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UPLC-QTOF-MS Analysis of Extracts from the Leaves of *Pouteria caimito* (Sapotaceae) and Their Antioxidant Activity

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Abstract

This study describes the phytochemical profile and antioxidant activity of an extract from the leaves of *Pouteria caimito* (Sapotaceae). The extract in ethanol was obtained by maceration at room temperature and subjected to the liquid-liquid partition to obtain fractions in hexane and ethyl acetate. Steroids, triterpenes, saponins, alkaloids and flavonoids were identified by the phytochemical prospection of extracts and fractions from the leaves. The analysis of the ethyl acetate fraction by UPLC-QTOF-MS allowed us to identify eight triterpenes, namely, euscaphic acid (1), hyptadienic acid (2), betulinic acid (3), oleanolic acid (4), ursolic acid (5), 3β-(*O*-*p*-coumaroyl)-alphitolic acid (6), 3β-(*O*-*p*-coumaroyl)-maslinic acid (7) and 3β-(*O*-*p*-coumaroyl)-2-hydroxy-urs-12-*en*-28-oic acid (8). The ethanol extract and ethyl acetate fraction presented total phenolic contents of 10.6 ± 0.1 and 11.4 ± 0.3 mg GAE g^{-1}, respectively, and considerable antioxidant activity in the DPPH assay with EC_{50} values of 299.4 ± 1.5 and 391.8 ± 0.9 μg·ml^{-1}, respectively.

Keywords

Abiu, UPLC, Triterpenes, DPPH, Antioxidants

1. Introduction

The genus *Pouteria* Aublet is a pantropical group comprising nine sections and 325 species [1]. In Brazil, the centres of dispersion of the genus are mainly Amazonia and coastal regions, often occurring in Bahia, Espírito Santo, Rio de

Janeiro and São Paulo [2]. The genus *Pouteria* is represented in Brazil by 114 species, of which 46 are endemic [3]. Many of these species produce high quality wood and edible fruits, such as *P. caimito* (abiu), *P. macrocarpa* (cutito), *P. macrophyla* (caimo) and *P. sapota* (sapota), which represent significant economic potential. In addition to their commercial importance, several species have been used in folk medicine to treat fevers, inflammations, ulcers, diabetes and nausea, among other applications [4] [5]. In addition, the extracts, semipurified fractions and isolated substances from the *Pouteria* species show anti-inflammatory activity [6], inhibition of the α-amylase and β-glucosidase enzymes [7] and antidiabetic activity [8]. Despite the occurrence of bioactive substances of pharmacological interest, such as carotenoids, flavonoids, triterpenes and cyanogenic glycosides, only about 15 *Pouteria* species have been investigated from a chemical point of view [5] [9] [10].

Pouteria caimito (abiu) is a fruit species belonging to the Sapotaceae family with a probable centre of origin in the Peruvian Amazon. The fruits and leaves of *P. caimito* are used in folk medicine in the treatment of coughs, bronchitis and diarrhoea [11]. Some studies report the pharmacological potential of this species, such as antioxidant activity [12] and inhibition of α-amylase and β-glucosidase enzymes, which are therapeutic targets in the search for new drugs for the treatment of diabetes [13]. The phytochemical study of *P. caimito* is of great importance, since there are few reports in the literature involving this species. The phytochemical study of the benzene extract of *P. caimito* fruits led to the isolation of α-amirin, lupeol, erythrodiol and dammarenediol. Taraxerol, taraxerol acetate, taraxerone and β-sitosterol were isolated from the stems of this plant [14]. In addition to the hexane extract from the leaves, spinasterol was isolated [5]. Continuing our search for antioxidant substances [15] [16] [17] in plant species of the Brazilian flora, we describe in this work, the chemical study and evaluation of the antioxidant activity of extracts from the leaves of *P. caimito* collected in the Tocantins state, Brazil.

2. Material and Methods

2.1. Materials

All the chemicals and reagents used in this study were of analytical grade. For thin layer chromatography (CCD), silica gel PF254 plates (Whatman plc, UK) were used, which were observed under UV light 254 nm and developed with solutions of ferric chloride and the Liebermann-Burchard reagent, followed by heating. Chromatographic column separations (CC) were performed using silica gel 60 (70 - 230 mesh, Vetec, Brazil).

