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Chemical constituents of *Lecythis pisonis* and cytotoxic activity

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Abstract: The phytochemical investigation of the ethanol extract from leaves of *Lecythis pisonis* Cambess., Lecythidaceae, resulted in the isolation of seven triterpenes: α- and β-amyrin, uvaol and erythrodiol, ursolic and oleanolic acids and 3β-friedelinol (friedelan-3β-ol), as well as a mixture of sitosterol and stigmasterol steroids and a diterpene (*E*)-phytol. The structures of these compounds were identified by ^{1}H and ^{13}C NMR spectral analysis and compared with literature data. The mixture of triterpenes ursolic and oleanolic acids isolated from the active ethereal fraction showed moderate cytotoxic activity. This paper describes for the first time the phytochemical and cytotoxic study of *Lecythis pisonis*' leaves.

Introduction

Lecythidaceae, known as the "Brazil nut family", is considered the third most abundant tree of the Amazon with about 25 genera and 489 species (Mori & Prance, 1990). Studies of plants in the Lecythidaceae family are still scarce and are restricted to 21 species in thirteen genera: *Barringtonia, Bertholletia, Careya, Cariniana, Couroupita, Eschweilera, Foetidia, Grias, Gustavia, Lecythis, Napoleonaea, Planchonia* and *Petersianthus*. *Barringtonia* is the most explored genus with five species (*B. acutangula, B. racemosa, B. maunwongyathiae, B. asiatica* and *B. speciosa*) (Oliveira, 2010).

The chemical constituents identified in plants of the Lecythidaceae family include pentacyclic triterpenoids and their glycosides, neo-clerodane diterpenoids, sesquiterpenoids, monoterpenoids, steroids, alkaloids, simple phenolic compounds, flavonoids, ellagic acid and derivatives, vitamin E, α-tocopherylquinone, 3-*O*-galloylepigallocatechin, epigallocatechin, sucrose, fatty acid, ethyl esters and waxes (Oliveira, 2010).

Several pharmacological activities have been report with plants of this family, such as antinociceptive, antibacterial, antitumor, anti-inflammatory, antifungal, antileishmanial, antioxidant, hepatoprotective and cytotoxic (Oliveira, 2010).

Lecythis pisonis Cambess, Lecythidaceae, is popularly known as sapucaia or sapucaia nut which is found among the states of Piauí, from Pernambuco to São Paulo and in the Amazon region (Corrêa, 1978). The leaves of sapucaia are popularly used in the form of baths for treatment of itching (pruritus) of the body and the oil extracted from the seeds is used as an emollient in reducing muscle pain (Franco & Barros, 2006; Agra et al., 2007).

Considering the scarcity of studies with plants of the family Lecythidaceae, and above all, the lack of chemical investigation of the species *L. pisonis*, this paper describes for the first time the phytochemical study of the leaves of this species and the evaluation results of cytotoxicity.

Materials and Methods

General procedures

The NMR spectra of ^{1}H and ^{13}C were obtained on Varian Inova spectrometer and Brüker Avance DRX-500 model, operating at 500 MHz (^{1}H) and 125 MHz (^{13}C). The chromatographic plates were prepared using a mixture of silica gel 60 G Vetec and 60 GF Fluka (1:1). Spots on TLC were observed by spraying the plates with solution of $Ce(SO_4)_2$, followed by heating at 100 °C. The chromatographic columns were prepared with silica gel

from 0.060 to 0.020 mm (Acros Organics) or Sephadex LH-20 (Pharmacia Biotech).

Plant material

The leaves of *L. pisonis* were collected in Teresina municipality, Piauí State, Brazil (southern latitude 05° 02' 53.2", western longitude 42° 47' 16.8", the sea level of 68 m), in July 2008. A voucher specimen (TEPB 26488) has been deposited in the Graziela Barroso Herbarium at UFPI.

Extraction and isolation

The leaves of *L. pisonis* were dried at room temperature and ground. The material (2 kg) was extracted exhaustively with ethanol six times, and each extraction had the duration of 48 h. The ethanol was removed under vacuum and lyophilized, giving 272 g (13%) of ethanol extract, which was suspended in a mixture of H_2O/MeOH (3:2) solution and extracted successively with *n*-hexane, diethyl ether and EtOAc, respectively, obtaining the *n*-hexane (60 g, 30%), diethyl ether (24 g, 12%), EtOAc (21 g, 10.5%) and water (70 g, 37.5%) fractions, and a precipitate formed in the phase EtOAC (ppt-EtOAc, 10 g, 5%).

