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Obtaining a Dry Extract of *Pterodon emarginatus* (Fabaceae) Fruits by Spray-Drying

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ABSTRACT

The *Pterodon emarginatus* presents some pharmacological properties that may be related to the presence of vouacapanes. The purpose of this work is to reach a standardized dry extract of the *P. emarginatus* fruit. The powder, ethanolic extract and dry extract showed the presence of vouacapanes and lupeol confirmed by IR and GC-MS. The drying process (spray drying) using the colloidal silicon dioxide showed to prevent the thermal degradation and increased approximately twice the terpenes content. The scanning electron microscopy (SEM) showed irregular and spherical particles. The analytical method by spectrophotometry for the quantification of total terpenes was validated and showed to be selective, linear, precise, accurate and robust. In the antinociceptive activity test (capsaicin), the pre-treatment with the dried extract reduced the reactivity time in 50.9%. The results may suggest that the technological processes employed to transform the *P. emarginatus* fruits in standardized dried extract were adequate to maintain quality chemistry and antinociceptive activity described for the fruits. This work represents the first description of the obtaining of the standardized dried extract of *P. emarginatus* and also the identification of lupeol in the fruits of this medicinal plant.

Key words: Medicinal plant, quality control, technological processes, spray drying, antinociceptive.

INTRODUCTION

The fruits of *Pterodon emarginatus* (Fabaceae family) are used for their larvicidal activity[1]; trypanocidal[2], leishmanicidal and phytopathogenic[3], allellopathic effect[4-6]; anti-inflammatory and analgesic[7-11]; antioxidant[12-13] and antiproliferative activities[14-16], which may be related to the presence of diterpenes, in particular, vouacapanes derivatives[11].

The vegetal raw materials used for the production of phytomedicines are commonly presented as dried extract[17-20] standardized for one substance or group of substances[17]. The purpose of this work was to obtain the dried extract standardized in the terpenes group from the fruits *P. emarginatus* by spray drying.

MATERIALS AND METHODS

Plant Material and Characterization.

The *P. emarginatus* fruits were collected in the state of Goiás (847 m, 17°02'1,1" S/48°49'0,3" W), Brazil. A voucher specimen was deposited in the herbarium of the Federal University of the State of Goiás (UFG), Brazil, under number 27.155. The fruits were naturally dried and crushed in knives mills. It were carried out sieve analysis, volatile content, the investigation of the presence of terpenes by thin layer chromatography (TLC)[21] and by infrared spectroscopy (IR) and by gas chromatography - mass spectrometry (GC / MS). For that, the powdered fruit was submitted to extraction with hexane in Soxhlet apparatus for a period of 4 hours and then it was concentrated at a rotary evaporator[22]. The relative density was adjusted to the approximate value of 0.93. It was stored under low temperature (-20° C) for 24 hours to form crystals[23] that were carefully removed and subjected to solid phase extraction (SPE) on silica cartridges of 1 g, to eliminate interference for the analysis of terpenes.

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Further it was developed a method for quantification of total terpenes in fruits, in which the extraction of 0.5 g powder of fruit in 150 mL ethanol 95% P.A was carried. Then, 1 mL of this solution was diluted with 25 mL of distilled water (stock solution). For color reaction, 1 mL of stock solution and 2 mL of a solution of sulfuric vanillin 1% was added in a test tube. The mixture was homogenized and allowed to stand in water bath at 20 °C for 15 minutes, and the readings were performed in the spectrophotometer (UV-VIS Meter®) at a wavelength of 520 nm[24]. The 6α,7ß-dihydroxyvouacapan-17ß-oate methyl ester was used as standard for the construction of the calibration curve (6,0 - 14,0 µg/mL). The assay was carried out in triplicate. This method was validated according to the specifications described in guidelines[25].The parameters of selectivity, linearity and range, precision (repeatability, intermediate precision), accuracy, limit of detection and limit of quantitation, robustness were evaluated.

Preparation and Characterization of the Ethanolic Extract.

