Journal of Applied Pharmaceutical Science Vol. 6 (07), pp. 184-188, July, 2016
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2016.60727
ISSN 2231-3354

Potential radical-scavenging activity of *Pouteria caimito* leaves extracts

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ARTICLE INFO

Article history:
Received on: 02/02/2016
Revised on: 16/03/2016
Accepted on: 24/05/2016
Available online: 28/07/2016

Key words:
Pouteria caimito, Sapotaceae, Cerrado, spinasterol, anti-oxidante activity.

ABSTRACT

Pouteria caimito (Sapotaceae) is widely distributed throughout Latin America, including Brazil. The yellow fruits, known as abiu, caimito, or abiurana, are eaten *in natura* or used to prepare desserts. In addition to being a commercially available Brazilian fruit, *P. caimito* has also been used as a traditional medicine. Therefore, hexane, ethanol, and aqueous extracts from its leaves were evaluated for radical-scavenging activity. Free radical-scavenging activity was tested using the DPPH assay. Total phenolic and proanthocyanidin contents were also determined. The aqueous extract comprised the highest total phenol and proanthocyanidin contents (173.6 μg/mL) and showed the highest radical-scavenging activity (ED_{50}= 36.1 μg/mL). Phytochemical analysis of the hexane extract allowed the isolation of spinasterol. We found a high correlation between total phenolic and proanthocyanidin contents and radical-scavenging activity. To our knowledge, this is the first time spinasterol has been isolated from *P. caimito* leaves.

INTRODUCTION

Oxidative stress has been considered as a major cause of aging and various chronic and degenerative diseases. These conditions include inflammation, diabetes mellitus, cancer, heart disease, and neuronal degeneration, such as Alzheimer's disease and Parkinson's disease (Ng, *et al.*, 2007). A general recommendation to reduce systemic oxidation processes involves increasing the intake of foods considered to be rich in antioxidant compounds (e.g. polyphenols and carotenoids) owing to their well-known health-giving effects. This recommendation led to the identification of many plants having potential antioxidant activities (Katalinic, *et al.*, 2006). Therefore, natural antioxidants from plant extracts have attracted growing interest due to consumer concern about the safety of the synthetic antioxidants in food. *Pouteria caimito* (Ruiz & Pav.) Radlk. belongs to the Sapotaceae family and is commonly known as abiu, caimito, or abiurana. This species is widespread in South America (Luna, 2004), and can easily be found in Brazilian home gardens. The yellow fruits are eaten *in natura* or used to prepare desserts and ice cream. Additionally, several parts of *P. caimito* have been used as folk remedies, mainly to relieve body pain and aid in wound healing (Kramer, *et al.*, 2002). The fruit pulp is mucilaginous and is eaten to relieve coughs, bronchitis, and other pulmonary complaints, while the latex is used as a vermifuge and a purgative. Also, it is applied to abscesses (Morton and Dowling, 1987). The Tikano people (Amazon native people) use *P. caimito* as a treatment to facilitate childbirth (Luz, 1996; Ruiz, *et al.*, 2011).

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The ethanol extract of *P. caimito* leaves showed antimicrobial activity against *Pseudomonas aeruginosa*, *Bacillus cereus*, and *Candida albicans* (Kramer, *et al.*, 2002). A bark extract was evaluated against *Artemia salina* larvae and showed no toxicity (Quignard, *et al.*, 2003). The methanol extract from the bark was tested against *Colletotrichum lindemuthianum*, a fungus causing bean anthracnose; however, it did not show any anti-fungal activity (Pinto, *et al.*, 2010). Peruvian rainforest inhabitants use the leaves of *P. caimito* to treat malaria. However, an *in vitro* evaluation of a crude hydro-ethanolic extract did not show any activity against *Plasmodium* (a chloroquine-resistant strain) or *Leishmania* (Kvist *et al.*, 2006; Ruiz *et al.*, 2011).

The aqueous extract of *P. caimito* leaves showed inhibitory activity against *α*-amylase (IC_{50} = 13.6 µg/mL), *α*-glucosidase (IC_{50} = 2.58 µg/mL) and tyrosinase (IC_{50} = 50.0 µg/mL) (Souza *et al.*, 2012a; Souza *et al.*, 2012b).

A crude ethanol extract from leaves inhibited germination in *Lactuca sativa* and *Lycopersicum esculentum*. The germination inhibition ratios (GIR) at 4 mg/mL were 76% and 75%, respectively (Condessa, *et al.*, 2013). However, the extract did not inhibit the growth (radicle and hypocotyl elongation) of *Phaseolus vulgaris* (Morikawa, *et al.*, 2012).

