



Phytochemical characterization of bioactive compounds on methanolic and ethanolic leaf extracts of *Myrciaria* sp.

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Abstract

Among the native species of importance in Brazil, *jaboticabeira* (*Myrciaria* sp.) is a native fruit tree from several Brazilian regions. Few studies report the chemical constituents of the leaves and its pharmacological and nutraceutical properties. The aim of this study was to identify the phenolic compounds of the methanolic (MeOH) and ethanolic (EtOH) leaf extracts of *Myrciaria* sp. Phytochemical profile of the extracts was carried-out using High Performance Liquid Chromatography (HPLC) analysis. Antioxidant potential was evaluated by radical scavenging capacity with 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and total phenolics were determined with Folin-Ciocalteu reagent. A total of nine different compounds were identified in the free and bound phenolics extractions: 2,4 dihydroxybenzoic, vanillin, *p*-coumaric, ferulic, sinapinic, rutin, epicatechin, trans-caffeic and myricetin. The extracts demonstrated high radical scavenging capacity (MeOH: 1.83 and EtOH: 8.05 mg/mL) and high phenolic content (MeOH: 1.15; and EtOH: 1.04 mg/g dry matter). The wide variability of compounds revealed and the amount of peaks not identified, gives us a background of a potential plant matrix for further investigations in order to develop a nutraceutical agent.

Keywords: *Myrciaria* sp, phenolic compounds, chemical profile, antioxidant capacity.

1. Introduction

Brazilian flora has a great diversity of native species, some of them medicinal plants widely used in folk medicine. Considering the variability provided by natural forests and the biological activities already observed in popular experience, this field of research has been increasing in order to characterize bioactive compounds from natural sources. Among the native plant species of importance in Brazil, *jaboticabeira* (*Myrciaria* sp.) is a fruit tree that belongs to the *Myrtaceae* family, which the synonym identification varies between *Eugenia cauliflora* Miq., *Eugenia cauliflora* DC., *Myrciaria trunciflora* Berg, among others. *Myrciaria* sp. occurs in several Brazilian regions such as northeast, south central and southeast (Henriques *et al.*, 2010).

The fruits of this family occurs generally twice a year and around 30 days after flowering stage. The fruits are globular berries of black color and presents a potential in taste, aroma and external appearance (Henriques *et al.*, 2010).

Regarding the pleasant flavor, the fruits have received attention for containing substances with medicinal characteristics, as well as, some authors already demonstrate that the fruit contain several substances with antioxidant activity, such as ascorbic acid, rutin, quercetin, quercitrin, among others (Borges *et al.*, 2014; Morales *et al.*, 2016).

Besides the fruits, the bark of *Myrciaria* sp. also appears to contain bioactive compounds. Brazilian people have used it, as a tea made with the bark, to treat diarrhea and skin irritations (Lucena *et al.*,

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2008). Researchers showed on an *in vitro* trial run, that the bark provided antifungal effect on oral microorganisms (Diniz *et al.*, 2010).

On the other hand, there are few studies concerning the chemical composition of the leaves of *Myrciaria* sp. Souza-Moreira *et al.* (2008) demonstrated a preliminary standardization of the leaves by histochemical and phytochemical methods. This investigation showed presence of phenols, tannins and alkaloids mainly in the ethanolic extract of the leaves. The same group also demonstrated biological activities as antioxidant and antimicrobial.

In addition, the characterization of the oil constituents, total phenol and tannin contents from *Myrciaria* sp. leaves was demonstrated by (Duarte *et al.*, 2010), as well as the environmental influence on chemical variability of the plant leaves.

Considering this scenario, few studies address a variety of information about the *Myrciaria* sp. leaves. Taking into account, the biological activities already demonstrated, the characterization of the chemical constituents of the leaves is a major issue to better understand its pharmacological and nutraceutical properties. The aim of this study was to identify the phenolic compounds in the methanolic (MeOH) and ethanolic (EtOH) leaf extracts of *Myrciaria* sp., and investigate its radical scavenging capacity and phenolics content.

2. Material and methods

2.1. Plant material

Myrciaria sp. leaves were collected in Mauá da Serra, Rio de Janeiro, Brazil, in March 2016. Plant material was dried at 40 °C for 16 hours, crushed and stored at room temperature, protected from light until required for the preparation of extracts.