The powdered fruits of *P. emarginatus* were extracted by percolation using ethanol 95% P.A (v/v). The extracts were concentrated under reduced pressure in a rotary evaporator to a ratio fruit: extract of 1:3 w/v. The following physicochemical parameters were measured for the extract: pH -digital potentiometer PHS-3B (Labmeter®), relative density, viscosity - rheometer DV-III (Brookfield®), alcohol level, solids content – moisture balance MB 35 (Ohaus®). The investigation of the presence of terpenes (TLC, IR) and total terpenes (%) were performed using the same methodology described for the powder of crushed fruit. For the quantification of total terpenes, 1 mL of the extract was diluted with 25 mL of ethanol 95% P.A. Then, an aliquot of 1 mL of this solution was diluted to 100 mL distilled water. The selectivity and repeatability was evaluated according to the specifications described in guidelines[25].

Preparation, Characterization of the Standardized Dried Extract.

The drying was carried out in Minispray Dryer, model MSD 1.0 (Labmaq®) with con-current flow regime constituted by a peristaltic pump and a two fluid atomizer (inlet orifice diameter of 1.2 mm). The colloidal silicon dioxide

(Aerosil®) was added to the extract in proportion of 15, 20, and 25% of solid content as adjuvant of drying. The operational conditions were 110°C inlet temperature, 97°C outlet temperature, and a feed flow rate of 35 L/min. The efficiency of the drying process and the percentage of the terpenes degradation rate were calculated.

The volatile content and the investigation of the presence of terpenes (TLC, IR, GC) were performed. For the quantification of total terpenes, 5 mg of the dried extracts were diluted with 5 mL of ethanol 95% P.A, sonicated for 5 min and filtered. Then, 1 mL of the filtrate was diluted with 10 mL distilled water and the color reaction was performed. The selectivity and repeatibility was evaluated according to the specifications described in guidelines[25].The particle morphology of the dried extract was evaluated using a scanning electronic microscope 5900 LV (JSM®) in laboratory of electron microscopy the National Laboratory Luz Síncontron, Brazil. The thermal analysis of the chemical marker (6a,7ß-dihydroxyvouacapan-17ß-oate methyl ester) was carried out with a Thermogravimetric DTG 60 (Schimadzu®) and was also analyzed by using a differential scanning calorimeter DSC 60A (Schimadzu®). The experiments were performed under a nitrogen flow to 50 mL/min. The sample was heated from 25 to 600 °C in aluminum crucibles with a linear heating rate of 10 °C/min and the reference material platinum.

The antinociceptive effect of the dried extract was carried out in male Swiss mice with 25-30 g body weight were kept at 25 ± 2°C in 12 h light-dark cycles with water and food ad libitum. The animals were separated in groups of mice. The analgesic test studies were carried out in accordance with current Brazilian College of Animal Experimentation (COBEA), and under the consent of the Ethics Committee for Research (number 104/08) of the Federal University of Goiás. The Capsaicin test used was according to Santos and Calixto[26]. The different groups of animals were treated p.o. with or vehicle (10 mL/kg and DMSO 20%, n = 10), and with standardized dry extract *P. emarginatus* (1000 mg/kg and DMSO 20%, n = 8), previously treated (30 minutes) i.p. with capsazepine (10 mg/kg and DMSO 20%, n = 8) and s.c with morfine (10 mg/kg, n = 4). After 60 or 30 minutes (respectively), 50 µL of capsaicin (1.6 µg/paw prepared in PBS) was injected in the ventral surface of the right hind paw. The time that the animals spent licking the injected paw, for the first 5 minutes post capsaicin injection, was recorded with a chronometer and considered as indicative of nociception. The results were submitted to one-way analysis of variance (ANOVA), considering p = 0.05 as critical level to evaluate significant difference between the control and treated groups, followed by the Tukey Test, using the GraphPad Instat® software.

RESULTS AND DISCUSSION

Characterization of the fruits of *P. emarginatus* fruits.

The material was classified as coarse powder, since less than 40% of the particles pass through the sieve with a nominal opening of 355 µm[24]. The presence of very fine particles hinders the processes of percolation by compaction and formation of preferential channels. The use of moderately coarse powder is recommended for the vast majority of drugs[27]. The volatile content was 1.2% being within the limits 8-14% recommended by the Brazilian Pharmacopeia[28].

Physicochemical Characterization of the Ethanolic Extract.

For this extract, the following physicochemical parameters obtained were: pH 5.10, relative density at 0.87, viscosity at 25.43 mPas, alcohol level at 71%, solids content at 15.20%. The determination of density and viscosity is essential for planning the drying process to prevent clogging of the spray nozzle, also influencing the droplet size and particle size of dried product. The solids content can significantly increase the yield of the dry product obtained. The solid content of plant extract is usually 15-40%[29].

