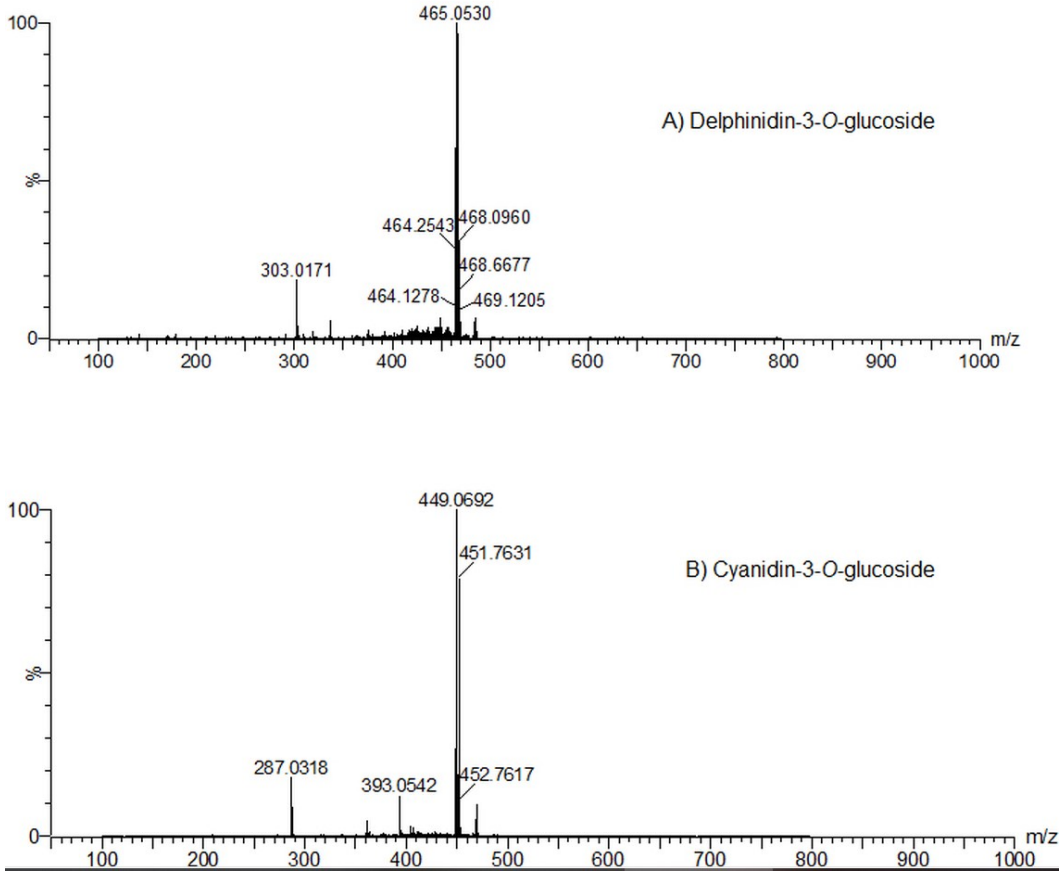

Figure 6. Mass spectra of *E. punicifolia* fruit isolated anthocyanins. A) Delphinidin-3-*O*-glucoside spectrum; B) Cyanidin-3-*O*-glycoside spectrum.

The fruit presented a similar anthocyanin profile to other Myrtaceae fruits such as jabuticaba, with total monomeric anthocyanin content of 280 mg 100^{-1} g^{-1} of dry whole fruit (Inada et al., 2015) and grumixama (*Eugenia brasiliensis* Lam), the latter belonging to the same genus as *E. punicifolia* with total monomeric anthocyanin content varying from 30 to 200 mg 100^{-1} g^{-1} of fresh weight of the flesh or 4837 mg 100^{-1} g^{-1} of dried weight of the peel (Teixeira et al., 2015; Nascimento et al., 2017).

Table 1 shows the compilation of the bioactive compounds amounts in the pulp and seeds of *E. punicifolia*.

Table 1. Bioactive compounds content in the pulp and seeds of *E. punicifolia*.

Bioactive compounds	Pulp	Seeds
Carotenoids	**µg g^{-1}**	**µg g^{-1}**
β-cryptoxanthin	32 ± 4.50	NA
β-carotene	5.70 ± 0.56	NA
Zeaxanthin	4.45 ± 4.30	NA
Lutein	3.20 ± 1.76	NA
Lycopene	503	NA
Ascorbic acid (vitamin C)	**mg 100 g^{-1}**	**mg 100 g^{-1}**
	74.2 ± 3.6	24.58 ± 5.40
Phenolic compounds	**mg g^{-1}**	**mg g^{-1}**
Gallic acid	1.46	1.54
Protocatechuic acid	ND	0.02
Syringic acid	0.01	0.03
p-coumaric acid	0.01	0.02
Ferulic acid	0.01	0.08
Ellagic acid	0.02	0.02
Anthocyanins	**mg 100 g^{-1}**	**mg 100 g^{-1}**
Delphinidin-3-*O*-glycoside	6.61	NA
Cyanidin-3-*O*-glycoside	5.00	NA

ND – Not detected; NA – Not analyzed.

4 Conclusion

The pulp of *E. punicifolia* fruits can be considered an excellent source of carotenoids, particularly lycopene, which proved to be the major compound at levels higher than other fruits considered good sources of this substance, including tomato products. They are a good source of vitamin C and present phenolic compounds like gallic acid and anthocyanins in their chemical composition. The results highlight the importance of composition studies on Brazilian biodiversity fruits to promote the underutilized species as a functional ingredient for natural and healthier products.

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