2.2. Extraction of phenolic compounds from *Myrciaria* sp. leaves

2.2.1. Free phenolics extraction

The dry matter was weighed (0.5 g) in a 50 mL falcon tube and to each tube was added

10 mL of ethanol:water solution (40:60) (EtOH) and methanol:water methanol solution (80:20) (MeOH) to perform the liquids extractions. The mixture was macerated with an Ultra-Turrax® mixer for 3 min and homogenized by vortex (Warmnest) for 10 min. Extraction of the homogenized samples was conducted by an incubation under temperature-controlled orbital shaker (KS 4000ic control; IKA, Germany) at 240 rpm, 60 °C for 1 hour. After incubation, samples were centrifuged (ST 16R Sorvall Centrifuge; Thermo Scientific, Germany) at 3000 × g for 5 min at room temperature. The supernatant was collected and transferred to a rotary evaporator balloon. The described steps was carried out twice and the supernatants were finally combined and dried in a rotary evaporator (Rotavapor R-215, BÜCHI, Switzerland) at 40 °C. The dried residue was reconstituted in 3 mL methanol:water solution (3:97, v/v) and stored at -20 °C in screw-capped amber flasks until the moment of analyzes.

2.2.2. Bound phenolics extraction

a. Alkaline hydrolyses

The alkaline hydrolysis was prepared based on the residue from the free phenolics extraction. It was added 6 mL of Milli-Q water and 2.5 mL of NaOH 10 M into the left precipitate from free phenolics extraction. Samples were homogenized by vortex and the procedure was conducted by an incubation under temperature-controlled orbital shaker at 240 rpm, 25 °C for 16 hour. After incubation, the pH was adjusted to 2 with hydrogen chloride (HCl). Then, it was added 7 mL of ethyl acetate and homogenized by vortex. Samples were centrifuged at 2500 × g for 5 min at 10 °C. The supernatant was collected and transferred to a rotary evaporator balloon. The described steps was carried out twice and the supernatants were combined and dried in a rotary evaporator at 40 °C.

b. Acid hydrolyses

Based on the residue from the alkaline hydrolyses, it was added 1.5 mL of HCl to

the left precipitate. Samples were incubated in a water bath for 30 min at 85 °C. It was added 7 mL of ethyl acetate and homogenized by vortex. Samples were centrifuged at $2500 \times g$ for 5 min at 10 °C. The supernatant was collected and transferred to a rotary evaporator balloon. The described steps was carried out twice and the supernatants were combined and dried in a rotary evaporator at 40 °C.

2.3. Determination of total phenolics

Total phenolics in the extracts was accessed with Folin-Ciocalteu reagent according to the method from (Singleton *et al.*, 1999), with modifications. Briefly, aliquots of 100, 200 and 500 μL from the EtOH extract and 50, 100, 200 μL from the MeOH extract of diluted samples (1:100) were added to 2.5 mL of Folin-Ciocalteu reagent. After 5 min, the volume was adjusted to 5 mL with the correspondent extractor and it was added 2 mL of a 4% sodium carbonate solution and mixed by vortex. The absorbance was measured at 750 nm using Shimadzu UV-1603 spectrophotometer after 2 hours. A standard curve was plotted using different concentrations of an aqueous solution of gallic acid. The amount of total phenolics compounds was expressed as milligrams of gallic acid equivalents per gram (mg GAE g^{-1}) of dry matter.

2.4. Radical scavenging capacity

Antioxidant activity of the extracts was evaluated using 0.06 mM DPPH (1,1-Diphenyl-2-picryl-hydrazyl) in methyl-alcohol using spectrophotometry at 515 nm. It was added 2.0 mL of DPPH solution to 100, 200, 500 μL of diluted samples (1:100), the volume was completed to 2.5 mL with the correspondent extractor, homogenized in a vortex and kept at dark place for 1 hour. Blank solutions were done replacing DPPH solution to methanol. Anti-radical activity was calculated as discolour percentage of DPPH according to the equation: $\% = (\text{DA} - \text{SA} / \text{DA}) \cdot 100$; where: DA = DPPH absorbance; SA = sample absorbance (Brand-Williams *et al.*, 1995) and the IC_{50}

values were calculated from the dose responses curves. All Assays were performed in duplicate.

2.5. Identification of phenolic compound profile by HPLC-DAD

Briefly, samples were diluted in 3% methanol aqueous solution (5% dilution), homogenized by vortex, sonicated (Shenzhen Codyson Electrical Co. Ltd.; Shenzhen, China) and filtered through a 0.45 μm PTFE membrane filter (Millex; Millipore, Germany) into an amber-colored vials (Allcrom). Samples and standards were analyzed in an HPLC system (Perkin Elmer, Shelton, USA) equipped with a degasser, a column oven and a photodiode array detector (PDA; Perkin Elmer) set at an acquisition data on 260 nm, 280 nm and 320 nm. Phenolic compounds were eluted from a reversed-phase C18 column (5 μm x 150 mm x 4.6 mm; Kromasil; Akzobel, Sweden) fitted with a Phenomenex (Torrance, CA, USA) security guard column (4 x 3.0 mm) operated at 40 °C (John and Shahidi, 2010). The mobile phase consisted of 0.3% (v/v) formic acid in water (A), methanol (B) and acetonitrile (C), the flow rate was 0.8 ml/min and the elution gradient is described in Table 1 (Gomes and Torres, 2016). Total run time was 33.2 min and the injection volume for all samples and standards was 20 μL .