The diethyl ether fraction (10 g) was fractionated by chromatography column on silica gel (53 x 5 cm, 250 g) eluted with $CHCl_3$, $CHCl_3$-MeOH in order of increasing polarity, yielding 103 fractions (240 mL each). The solvent was removed under vacuum and analyzed by TLC. The fractions were combined into fifteen groups. Fraction F7 (242 mg) was suspended in *n*-hexane and yielding an amorphous precipitate corresponding to compound 7 (226 mg, 2.26%).

The group F17 (17-23, 603 mg) was suspended in MeOH to yield a mixture of compounds **5** and **6** (475 mg, 4.75%).

The group F8 (8-15, 1.02 g) was fractionated on a silica gel column eluted with *n*-hexane, *n*-hexane-EtOAc in increasing order of polarity giving 162 fractions (50 mL each). After evaporation of the solvent and analysis by TLC, the fractions were combined into fourteen groups. The groups G22 (33 mg) and G25 (25-45, 78 mg) were rechromatographed on Sephadex LH-20 column (54 x 1.5 cm) using hexane-CH_2Cl_2 (1:4) as eluent, resulting in 20 mg (0.20%) of compounds **1, 2** and **8**, and 13 mg (0.13%) of a mixture of sitosterol and stigmasterol. Group G92 (92-100, 54 mg) was purified on Sephadex LH-20 column using as eluent CH_2Cl_2- *n*-hexane (1:4) and CH_2Cl_2-acetone (3:2) yielding 19 mg (0.19%) of a mixture of compounds **3** and **4**.

Cytotoxicity studies

The cytotoxicity of the ethanol extract and diethyl ether fraction, compound **7** and mixture of compounds (**5**+**6**) was investigated against HL-60 (human leukemia), SF-295 (glioblastoma), HCT-8 (human colon carcinoma) and MDA-MB-435 (human melanoma) tumor cell strains (National Cancer Institute, Bethesda, MD, USA). All lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U mL^{-1} penincillin and 100 µg mL^{-1} streptomycin at 37 °C with 5% CO_2. Cells were plated in 96-well plates (10^5 cells/well for adherent cells or 0.3 x 106 cells/well for suspend cells in 100 µL for medium). After 24 h, samples (0.39 to 25 µg mL^{-1}) dissolved in DMSO (1%) were added to each well and incubated for 72 h. Control groups received the same amount of DMSO. Doxorubicin (0.01 to 0.58 µg mL^{-1}) was used as positive control. Tumor cell growth was quantified by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a blue formazan product (Mosmann, 1983).

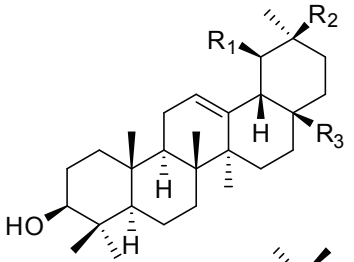
The percentage of cell growth inhibition (IC%) was calculated according to the report by Mahmoud et al. (2011). The IC50 values and their confidence intervals were obtained by nonlinear regression using the GraphPad program (Intuitive Software for Science, San Diego, CA).

Results and Discussion

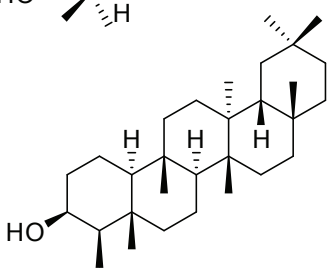
The phytochemical investigation of the ethanol extract from leaves of *Lecythis pisonis* Cambess, Lecythidaceae, resulted in the identification of seven triterpenoids; a mixture of α- and β-amyrin (**1**+**2**) and the diterpenoid (*E*)-phytol (**8**), a mixture of uvaol and erythrodiol (**3**+**4**), a mixture of ursolic and oleanolic acids (**5**+**6**), friedelan-3β-ol (**7**), as well as a mixture of steroids sitosterol and stigmasterol. The structural identification of these compounds was performed by analysis of spectral data of ^1H and ^{13}C NMR (including DEPT 135° and 90°), as well as by comparison of their spectral data with those reported in the literature (Olea & Roque, 1990; Ayres et al., 2008; Rahman & Ahmad, 1992; Mahato & Kundu, 1994; Junges et al., 2000; Salazar et al., 2000; De-Eknamkul & Potduang, 2003).