2.2. Preparation of Extract and Fractions

Leaves of *P. caimito* were collected on the campus of the Federal University of Tocantins, Gurupi, Tocantins, Brazil. An exsicta was deposited in the herbarium HUTO-UNITINS. Dry leaves (23.0 g) were comminute and extracted with EtOH

by maceration at room temperature. The resulting solution after filtration was concentrated under reduced pressure to give the crude extract in EtOH (PFE, 3.8 g). Part of the PFE extract (3.5 g) was solubilised in a MeOH/H_2O solution (7:3) and submitted to liquid/liquid partition to yield the fractions in n-hexane (PFH, 0.9 g) and AcOEt (PFAE, 1.3 g), respectively.

2.3. Phytochemical Prospecting

The phytochemical screening of leaf extracts and fractions was assessed by standard phytochemical methods [18]. Phytochemical screening was carried out by the qualitative chemical composition of leaf extracts using different solvents and using colour change, foaming and precipitate to identify the major natural chemical groups, such as tannins, saponins, flavonoids, catechins, alkaloids, xanthones, triterpenes and steroids. General reactions in this analysis revealed the presence or absence of these classes of compounds in the tested leaf extracts.

2.4. Separation and Identification of Chemical Constituents

Part of the ethyl acetate fraction (PFAE, 1.0 g) was subjected to fractionation using silica gel column chromatography with hexane, chloroform, ethyl acetate and methanol in isocratic mode as eluents. The four sub-fractions obtained were analysed by thin layer chromatography. The sub-fractions eluted with chloroform and ethyl acetate were selected for analysis by ultra-high performance liquid chromatography (UPLC) with a photodiode array detector (PDA) coupled to quadrupole time-of-flight mass spectrometry (QTOF-MS) to obtain the chemical profile, which allowed the identification of triterpenes, namely, euscaphic acid (1), hyptadienic acid (2), betulinic acid (3), ursolic acid (4), oleanolic acid (5), 3β-(*O*-*p*-coumaroyl)-alphitolic acid (6), 3β-(*O*-*p*-coumaroyl)-maslinic acid (7) and 3β-(*O*-*p*-coumaroyl)-2-hydroxy-urs-12-*en*-28-oic acid (8).

2.5. Qualitative UPLC-QTOF-MS Analysis

Chromatographic separation of compounds was performed on an ACQUITY UPLC system (Waters, Milford, MA, USA) with a conditioned auto sampler at 4˚C, using an Acquity BEH C18 column (50 mm × 2.1 mm i.d., 1.7 µm particle size) (Waters, Milford, MA, USA). The column temperature was maintained at 40˚C. The used mobile phase was water with 0.1% formic acid in water (solvent A) and acetonitrile (solvent B), which was pumped at a flow rate of 0.4 ml·min^{-1}. The gradient elution program was as follows: 0 - 5 min, 5% - 10% B; 5 - 9 min, 10% - 95% B. The injection volume was 10 µl. MS analysis was performed on a Xevo G2 QTOF (Waters MS Technologies, Manchester, UK). Source conditions as follows: capillary voltage, 2.0 kV; sample cone, source temperature, 100˚C; desolvation temperature 250˚C; cone gas flow rate 20 l·h^{-1}; desolvation gas (N_2) flow rate 600 l·h^{-1}. All analyses were performed using a lockspray, which ensured accuracy and reproducibility. Leucine enkephalin (5 ng·ml^{-1}) was used as a reference compound to calibrate mass spectrometers during analysis and intro-

duced by a lockspray at 10 μl·min^{-1} for accurate mass acquisition. All the acquisition and analysis of data were controlled using Waters MassLynx v 4.1 software.

2.6. Antioxidant Activity

2.6.1. Determination of Total Phenolic Content

The total phenolic content determined the modified Folin-Denis method [19]. Briefly, 0.5 ml of the extract (1.0 mg·ml^{-1} in methanol) was mixed with 2.5 ml of the Folin-Denis reagent, and after 5 min, 2 ml of a 14% sodium carbonate (Na_2CO_3) solution was added. After incubation at room temperature for 2 h, the absorbance of the reaction mixture was measured at 760 nm (T60 UV-Visible Spectrophotometer, PG instruments, UK) against a methanol blank. A standard curve was constructed using gallic acid as a reference substance. The total phenolic content expressed in gallic acid equivalents (mg GAE g^{-1} of extract).