Table 1

Mobile phase gradient to phenolic compounds elution from HPLC- DAD reversed-phase C18 Column

Time (min)	Solvent (%)		
	Formic acid (0.3%)	Methanol	Acetonitrile
0.1	85.0	14.5	0.5
7.0	55.0	43.5	1.5
7.0	5.0	93.0	2.0
6.0	1.0	97.0	2.0
3.0	15.0	83.0	2.0
0.1	85.0	14.5	0.5
10.0	85.0	14.5	0.5

All data acquisition and monitoring were carried out at 260 nm, 280 nm and 320 nm and was performed using the software

Chromera Data System (Perkin Elmer). Peak identities were determined by comparing the relative retention times and PDA spectra of commercial standards.

3. Results and discussion

3.1. Total phenolic compounds

Total phenolic contents in the extracts was measured with Folin-Ciocalteu reagent. A little higher phenolic level was found in the MeOH extract (1.15 mg/g dry matter) whereas the EtOH extract presented 1.04 mg/g dry matter. These values are compared with values of traditional and exotic fruits (Table 2). It was found similar values in avocado pulp (1.42 mg/g of fresh fruit weight) (Wang *et al.*, 2010) and banana pulp (2.3 mg/g of dry fruit weight weight) (Someyaa *et al.*, 2002). On the other hand, much higher amounts was found in the peel of these fruits (~8.9 and 9.07 mg/g, respectively). Some other fruits showed greater amounts of total phenolic compounds such as mango (Arbos *et al.*, 2013) and pomegranate (Li *et al.*, 2006) (31.23 and 24.4 mg/g, respectively). It should be considered that all these data were performed separately, and generally, the submission of data varies between different forms of presentation as, mg of gallic acid equivalents (GAE), catechin equivalent (CE), trolox equivalent (TE), among others. Therefore, the range of comparison becomes restricted.

3.2. Antioxidant capacity

The antioxidant capacity of the leaf extracts of *Myrciaria* sp. was accessed with DPPH reagent, which is based on a decolorization assay that measures the capacity of antioxidants to directly react with DPPH radicals. The highest antioxidant capacity was obtained from the MeOH extract (1.83 mg/mL IC₅₀) and the MeOH extract showed 8.05 mg/mL IC₅₀ of antioxidant capacity (Table 2). Another authors present the antioxidant capacity of ethanolic extracts (50 and 70 %) from *Myrciaria* sp. leaves beyond 90% (Souza-Moreira *et al.*, 2008), considering data expressed in percentage, we found similar values in the present study, around 93% for EtOH extract (Table 2). Similar values to the MeOH extract were found in the peel and kernel of mango extracts, 1.01 and 1.33 mg/ mL IC₅₀, respectively (Arbos *et al.*, 2013) showing that the *Myrciaria* sp. leaves has the potential to further investigations.

A H₂O:MeOH fraction from *Myrciaria* sp. fruit presents roughly 0.0062 mg/mL (Einbond *et al.*, 2004) demonstrating that the fruit has a great antioxidant capacity. The high phenolic compound content founded in the leaves may be responsible for the high antioxidant activity of the extracts (Arbos *et al.*, 2013).

Table 2

Total phenolics content and radical scavenging capacity found in *Myrciaria* sp. leaves extracts and in different parts of exotic and tropical fruit

Plant/ Fruit extract	Phenolics (mg/g)	Antioxidant capacity (mg/mL IC ₅₀)	Antioxidant capacity (%)	Reference
MeOH extract	1.15	1.83	93.91	
EtOH extract	1.04	8.05	78.08	
Mango peel	66.44	1.01	88.5	Arbos <i>et al.</i> , 2013
Mango kernel	31.23	1.33	69.8	Arbos <i>et al.</i> , 2013
MeOH fraction of <i>Myrciaria</i> sp fruit	-	0.0062	-	Einbond <i>et al.</i> , 2004
Avocado pulp	~1.42*	-	-	Wang <i>et al.</i> , 2010
Avocado peel	~8.9*	-	-	Wang <i>et al.</i> , 2010
Banana pulp	2.32	-	-	Someyaa <i>et al.</i> , 2002
Banana peel	9.07	-	-	Someyaa <i>et al.</i> , 2002
Pomegranate peel	249.9	-	-	Li <i>et al.</i> , 2006
Pomegranate pulp	24.4	-	-	Li <i>et al.</i> , 2006

*Mean of different cultivars.