Lupeol, α-amyrin, erythrodiol, and dammarendiol II were isolated from the benzene extract of the fruit (Pellicciari, *et al.*, 1972). The fruits gave α–copaene, hexadecyl acetate, palmitic acid (Maia, *et al.*, 2003), and 5-caffeyoylquinic acid (Pontes, *et al.*, 2002). The fruit contained 2-10 mg/100 g of ascorbic acid (Canuto, *et al.*, 2010; Jáuregui, *et al.*, 2015). Taraxerol, taraxerol acetate, sitosterol, and erythrodiol were isolated from *P. caimito* bark (Ardon and Nakano, 1973). The major fatty acids derived from the fruit seed oil were palmitic and oleicacids (Schuch, *et al.*, 1984).

Thus, *P. caimito* is an interesting species owingto its antioxidant and biological properties and should be thoroughly examined. The objective of this work therefore was to evaluate the hexane, ethanol, and aqueous extracts from *P. caimito* leaves byusing pre-screening procedures for *Artemia salina* toxicity and *in vitro* antioxidant models.

MATERIAL AND METHODS

Plant material

The leaves of *P. caimito* were collected at Pocrane, Minas Gerais, Brazil, in December 2005 and identified by Prof. S. M. Gomes. A voucher specimen was deposited at Herbarium of Universidade de Brasília (UB) (voucher number UB 27284).

Extraction procedures

The dried and powdered plant material (506.0 g) was macerated at room temperature for seven days (repeated three times) with hexane, followed by ethanol. After filtration, the solvents were removed under reduced pressure, at a temperature below 40 ^{o}C, yielding 14.0 g of hexane and 28.4 g of crude ethanol extracts (2.8% and 5.6% yield, respectively). The aqueous extract from 300.0 g of the plant material was obtained by infusion by using 3.0 L of distilled water. After filtration, the obtained infusion was lyophilized, yielding 23.2 g of aqueous extract (7.7% yield).

Isolation procedures

Hexane extract (8.5 g) was chromatographed on silica gel 60 Merck, by using a hexane: ethyl acetate: methanol gradient. The obtained fractions were monitored by thin layer chromatography (TLC), performed on precoated ALUGRAM sil G Machery-Nagel silica gel (60/0.2 mm) plates, using anisaldehyde reagent to visualize the spots (Wagner and Bladt, 1996). After analysis by TLC, the obtained fractions were collected and 15 groups were formed. After purification, groups 1, 3, 5, and 6 were characterized by ^{1}H and ^{13}C NMR spectra analysis. ^{1}H and ^{13}C NMR spectra were obtained using Varian (7.05 T) MercuryPlus spectrometers, operating at 300 MHz and by using $CDCl_3$.

Group 1 (93.0 mg), eluted with hexane, gave a hydrocarbon mixture. Group 3 (150.0 mg), eluted with hexane:ethyl acetate (9:1), was extracted with acetone, yielding 15.0 mg of an amorphous white solid, which was characterized as a long-chain ester mixture. Groups 5 and 6 (438.0 mg) were extracted with acetone: methanol (1:1), yielding 30 mg of **1**.

Brine shrimp lethality test (BST)

The crude extracts were tested for brine shrimp lethality. The assay was performed according to a simplified Meyer's method (Meyer *et al.*, 1982), with modifications. Briefly, encysted eggs of the brine shrimp *Artemia salina* L. (Maramar) were incubated in artificial sea water at 28 °C. Samples were dissolved in 200 µL of DMSO plus 20 mL of artificial seawater. Serial dilutions (triplicate) were prepared in the same solution. Metanauplius (10 units) was added to each set of tubes containing samples and the cultures were further incubated for 24 h. Controls containing DMSO were included in each set of experiments. Potassium dichromate was used as the reference standard. LD_{50} (after 24 h) was calculated by Probit analysis (Perfeito, *et al.*, 2005).

DPPH assay

The free radical-scavenging activity of the plant extracts and standards was assessed based on Blois' method with slight modification (Yildirim, *et al.*, 2001), using the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. Butylated hydroxytoluene (BHT), a well-known and widely used synthetic antioxidant, was used as a positive control. DPPH (2.0 mg) was dissolved in ethanol (10 mL) to obtain a final concentration of 500 µM. This solution was preserved in a dark flask at room temperature.

DPPH solution (300 µL), prepared daily, was added to 300 µL of each extract or BHT solution in various concentrations and placed in the dark at 25 ^{o}C. After incubation for 30 min, the absorbance of each solution was measured at 517 nm.

A negative control was prepared using 300 µL of the solution, previously obtained from each extract and 300 µL of

ethanol, to account for the pigments of the crude extracts. Absorbance values of the solutions were measured and subtracted from those obtained with DPPH.

Data were used to calculate the concentration necessary to consume one-half of the initial amounts of DPPH (EC_{50}). Each of the measurements described above was performed in at least three replicated experiments, and the results were reported as mean and standard deviation values. The results were expressed in μg/mL.