The ^1H and ^{13}C NMR spectra of mixtures (**1**+**2**+**8**), (**3**+**4**) and (**5**+**6**) showed signals between δ 3.11-3.16 (*dd*, *J* = 4.5-7.5 and 9.0-11.0 Hz, H-3), and 78.5-79.3 (C-3) characteristic of 3β-OH triterpenoids. In addition, the ^{13}C NMR spectra presented two pairs of signals between δ 125.1-124.7, and 137.9-139.8; δ 122.0-122.4 and 143.6-145.4 assigned to olefinic carbons C-12 and C-13 skeletons ursane and oleanane, respectively (Olea & Roque, 1990).

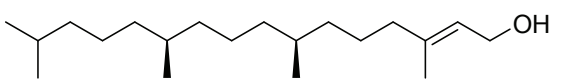
The proportion of substances in the mixtures was



1 R_1=R_3=CH_3; R_2=H
2 R_1=H; R_2=R_3=CH_3
3 R_1=CH_3; R_2=H; R_3=CH_2OH
4 R_1=H; R_2=CH_3; R_3=CH_2OH
5 R_1=CH_3; R_2=H; R_3=CO_2H
6 R_1=H; R_2=CH_3; R_3=CO_2H



7



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determined by comparing the integrations of signals from oximethinic hydrogen (H-3) of triterpenoids, steroids or oximethilenics of **8** with olefinic hydrogens (Ayres et al., 2008).

The mixture of triterpenoids α-amyrin (**1**), β-amyrin (**2**) and the diterpenoid (*E*)-phytol (**8**) was identified at a ratio of 40:36:24. (*E*)-phytol was evidenced in the ^{1}H NMR spectrum by signals at δ 4.16 (*dd, J* = 0.5 and 7.0 Hz) assigned to the hydrogens oximethilenics and allylics, and δ 5.42 (*tq, J* = 7.0 and 1.5 Hz), referring to the olefinic hydrogen. The presence of (*E*)-phytol was confirmed by observation in the ^{13}C NMR spectrum of the signals in δ 59.1 (CH_2O), 123.4 and 140.5 (olefinic CH and C) and by comparison with literature data (Rahman & Ahmad, 1992; Ayres et al., 2008).

The triterpenoid mixture (**3**+**4**) was identified at a ratio of 66:34 and their structures were determined by the additional presence in the ^{1}H NMR spectrum of two doublet with *J* = 11.0 Hz, located in δ 3.14 and 3.56, assigned H-28a and H-28b, in accordance with the signals at δ 69.7 and 69.9 in the ^{13}C NMR spectrum assigned to C-28 of both substances. The NMR data obtained were consistent with those reported for uvaol (**3**) and erythrodiol (**4**) (Mahato & Kundu, 1994).

The mixture of compounds **5** and **6** was evidenced by the signals at δ 180.4 and 180.6, in the ^{13}C NMR spectrum, assigned to carboxylic carbon (C-28), compared to the spectrum of mixture of α-and β-amyrin (1 and 2). The NMR data obtained were consistent with those reported for ursolic (**5**) and oleanolic (**6**) acid at a ratio (59:41) (Junges et al., 2000).

The analysis of ^{13}C NMR and DEPT 135° and 90° of the substance 7 showed signals corresponding to six non-hydrogenated carbons, five methine, eleven methylene and eight methyl groups. We also observed a characteristic signal at δ 72.8 from oximethinic carbon (C-3), consistent with the signal in δ 3.67 (H-3) in the ^{1}H NMR spectrum. The NMR data obtained are in agreement with the structure of friedelan-3β-ol (**7**) (Salazar et al., 2000).

