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Histochemical and Phytochemical Study of *Amburana cearensis* (Fabaceae) Seed Oil

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Amburana cearensis seed oil is used in traditional medicine to treat respiratory diseases. However, investigations of the chemical compounds present in this oil are necessary to prove their effectiveness. Thus, we aimed to analyze the compounds present in *A. cearensis* seeds it was analyzed by histochemical and phytochemical tests. Fatty acids profiles were analyzed by gas chromatography coupled with flame ionization detector, which detected the presence of high concentrations of oleic (50.1%), palmitic (15.7%), linoleic (9.7%) acids and linolenic (5.0%). Volatile components were analyzed by headspace extraction and gas chromatography coupled with mass spectrometry, with emphasis on the presence of a coumarin, which may be associated with the bronchodilator effect of the oil. Histochemical analysis of the seed oil revealed presence of alkaloids in the cell vacuoles and lipophilic components in drops in the cell cytoplasm. The analysis of *A. cearensis* seed oil contributed to the understanding of the therapeutic actions of the species and to the prospection of promising compounds for the development of new drugs.

Keywords: Coumarin, Fatty acids, GC-MS, Secondary metabolites, Oleic acid, HS extraction, Traditional medicine.

Amburana cearensis (Allemão) A.C.Sm. [1] occurs in Caatinga (Brazilian dry land) and throughout South America. It is a tree up to 20 m height and produces flat and elongated legume containing wrinkled seeds [2].

The seed oil is used in traditional medicine to treat respiratory diseases, and it is valued for their aroma. A fine powder made from the seed is used as snuff to induce sneezing and alleviate nasal congestion [3-5]. The bronchodilator effect of the oil is attributed to coumarin [5] and it was confirmed in preclinical studies [6].

Compounds related to the therapeutic properties of natural products can be detected by phytochemicals and histochemical tests. These bioproducts prospecting techniques are quick and inexpensive and can guide studies to identify promising compounds for the synthesis of new drugs [7-9]. The work is about the chemical investigation of seed oil of *A. cearensis*, which is traditionally used for respiratory diseases and it aimed to identify the main classes of compounds to determine the profile of fatty acids and to analyze volatile compounds.

Histochemical tests performed in the protoderm and ground meristem were positive for alkaloids (Figures1a-b) in the central cell vacuole, and for neutral lipids (Figure 1c) and terpenes (Figure 1d) in drops in the peripheral cytoplasm. Tannins and flavonoids were not detected. The phytochemical tests of the oil were positive for alkaloids (Figures 2a-b), lipids in general (Figure 2c) and terpenes (Figure 2d), and negative for tannins and flavonoids.

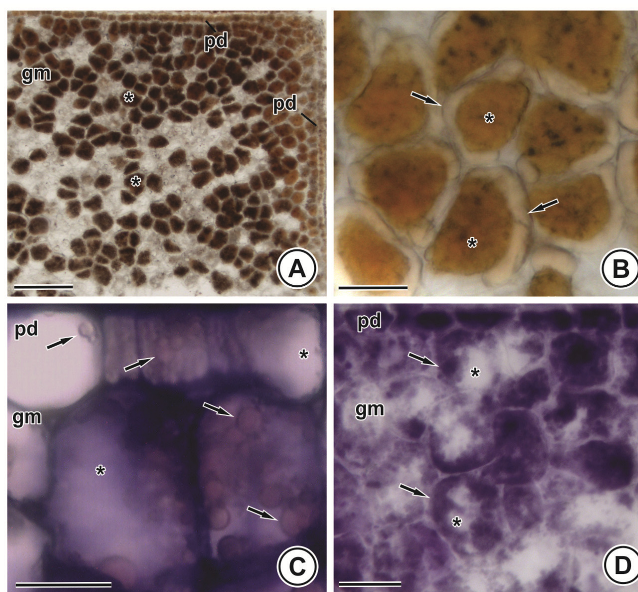

Figure 1: Histochemical tests in transversal sections of *A. cearensis* seeds. a. Alkaloids (brown, Dragendorff Reagent). b. Alkaloids (brown, Dittmar reagent). c. Neutral lipids (pink, Nile Blue sulfate). d. Terpenes (light purple, NADI). Legends: pd, protoderm; gm, fundamental meristem. Arrow, cytoplasm with terpene droplets. Asterisk vacuoles with alkaloids. Bars: a-b, d=10μm, c=20μm.