2.6.2. 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Assay

The scavenging activity for the radical DPPH was measured as described by Zhang and Hamauzu (2004) [20]. A volume of 2.7 ml of a 23.6 μg·ml^{-1} DPPH methanolic solution was added to 0.3 ml of various concentrations of extracts (100 to 1200 μg·ml^{-1} in methanol) or ascorbic acid standard (0.5 at 4 μg·ml^{-1} in methanol). The mixtures were kept in the dark for 30 min at room temperature, and the absorbance of the remaining DPPH was determined at 517 nm (T60 UV-Visible Spectrophotometer, PG instruments, UK). A 3.0 ml of methanol was used as a blank, and a mixture of 2.7 ml of DPPH solution with 0.3 ml of methanol was used as a negative control. The radical scavenging activity was calculated as a percentage of DPPH discoloration using Equation (1):

$$AA\% = \left(\text{the absorbance of the negative control} - \text{the absorbance of an extract or standard} / \text{the absorbance of the negative control}\right) \times 100 \quad (1)$$

where AA% is the inhibition percentage. The effective concentration providing 50% inhibition (EC_{50}) was calculated from the graph of scavenging effect percentage against extract or ascorbic acid standard concentration.

2.7. Statistical Analysis

Each experiment was performed at least three times. All values are expressed as means ± standard deviation. All statistical analyses were performed with GraphPad Prism 5 DEMO.

3. Results and Discussion

3.1. Phytochemical Prospecting and Peak Assignment by UPLC-QTOF-MS

In phytochemical prospecting, it was verified that only triterpenes and steroids were present in the hexane fraction (PFH) of leaves of *P. caimito*. However, triterpenes, steroids, saponins, flavonoids and catechins were detected in the

ethanolic extract (PFE) and the ethyl acetate fraction (PFAE) (**Table 1**). The study evidenced the presence of secondary metabolites, which may be related to the medicinal properties of *P. caimito* leaves.

UPLC-PDA-MS is a newly developed technique that provides a great amount of information rapidly and efficiently compared with other techniques. The high selectivity and sensitivity of UPLC-QTOF-MS makes it a widely applied technique in quantitative and qualitative analysis, as well as in metabolite analysis and the identification of complex compounds in natural products [21].

Table 2 lists the tentatively identified compounds in PFAE. A total of eight compounds were identified by UPLC-QTOF-MS (**Figure 1**) based on database interrogation and references, as shown in **Figure 2**. Peaks 1, 2, 3, 4, 5, 6, 7 and 8 were identified as euscaphic acid, hyptadienic acid, betulinic acid, oleanolic acid, ursolic acid, 3*β*-(*O*-*p*-coumaroyl)-alphitolic acid, 3*β*-(*O*-*p*-coumaroyl)-maslinic acid and 3*β*-(*O*-*p*-coumaroyl)-2-hydroxy-urs-12-*en*-28-oic acid, respectively, based on UV, MS and MS/MS fragment ions [21] [22]. This is the first report of the occurrence of triterpenes 1, 2, 6, 7 and 8 in the Sapotaceae family. Euscaphic acid (1) isolated from *Cecropalyratiloba* presented cytotoxic activity against leukemic cell lines [23] and 3*β*-(*O*-*p*-coumaroyl)-alphitolic acid (6) leads to apoptotic cell death in human leukemia cells [24]. Betulinic acid (3) was previously identified in the species *P. gardnerii*, *P. tomentosa* and *P. torta* [5]. Oleanolic acid (4) was isolated from *P. gardnerri* [5]. Ursolic acid (5) was isolated from the species *P. gardnerii*, *P. venosa*, *P. tomentosa* and *P. torta* [5]. The triterpenes derived from oleanans and ursans present antitumor, anti-inflammatory and antioxidant activities [25]. Thus, the identification of these triterpenes in leaf extracts of *P. caimito* may contribute to the pharmacological potential of this species.

Table 1. Phytochemical prospecting of extracts and fractions of *P. caimito* leaves.

Constituents	extract in ethanol	fraction in hexane	fraction in ethyl acetate
Triterpenes/steroids	+	+	+
Saponins	+	-	+
Alkaloids	-	-	-
Tannins	-	-	-
Anthocyanins and anthocyanidins	-	-	-
Flavones, flavonols and xanthones	+	-	+
Chalcones and auronas	-	-	-
Flavanonols	-	-	-
Leucoantocianidines	-	-	-
Cathechins	+	-	+
Flavanones	-	-	-

Table 2. Identified compounds in the extracts from leaves of *P. caimito*.