3.3. Phenolic compound profile

Characterization of phenolic compounds was performed by HPLC-DAD. The peak identities of the phenolic compounds were determined by comparison of corrected retention times, UV spectra from PDA and spiking samples with 16 commercial standards (Table 3).

The leaves extract of *Myrciaria* sp. revealed nine different compounds in the free and bound phenolics extraction, for the peak identification, see Table 3 and Figure 1. Seven of the ten compounds were identified in the extraction for free phenolics: 2,4 dihydroxybenzoic, vanillin, *p*-coumaric, ferulic, sinapinic acids and rutin in both extracts and the EtOH extract also revealed epicatechin. Even though, the extraction for bound phenolics showed more two different compounds: trans-caffeic and myricetin (EtOH) (Table 3).

The phenolic profile of *Myrciaria* sp. leaves extracts was similar to those found in other studies with fruits, considering that there are no previous studies relating the phenolic profile of *Myrciaria* sp. leaves. Phenolic acids including *p*-coumaric, sinapinic and ferulic are secondary aromatic plant products suggested to possess many physiological

and pharmacological functions. They have been anteriorly found in several fruits such as banana, persimmon, durian, grape, strawberry, apple pulp, kiwi, mangosteen, pomelo, grapefruit and lemon (Parka *et al.*, 2015), and in both analyzed extracts, they were the most abundant compounds identified, presenting the highest peaks (Figure 1). Czyżewska *et al.* (2016) showed that *p*-coumaric and ferulic acids were able to induce cytotoxicity on human tongue carcinoma cells (Czyżewska *et al.*, 2016).

In addition, the protection of vascular function by sinapinic acid was examined on experimental hypertensive animal model and the protection was attributed to antioxidant potential and attenuating fibrosis capacity of the compound (Silambarasan *et al.*, 2014). Vanillin also presented a great peak in both extracts (Figure 1), the compound is a oxidized form of vanillic acid, and was recently demonstrated that provides neuro-protection through its potential pharmacological functions including antioxidant, anti-inflammatory, mitochondrial protective and anti-apoptotic properties (Dhanalakshmi *et al.*, 2016).

Table 3

Retention time (Rt), total commercial standards utilized and identification of free and bound phenolic compounds in EtOH and MeOH extracts of *Myrciaria* sp. leaves by HPLC-DAD analysis

Phenolic Compound	Free phenolics			Bound phenolics		
	EtOH	MeOH	Rt (min)	EtOH	MeOH	Rt (min)
Gallic acid	-	-	-	-	-	-
Pyrogallol	-	-	-	-	-	-
3,4-dihydroxyphenylacetic acid	-	-	-	-	-	-
Catechin	-	-	-	-	-	-
4-hydroxybenzoic	-	-	-	-	-	-
Epicatechin	✓	-	10.51	-	-	-
Trans-caffeic	-	-	-	✓	✓	9.94
Vanillic acid	-	-	-	-	-	-
2,4 dihydroxybenzoic	✓	✓	10.4	-	-	-
Vanillin	✓	✓	12.42	-	-	-
<i>p</i> -coumaric	✓	✓	12.1	✓	✓	11.95
Ferulic	✓	✓	12.28	✓	✓	12.43
Sinapinic	✓	✓	12.34	✓	✓	12.52
Rutin	✓	✓	13.90	-	-	-
Myricetin	-	-	-	✓	-	1.42
2-hydroxybenzoic acid	-	-	-	-	-	-