The ^{1}H NMR spectrum of the mixture of steroids sitosterol and stigmasterol showed signals between δ 0.6 and 0.9 corresponding to methyl groups of steroids, a multiplet in δ 3.53 (H-3) corresponding to oximethinic carbon and a singlet in δ 5.35 assigned to H-6 in steroid-Δ^5. In the ^{13}C NMR spectrum signals in δ 72.0 (C-3) of oximethinic carbon, δ 121.9 and 141.0 referring to olefinic carbons C-6 and C-5 were observed, respectively. The presence of two signals of lower intensity at δ 129.5 and 138.5 assigned to C-23 and C-22 of the stigmasterol confirmed the presence of this substance. The evidences above allowed identification of the steroids: sitosterol and stigmasterol (De-Eknamkul & Potduang, 2003) at a ratio of 85:15.

The substances α-amyrin (**1**) and β-amyrin (**2**), friedelan-3β-ol (**7**), ursolic (**5**) and oleanolic (**6**) acid, sitosterol and stigmasterol had been reported in species of Lecythidaceae, however triterpenoids uvaol (**3**), erythrodiol (**4**) and diterpenoid (*E*)-phytol (**8**) have been reported for the first time in this family.

In the test with tumor cells, the EtOH extract and triterpenoid **7** (25 μg mL^{-1}), showed a low percentage of cell growth inhibition (IC%), while the diethyl ether fraction had values ranging from 43.7 to 95.5% (Table 1). The mixture composed of ursolic (**5**) and oleanolic acid (**6**) isolated from the diethyl ether fraction, showed 100% inhibition for the strains of HC-T8 (human colon carcinoma), SF-295 (glioblastoma) and MDA-MB-435 (human melanoma) when tested in the concentration of 25 μg mL^{-1}. However, the values of mean inhibitory concentration (IC50) of the mixture of **5** and **6** were greater than 1 μg mL^{-1} and showed a moderate activity

compared to doxorubicin, used as positive control (Table 2).

Table 1. Percentage of growth inhibition (IC%) in the panel of three cancer cell lines after treatment for 72 h with samples of *Lecythis pisonis* concentration of 25 μg mL^{-1}. The results are expressed as mean and standard deviation.

Samples	IC%		
	HCT-8	SF-295	MDA-MB-435
EtOH extract	1.6±0.9	51.6±0.4	40.9±2.4
Diethyl ether fraction	43.7±1.8	95.5±1.7	60.8±5.5
5+6	100±0.1	100±0.1	100±0.6
7	11.7±2.8	13.1±5.7	0.0±0,0
Doxorubicin *	96.9±4.8	84.3±3.2	99±0.92

HL-60: human leukemia; HCT-8: human colon; SF-295: glioblastoma; MDA-MB-435: human melanoma * positive control.

Table 2. Growth inhibition (IC50 in μg mL^{-1}) with its respective confidence interval in tumor cell lines treated for 72 h by mixing **5+6**.

Samples	IC50			
	HL-60	HCT-8	SF-295	MDA-MB-435
5+6	3.4 1.6-7.0	7.1 5.5-8.9	2.4 1.5-3.9	10.6 8.3-13.5
Doxorubicin	0.04 0.03-0.05	0.02 0.02-0.03	0.48 0.34-0.72	0.96 0.68-1.32

HL-60: human leukemia; HCT-8: human colon; SF-295: glioblastoma; MDA-MB-435: Human Melanoma * positive control.

In the literature there are reports of tests with the cell line HCT-15 (human colon carcinoma), with inhibitory concentration of 30 μM to ursolic acid and 60 μM to oleanolic acid (Vechia et al., 2009). The M4Beu cell line (human melanoma) was tested only with ursolic acid and the IC50 value ranged from 12.5 to 15.0 μM (Vechia et al., 2009). There are no reports in the literature on the cytotoxicity of these triterpene acids with strains of human glioblastoma, a type of the most lethal malignant brain tumors. Patients with this type of tumor have a median survival of fifteen months after diagnosis (Horvath et al., 2006), justifying the search for new molecules with potential antitumor activity.

In conclusion, we believe that the above mentioned data are of importance from the chemotaxonomy and pharmacological point of view. On the other hand, further phytochemical studies on *L. pisonis* are necessary to obtain better knowledge of this species.

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