Peak No.	T_R (min)	Assigned identity	[M-H]⁻ m/z Meanmeasured mass (Da)	Theorical exact mass (Da)	ppm	References
1	2.69	euscaphic acid	487.3417	487.3423	−1.2	[21]
2	2.75	hyptadienic acid	469.3327	469.3318	1.9	[21]
3	4.64	betulinic acid	455.3520	455.3525	−1.1	[21]
4	4.66	oleanolic acid	455.3518	455.3525	−1.5	[21]
5	4.68	ursolic acid	455.3528	455.3525	0.6	[21]
6	5.75	3*β*-*O*-(*p*-coumaroyl)-maslinicacid	617.3823	617.3842	−3.0	[22]
7	5.89	3*β*-O-(*p*-coumaroyl)-maslinic acid	617.3820	617.3842	−3.6	[22]
8	5.98	3*β*-*O*-(*p*-coumaroyl)-2-hydroxy-urs-12-*en*-28-oic acid	617.3819	617.3842	−3.7	[21]

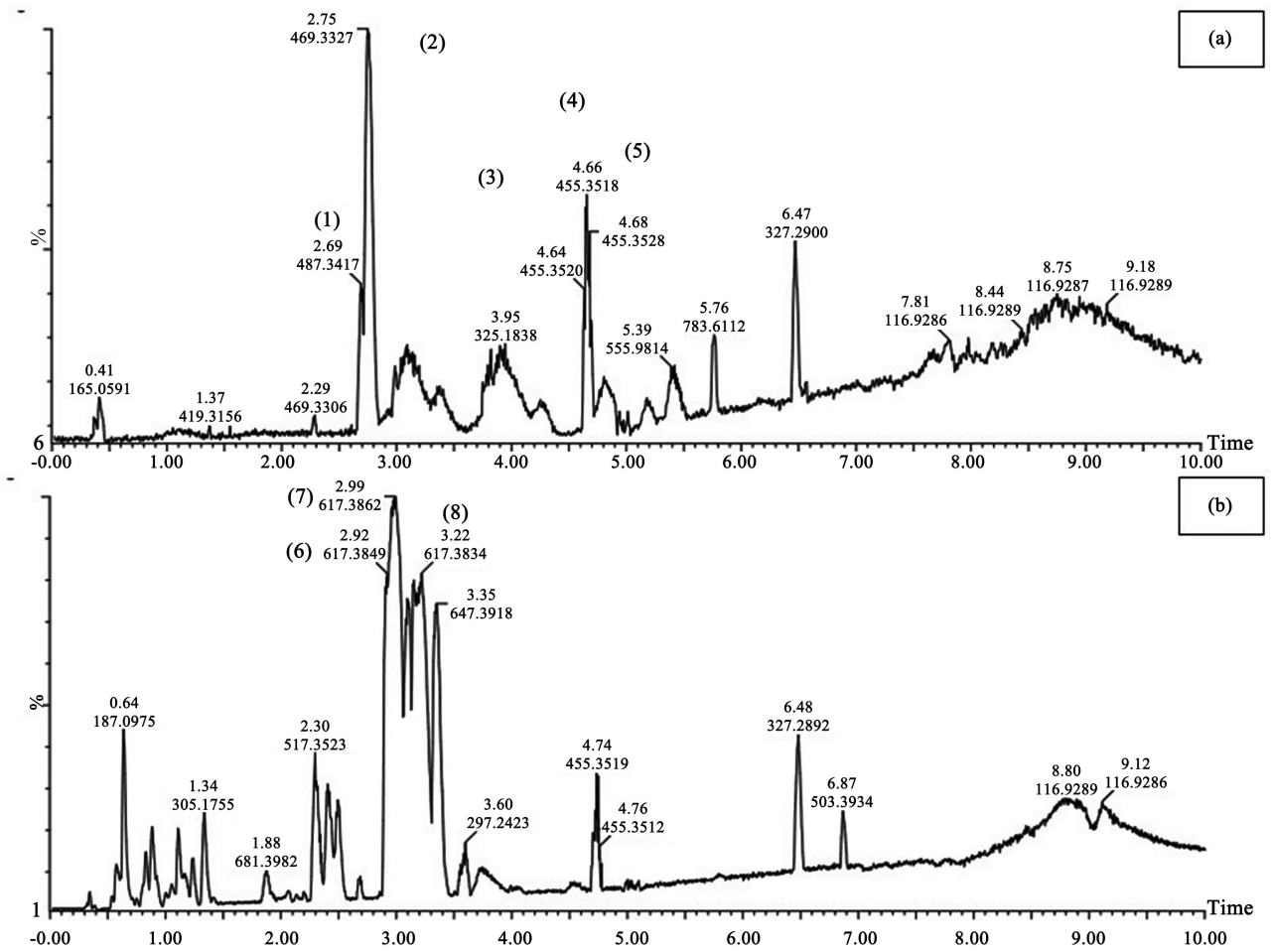

Figure 1. The total current ion chromatograms (UPLC-QTOF-MS) of the PFAE fraction of *P. caimito* leaves in negative ion mode: sub-fractions chloroform (a) and ethyl acetate (b).