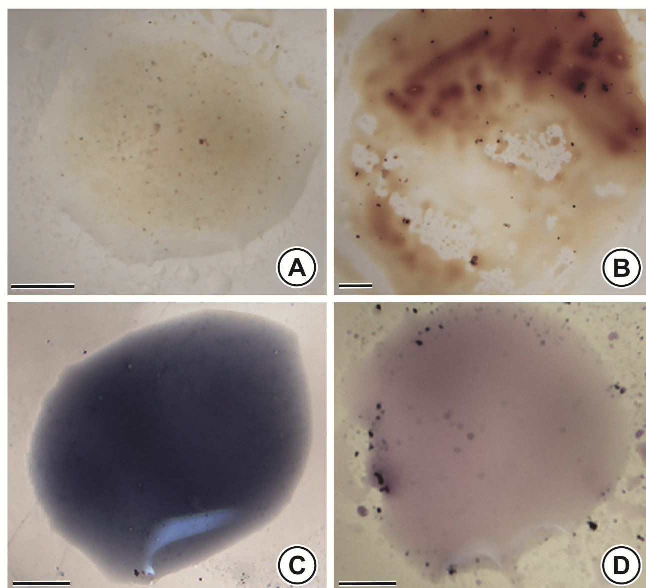

Figure 2: Phytochemical tests of *A. cearensis* seed oil performed on glass slides. A. Alkaloids (brown, Wagner Reagent). B. Alkaloids (brown, Dittmar reagent). C. Lipids in general (blue, Sudan Black). d. Terpenes (light purple, NADI). Figure width = 1 mm.

GC-FID analyses indicated that unsaturated fatty acids were more abundant (67.1%) than saturated (30.6%). Among the saturated fatty acids, palmitic (15.7%), stearic (5.3%), behenic (3.9%), arachidonic (2.4%), lignoceric (2.2%), lauric (0.6%), myristic (0.3%), and margaric (0.2%) acids were identified. Among the mono-unsaturated fatty acids oleic was the most abundant (50.1%), and eicosanoic (2.1%) and palmitoleic (0.2%) acids were also detected. The polyunsaturated linoleic (9.7%) and linolenic (5%) fatty acids were detected (Table 1). Coumarin (1,2-benzopyrone) determined via headspace (HS) is the only volatile component present in the oil.

Table 1: Fatty acids from *Amburana cearensis* seed oil analyzed by gas chromatography coupled to a flame ionization detector (GC-FID).

N°	*$RT_{(min)}$	Compounds	Saturation	Relative area (%)
1	0.68	Lauric (C12:0)	Saturated	0.6
2	1.32	Myristic (C14:0)	Saturated	0.3
3	2.49	Palmitic (C16:0)	Saturated	15.7
4	2.63	Palmitoleic (C16:1)	Mono-unsaturated	0.2
5	3.20	Margaric (C17:0)	Saturated	0.2
6	4.08	Stearic (C18:0)	Saturated	5.3
7	4.31	Oleic (C18:1)	Mono-unsaturated	50.1
8	4.65	Linoleic (C18:2)	Di-unsaturated	9.7
9	5.22	Linolenic (C18:3)	Tri-unsaturated	5.0
10	5.91	Arachidonic (C20:0)	Saturated	2.4
11	6.06	Eicosanoic (C20:1)	Mono-unsaturated	2.1
12	7.85	Behenic (C22:0)	Saturated	3.9
13	9.74	Lignoceric (C24:0)	Saturated	2.2
		Not identified	Saturation	2.3
Total				**100.0**

*$RT_{(min)}$: Retention time, in minutes

Fabaceae species store a wide variety of secondary metabolites known to have biological activity [10]. Alkaloids often are accumulated in seeds and show antibacterial and anti-inflammatory functions [11-13]. The alkaloids present in significant amounts in the oil from *A. cearensis* seed probably act together with the fatty acids and coumarin expanding the anti-inflammatory effect in the treatment of respiratory diseases [14, 15]. The phytochemical method used in this work realized on glass slides to detect the alkaloids [16] is a simple and inexpensive innovation in prospecting secondary compounds in medicinal oils.

Fatty acids are essential to humans as some of them can only be obtained in our diets [17]. Oleic acid/omega 9 was the most abundant compound present in the oil from *A. cearensis* seed (50.1%). This percentage is quite high when compared to other oils rich in omega 9, such as olive oil (70 %) and peanut oil (62 %) [18]. Palmitic acid is the second major component of the fatty acids (15.7%) and it was related to the increase of anti-inflammatory eicosanoid precursors in the erythrocyte membrane during the development of respiratory diseases [15]. Linoleic acid/omega 6 and linolenic acid/omega 3 were involved in the synthesis of polyunsaturated fats, such as arachidonic, eicosapentaenoic, and docosahexaenoic acids [19] known to mediate inflammatory processes [20, 21].

Coumarin 1, 2-benzopyrone is the main component of the volatile oil from *A. cearensis* seed (see Figure 1). It is responsible for the pleasant smell of the seed and it was also detected in the bark [22, 4]. Many pharmacological properties have been attributed to coumarin for the treatment of respiratory diseases [23, 14] as the expectorant, anti-inflammatory and anti-allergic activities [24]. It induced relaxation of the respiratory muscles and favored the fluidization of tracheobronchial exudates [25]. Its anti-inflammatory activity was attributed to inhibiting vascular permeability and migration of leukocytes and neutrophils [26, 27].

The oil from *A. cearensis* seed is rich in coumarin, alkaloids and oleic, palmitic, linoleic, and linolenic fatty acids. The compounds observed have the potential to be used in the development of pharmaceuticals.