Proanthocyanidin content

Proanthocyanidin quantification was performed using an adapted vanillin/sulfuric acid method (Morais, *et al.*, 1999). Briefly, extracts were dissolved in a 10% hydroethanolic solution at 1000 μg/mL concentration. An aliquot (1 mL) of these solutions was mixed with 2 mL of freshly made vanillin/sulfuric acid solution (0.2 g of vanillin in 100 mL of 30% sulfuric acid) and incubated for 15 minutes at 25 °C. The absorption was measured at 500 nm. A calibration curve was constructed with catechin as a reference standard in the 1000–62.5 μg/mL concentration range. Results were expressed in mg equivalents of catechin per g of extract.

Total phenolic content

The total phenolic content (TPC) was determined by the Folin-Ciocalteu method as previously described (Morais, *et al.*, 1999; Souza, *et al.*, 2007), with slight adaptation to the semi-micro scale. A 5% aqueous solution of Folin-Ciocalteu reagent was prepared and 300 μL was transferred to disposable semi-micro cuvettes, which contained aliquots of the extract solutions (100 to 200 μL). After 5 minutes, 300 μL of an aqueous solution of sodium carbonate (7.5%) was added and the final volume was adjusted to 900 μL with water.

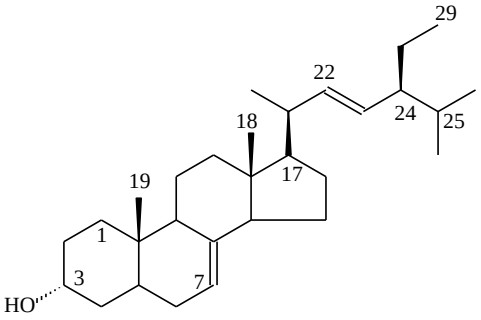
This mixture was incubated for 2 hours at 25 °C. Absorbance was measured at 760 nm. A calibration curve was constructed with gallic acid solutions in the 10–40 μg/mL concentration range.

The TPC was determined by interpolation of absorbance values with the calibration curve obtained (TPC = Abs*0.0072 + 0.0029), with a correlation coefficient of 0.9956, and detection and quantification limits of 0.5 and 1.5 μg/mL gallic acid, respectively. Results were expressed as mg equivalents of gallic acid (GAE) per g of extract.

RESULTS AND DISCUSSION

The NMR spectra of compound ***1*** showed a typical steroidal profile. In ^{1}H NMR spectrum, in addition to other signals, a multiplet at δ 3.57, from hydrogen linked to C3 of the steroidal skeleton and two double doublets at δ 5.02 and δ 5.16, attributed to olefinic hydrogens,were observed. The ^{13}C NMR spectrum showed 29 signals (Table 1). A comparison of NMR spectra data with those reported in the literature (Villasenor *et al.*, 1996) allowed identification of compound ***1*** as spinasterol.

Spinasterol (***1***): ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 0.54, 3H, s (H-18); 0.79, 3H, s (H-19); 0.80, 3H, t, J=7.3 Hz (H-29);0.84, 3H, d, J= 6.34 Hz (H-26 or 27); 1.02, 3H, d, J=6.58 Hz (H-21); 3.57, 1H, m (H-3); 5.02, 1 H, dd, J = 8.6 Hz (H-22 or 23); 5.14, IH, m (H-7); 5.16, IH, dd, J = 8.5 Hz (H-22 or 23). ^{13}CNMR (75MHz, $CDCl_3$)**:** see table 1.



spinasterol (***1***)

Table 1: 13C NMR data (δ, CDCl3) from compound 1 in comparison with literature (Villasenor *et al*., 1996).

13C	spinasterol	1	13C	spinasterol	1
1	37.11	37.11	16	28.49	28.51
2	31.44	31.45	17	55.86	55.85
3	71.04	71.06	18	12.03	12.04
4	37.95	37.96	19	13.02	13.04
5	40.23	40.22	20	40.82	40.84
6	29.63	29.62	21	21.08	21.10
7	117.43	117.45	22	138.15	138.17
8	139.53	139.56	23	129.45	129.41
9	49.42	49.41	24	51.22	51.23
10	34.19	34.20	25	31.85	31.87
11	21.52	21.53	26	21.34	21.37
12	39.44	39.43	27	18.97	18.98
13	43.27	43.27	28	25.28	25.4
14	55.09	55.11	29	12.23	12.26
15	22.98	23.00			