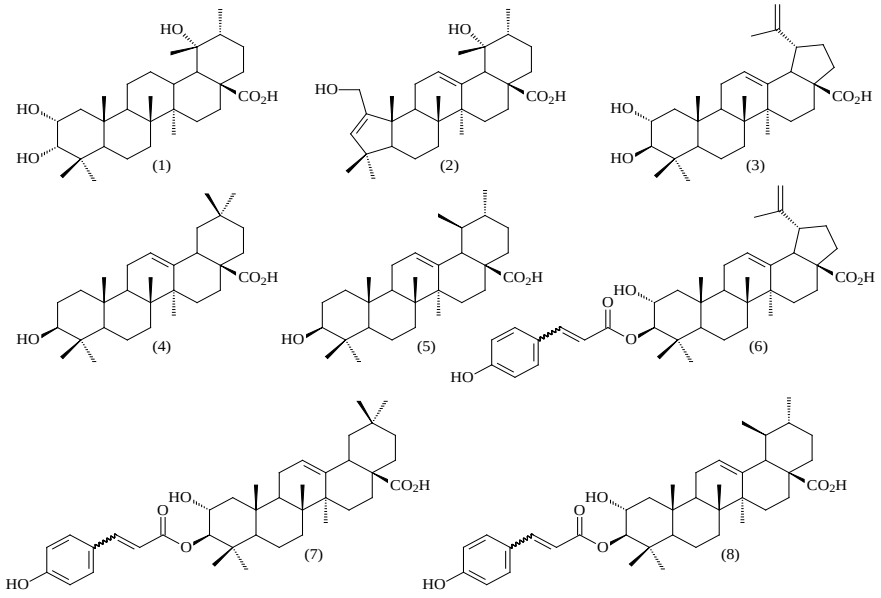

Figure 2. Structures of substances identified in extracts of *P. caimito* leaves.

Table 3. Antioxidant activityof extracts of *P. caimito* measured by total phenolic content and DPPH assay.

Extracts and standard substance	Total phenolic content (mg GAE g^{-1} extract)	DPPH EC_{50}
PFE	10.6 ± 0.1	299.4 ± 1.5
PFH	7.7 ± 0.6	1439.4 ± 2.7
PFAE	11.4 ± 0.3	391.8 ± 0.9
Ascorbic acid	-	2.05 ± 0.02

3.2. Antioxidant Activity

The Folin-Denis assay is a fast and simple method for determining the phenolic compound contents in plant samples. The fraction in AcOEt of the leaves of *P. caimito* presented the highest phenolic content in comparison to the other fractions of the leaves analysed (11.37 ± 0.3 mg GAE g^{-1}), followed by the extract in EtOH (**Table 3**). Extracts from leaves of *P. caimito* presented total phenolic contents similar to other species of this genus [12] [26] [27].

The scavenging activity for free radicals of DPPH (1,1-Diphenyl-2-picrylhydrazyl) has widely been used to evaluate the antioxidant activity of natural products from plant and natural sources [28]. The results of the DPPH radical scavenging activity of the extract, fractions and ascorbic acid are presented in **Table 3**. PFE and PFAE had higher antioxidant activity than PFH ($p < 0.05$), but the antioxidant activity was significantly lower than the standard ascorbic acid ($p < 0.05$). The ethanol extract and ethyl acetate fraction showed a higher DPPH radical scavenging activity than that reported for the acetone extract of the pulp of *P. caimito* [12]. Part of the observed antioxidant activity can be attributed to phenolic compounds detected by the phytochemical

analysis and triterpenes identified by UPLC-QTOF-MS.

4. Conclusion

The phytochemical screening and antioxidant activity of medicinal plants are very important in identifying new sources of therapeutically and industrially important compounds. The analysis of the ethyl acetate fraction (PFAE) by UPLC-QTOF-MS allowed the identification of eight triterpenes that are being reported for the first time in *P. caimito*. In addition, the *in vitro* antioxidant activity of the extract and fractions obtained from the leaves of *P. caimito* using the DPPH assay is reported.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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