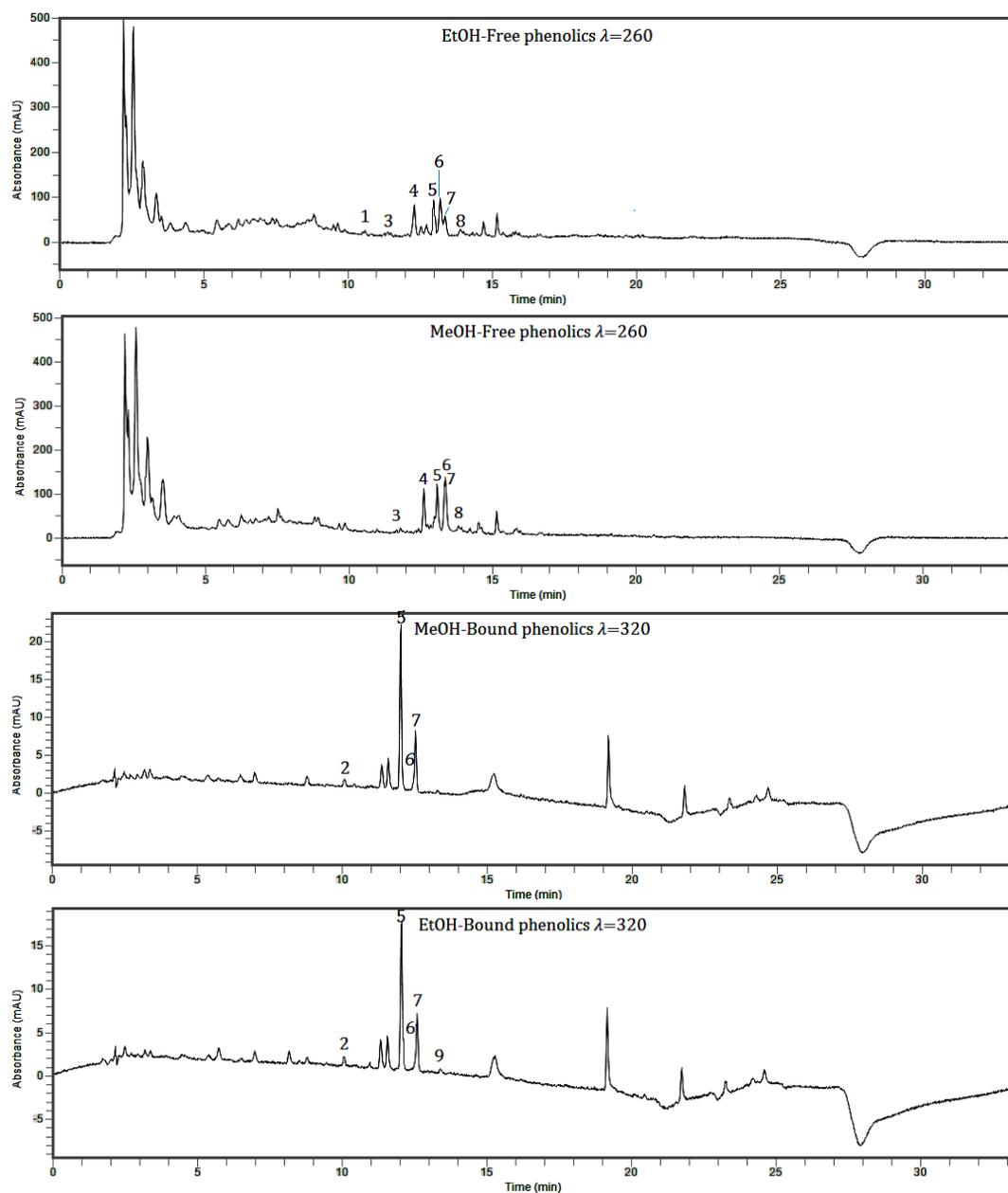


Figure 1. HPLC phenolic profile of the EtOH and MeOH extracts obtained from the leaves of *Myrciaria* sp., recorded at wavelengths of maximum absorption in visible region (λ). Epicatechin (1), trans-caffeic (2), 2,4-dihydroxybenzoic acid (3), vanillin (4), *p*-coumaric acid (5), ferulic acid (6), sinapinic acid (7), rutin (8) and miricetin (9).

The phenolic compound profile of the extracts might influence not only its antioxidant capacity as widely described (Parka *et al.*, 2015), but other biological functions as mentioned above.

In addition, the diversity of compounds founded and the amount of peaks not identified yet, gives us a background of a

potential plant matrix to further investigate its characteristics in order to develop a nutraceutical agent rich in natural compounds.

4. Conclusions

The high antioxidant activity of MeOH and EtOH extracts appeared to be attributed to

the high phenolics content and to the diverse phenolic profile of the *Myrciaria* sp. leaves. We consider that these extracts deserve more intensive study, including identification of all the peaks founded and its quantifications. Characterize the antioxidant composition and bioavailability to explore the possibility of developing a nutraceutical agent rich in natural antioxidants. In addition, investigate if the foliar extracts composition has some variation within seasons and environmental variables. Besides, considering that plants matter are not consumed and rarely approached, the high amount of bioactive compounds presented in these non-edible parts could be used for different purposes in the food industry such as enrichment or development of new products.

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