Experimental

Oil extraction: Certified seeds of *A. cearensis* (10 g) obtained from the Brazilian Institute of Forest (São Paulo, Brazil) were ground in a TE-648 grinder (Tecnal, Ourinhos, Brazil), packed in filter paper and bound with fat free string, and the oil was extracted via Soxhlet. Petroleum ether (150 mL) was used as extracting solvent and its volume maintained by addition of more solvent to compensate evaporative losses. The solvent was evaporated for three hours at 50°C and the oily residue was transferred to a plastic tube (2 mL) and held at -20°C.

Histochemical tests: Histochemical tests were performed for metabolites in transversal sections hand-cut with a razor blade (20 μm thickness) including the protoderm and ground meristem. Dittmar [28] and Dragendorff [29] reagents were used to detect alkaloids, vanillin-HCl [30] to detect tannins, p-dimethyl-amino-cinannamaldehyde and caffeine (DMACA) [31] to detect flavonoids, naphthol + dimethyl-paraphenylene-diamine) (NADI) to detect terpenes [32], Sudan Black to detect general lipids, and Nile Blue sulfate [33] to detect neutral lipids.

Phytochemical tests on glass slides: Glass capillary tubes (0.5 mm diameter) were used to deposit oil droplets on glass slides held at -5°C for 10 minutes. This process was repeated three times to increase the oil volume on the slides. The phytochemical tests were undertaken using the same reagents described for the histochemical tests [34]. Photographic documentation of histochemical and phytochemical tests was performed using an A620 digital camera (Canon, Tokyo, Japan) coupled to an Eclipse E200 optical microscope (Nikon, Tokyo, Japan).

Fatty acids profile: The oil samples (12 mg) were dissolved in an ethanol solution (KOH 1.0 mol·L^{-1} ethanol 100 μL), homogenized in a vortex mixer for 10 seconds, heated in a microwave oven (80 W potential) for 5 minutes, cooled to room temperature, and

homogenized for 10 seconds in a solution composed of HCl (400 µL, 20%) + NaOH (20 mg) + ethyl acetate (600 µL). After 5 minutes the organic layer (300 µL) was removed, agitated in a vortex mixer for 10 seconds, and left standing for 5 minutes. The free fatty acids were methylated with BF_3/methanol (100 µL, 14%), warmed to 60°C (in a water bath) for 10 minutes, and diluted in methanol (400 µL).

Gas chromatography was performed in an Agilent HP7820A equipment with an EZChrom Elite Compact data acquisition program (Agilent Technologies, Santa Clara, CA) coupled to an HP-INNOWAX column (Hewlett-Packard 15 m x 0.25 mm x 0.20 µm) and to a flame ionization detector (GC-FID). The initial column temperature was 150°C and it was increased to 240ºC by 7 ºC·min^{-1}. The carrier gas was hydrogen (3 mL·min^{-1}), the injector (1 µL) operated at 250°C with split mode (1:50) and the FID detector maintained at 260°C. The peaks were identified by comparisons with FAME C_{14}-C_{22} methylated fatty acids patterns (Supelco cat no 18917).

Volatile components: *A. cearensis* seeds (2 g) were stored individually in glass vials (20 mL), placed in an auto sampler HS Combi-PAL (Agilent Technologies, Santa Clara, USA), homogenized at 500 rpm and incubated at 75°C for 5 minutes. The volatile components were extracted by headspace (HS) and analyzed by gas chromatography coupled with mass spectrometry (GC-MS) under the conditions described in Table 2. GC-MS was performed using a gas chromatograph 7890A, coupled with a mass spectrophotometer MS 5975C, equipped with a fused silica capillary column DB-5MS (30 m x 0.25 mm x 0.25 µm) using helium as the carrier gas (1 mL min^{-1}). Sample injection (1000 µL) was performed by splitless injection using an auto injector Combi PAL (Agilent Technologies, Santa Clara, CA). The rate of temperature increase was 2ºC min^{-1} from 35°C to 80°C, and 4°C min^{-1} to 150°C, with a total run time of 47 minutes. The system was operated in the scan mode (monitoring) with electron impact ionization at 70eV, and scan mass range from 40 to 550 (*m/z*) [35]. The resulting data were analyzed using the software MSD Chemstation and the NIST mass spectral library [36]. The relative abundance (%) of the constituents was calculated from peak areas of the gas chromatogram and organized according to the elution order. The percentage of each component was calculated using the normalized means of the chromatogram areas, and the compounds were identified through a comparison with the spectra of compounds deposited in the NIST mass spectral library [36].

Table 2: Auto-sampler System for Headspace Extraction (HS Combi-PAL) conditions.

Injection volume	1000 µL
Incubation temperature	75°C
Incubation time	5 min
Syringe temperature	75°C
Agitation speed	500 rpm
Fill speed	500 µLs^{-1}
Fill strokes	0
Pull up delay	500 s
Injection speed	500 µLs^{-1}
Pre-injection delay time	0 ms
GC run time	47 min
Sample weight	2.0 g

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