Spinasterol has been investigated for its biological activities. At a concentration of 7.0 μg/0.1 mL acetone, this phytosterol decreased the incidence of skin tumours in mice by 55.6% (Villasenor and Domingo, 2000). Additionally, it showed antigenotoxic activity in both a micronucleus test and an *in vivo* method (Villasenor, *et al.*, 1996), and increased the resistance of murine hippocampal HT22 cells to oxidative injury (Jeong, *et al.*, 2010). Spinasterol was able to decrease plasma and liver cholesterol levels, causing no significant change in phospholipid levels (Uchida, *et al.*, 1983). In addition, spinasterol showed inhibition of glomerular mesangial cell proliferation caused by high-ambient glucose, about 1,000 times higher than that of simvastatin, and reduced the serum triglyceride increase, renal weight, and urinary protein excretion in streptozotocin-induced diabetic mice (Jeong, *et al.*, 2004). Furthermore, spinasterol suppressed pro-inflammatory enzymes and inflammatory mediators in BV2 microglial cells. This steroid also suppressed nitric oxide (NO), prostaglandin E2 (PGE2), tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (Jeong, *et al.*, 2010). As expected, TPC, expressed as mg of gallic acid equivalent (GAE)/g extract, and proanthocyanidin content expressed as mg of

catechin equivalent/g extract were affected by the extracting solvents, with the following order: hexane ≤ ethanol < water (Karabegović, *et al.*, 2014). The results are presented in Table 2.

Table 2: Total phenol and proanthocyanidin content and radical scavenge potential of *Pouteria caimito* leaves extracts.

Sample	DPPH[a]	Proanthocyanidins[b]	Total Phenol Content[c]
Hexane extract	597.7 ± 9.3	< L. D.[d]	11.5 ± 1.0
Ethanol extract	223.1 ± 2.8	< L. D.[d]	46.5 ± 6.5
Aqueous extract	36.1 ± 0.8	266.3 ± 6.3	173.6 ± 4.6
Ascorbic acid	12.9 ± 3.5		
BHT	14.9 ± 2.2		

[a]EC_{50} (µg/mL); [b]catechin equivalent (mg/g); [c]gallic acid equivalent (GAE) (mg/g); [d] L.D. = 20.4 mg/mL of catechin.

The TPC varied from 11.5 to 173.6 GAE mg/g. The aqueous extract had the highest phenol content. It is interesting to observe that the TPC value found in the leaf extract was greater than that found in fruit pulp. Contreras-Calderon et al. (2011) found 83 GAE mg/100 mg for the hydro-methanol (50%) extract from fruit (Contreras-Calderón, *et al.*, 2011), while Tuesta et al. (2014) found 7.81 GAE mg/100 mg for thehydromethanol (90%) extract (Tuesta, *et al.*, 2014).

The *Artemia salina* lethality test presents a correlation with an *in vivo* oral acute toxicity model (Parra, *et al.*, 2001). Mathews (1995) proposed the use of the Artemia salina test as a model to evaluate compounds for their ability to protect against superoxide-mediated toxicity (Matthews, 1995). For both evaluations, an LD_{50} value lower than 1 mg/mL was considered active (Meyer, *et al.*, 1982). Based on this classification, *P. caimito* leaf extracts can be regarded as non-toxic, as well as presenting no protective activity against superoxide-mediated toxicity.

On the other hand, by using a DPPH radical-scavenging test, one of the shortest tests available to investigate the overall hydrogen/electron-donating activity of single antioxidants, the scavenging ability of the aqueous extract, despite being lower than that of the controls (ascorbic acid and BHT), was found to be good. Moreover, it seemed that the inhibition percentage (antioxidant activity) and total phenolics could be correlated.

By comparison of the obtained values for antioxidant activity and phenolic content, it is possible to observe that as phenol content rises, radical-scavenging activity increases, as shown by other authors (Chaouche, *et al.*, 2014; Haddouchi, *et al.*, 2014; Karabegović, *et al.*, 2014)

CONCLUSION

While studies of the antioxidant activity of *P. caimito* fruit can be found (Canuto, *et al.*, 2010; Nascimento, *et al.*, 2008), there are few studies involving *P. caimito* leaves. With regard to chemical composition, although spinasterol has been described as a common phytosterol of the Sapotaceae family (Khallouki, *et al.*, 2003), as far we know, this is the first time this compound has been found in *P. caimito.* The presence of this compound can justify, at least in part, the biological activity shown by this plant species.

ACKNOWLEDGEMENTS

Financial support from The National Council of Technological and Scientific Development (CNPQ), The Brazilian Innovation Agency (FINEP), and The Foundation for Scientific and Technological Enterprises (FINATEC).

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How to cite this article:

França CV, Perfeito JPS, Resck IS, Gomes SM, Fagg CW, Castro CFS, Simeoni LA, Silveira D. Potential radical-scavenging activity of *Pouteria caimito* leaves extracts. J App Pharm Sci, 2016; 6 (07): 184-188.