Investigation of the presence of terpenes in fruits, ethanolic extract and dry extract.

In this study we observed that the drying process by spray-dryer, on the established operational conditions, did not have influence on the characteristics of the powders fruit and extract constituents in relation to the dry extract by the used techniques (Tables 1 and 2).

After the procedure of solid phase extraction on silica cartridges, the fractions collected masses were respectively: 2.9 mg (hexane fraction), 4.1 mg (chloroform fraction), and 14.4 mg (chloroform-methanol fraction). In the analysis by GC / MS, the chromatographic peaks obtained from chloroform-methanol fraction for the marker 6α,7ß-dihydroxyvouacapan-17ß-oate me

Table 1. Analysis of the chromatographic profile by TLC of the *P. emarginatus*

Chromatographic spot	Rf (fruit powder)	Rf (Ethanolic extract)	Rf (dry extract)	Chemical Markers
1	0,16	0,19	0,16	VA
2	0,33	0,29	0,30	*
3	0,44	0,44	0,41	VE
4	0,48	0,46	0,45	*
5	0,59	0,56	0,56	*
6	0,71	0,70	0,70	VL
7	0,78	0,79	0,78	L P
8	0,85	0,85	-	*
9	0,89	0,90	-	*

Methanolic extract of fruit, chemical markers - 6a,7ß-dihydroxyvouacapan-17ß-oic acid (VA); 6a,7ß-dihydroxyvouacapan-17ß-oate methyl ester (VE); 6a-hydroxyvouacapan-7ß-17ß-lactone (VL) - and lupeol were applied (10 µL) in TLC plates coated with silica gel 60 F254. Mobile phase: hexane: ethyl acetate: glacial acetic acid (60:40:1).

Table 2. Analysis by infrared spectroscopy of the *P. emarginatus* Vogel

Bands (cm^{-1})	Fruit powder	Ethanolic extract	Dry extract	Marker (VA)	Marker (VE)	Marker (VL)	Chemical grouping
1	3292	3424	3430	3502-3428	3539-3520	3499	O H
2	2926-2855	2931	2931	2948-2862	3002 - 2854	2934-2824	C - H
3	1745	1736	1732	1718	1727	1769	C = O
4	1652	~1600	~1600	1686-1636	1649-1511	1683-1646	C = C
5	1246	1247	~1100	1250	1195	1131	C - O

Markers: 6a,7b-dihydroxyvouacapan-17â-oic acid (VA), 6a,7b-dihydroxyvouacapan-17ß-oate methyl ester (VE), 6a-hydroxyvouacapan-7ß-17ß-lactone (VL) were analysed in potassium bromide (KBr) pellet in spectroscopy infrared Spectrum BX (Perkin Elmer®) in the range between 4000-400 cm^{-1}.

thyl ester showed a retention time of 54.478 minutes and also showed the mass / charge (m / z) in a very similar relative abundance presented by Fascio et al.[30] (Table 3). The spectrum GC / MS fraction obtained from chloroform-methanol for the sample has several chromatographic peaks, with greater intensity to the retention time of 55.045 and 55.898 minutes. The mass / charge (m / z) presented to the major chromatographic peaks shows a relative abundance very similar to compounds 14 (methyl-6α-acetoxy-7β-17β-oate dihidroxyvouacapane) and 15 (methyl 6α-hydroxy-7β-acetoxy-17β-oate-vouacapane), respectively, as Fascio et al.[30] (Table 4). It may be suggested by the intensity for the chromatographic peak (rt = 55.89 min) that the same compound may represent the major diterpene present in the sample.

The chromatographic peaks obtained from the chloroform fraction and the mass spectrum compared to literature data suggest the isolation of triterpene lupeol described by the first time in the fruits of *P. emarginatus*. The comparison with library data offering 92% similarity with lupeol.

Table 3. Results of the analysis by GC / MS for the marker 6a,7ß-dihydroxyvouacapan-17ß-oate methyl ester

Retention time (min)	Fragments (Abundance) Marker 6a,7ß-dihydroxyvouacapan-17ß-oate methyl ester	Fascio et al. (*30*) 6a,7ß-dihydroxyvouacapan-17ß-oate methyl ester
	362 (08)	362 (45)
	326 (20)	326 (50)
	303 (07)	303 (17)
	285 (22)	285 (68)
	284 (22)	284 (42)
	268 (18)	268 (20)
	267 (72)	267 (98)
	197 (13)	197 (11)
54.478	191 (11)	191 (18)
	185 (19)	185 (15)
	161 (20)	161 (25)
	149 (28)	149 (29)
	147 (34)	147 (33)
	145 (48)	145 (39)
	137 (32)	137 (53)
	133 (38)	133 (37)
	131 (88)	131 (85)

Conditions used for elucidation of terpenes: column DB-05 MS, 30m x 0.25mm x 0.25um; temperature programming starting at 100 ° C - 1 min and increased to 15 ° C / min to 300 ° C, 300 ° C - 40 min, oven temperature 100 ° C, injector temperature 240 ° C, split, ratio 1:20.

Table 4. Results of the analysis by GC / MS for the sample of the *P. emarginatus*

Retention time (min)	Fragments (Abundance) Sample extract	Fascio et al. (*30*) "Compound 14"	Retention time (min)	Fragments (Abundance) Sample extract	Fascio et al. (*30*) "Compound 15"
	404 (04)	404 (13)		404 (02)	404 (14)
	372 (05)	372 (07)		345 (04)	345 (14)
	344 (10)	344 (57)		344 (10)	344 (56)
	326 (04)	326 (20)		330 (08)	330 (15)
	312 (45)	312 (100)		312 (40)	312 (100)
	285 (12)	285 (15)		311 (02)	311 (23)
55.898	267 (18)	267 (13)	55.045	285 (05)	285 (16)
	178 (11)	178 (81)		267 (08)	267 (13)
	145 (20)	145 (14)		179 (05)	179 (10)
	137 (20)	137 (16)		178 (30)	178 (81)
	131 (38)	131 (42)		177 (07)	177 (16)
	123 (22)	123 (24)		133 (09)	133 (10)
	119 (28)	119 (24)		131 (40)	131 (42)
	109 (19)	109 (23)		123 (04)	123 (24)
				119 (30)	119 (24)
				109 (20)	109 (23)
				55 (34)	55 (27)
				43 (100)	43 (71)
				41 (39)	41 (21)

Conditions used for elucidation of terpenes: column DB-05 MS, 30m x 0.25mm x 0.25um; temperature programming starting at 100 ° C - 1 min and increased to 15 ° C / min to 300 ° C, 300 ° C - 40 min, oven temperature 100 ° C, injector temperature 240 ° C, split, ratio 1:20. To identify other substances present it was used the comparison of mass spectra of each peak with the library's software unit.

Development and Validation of the method for quantification of total terpenes.

Initially, a scan of 6α, 7ß, di-hidroxyvouacapane-17-ß-oate was performed and there was no absorption peak in the range of UV-VIS. Then, an investigation was conducted in a colorimetric reaction indicative of terpenes employing the reaction with vanillin sulfuric[24]. The stability of the color reaction was also investigated, where reaction time was evaluated in the first hour and after 24 hours of preparation. The relative standard deviation (RSD%) between readings in the first hour was 1.0%, showing great stability and security of the solutions of reaction time in the proposed method developed. On the analytical curves previously constructed in triplicate in the range of 6.0 – 14.0 µg/mL, an equation was obtained (y = 0.051x - 0.064, y = 0.055x – 0.132 and 0.049x – 0.073) and r of 0.999, 0.998 and 0.997, respectively. The method showed to be selective, linear, precise (RSD < 5% for repeatability, intermediate precision), accurate, and robust (Table 5).

Table 5. Results of means values obtained in the validation of the method of quantification of total terpenes in the *P. emarginatus* powder.

Parameters	Limits required	Fruit powder
Selectivity	No interferent	No interferent
Linearity and range	r > 0.99	0.998
Repeatability	RSD = 5%	3.90%
Intermediate precision	RSD = 5%	0.93%
Limit of detection	*	0.23 µg/mL
Limit of quantitation	*	0.78 µg/mL
Accuracy	*	1.97%
Robustness	*	0.14%

Correlation coefficient (r); Relative Standard deviation (RSD).

The method showed to be selective and precise (repeatability) for quantification of total terpenes in ethanolic extract and dry extract, presenting relative standard deviation of 1.85% and 2.55%, respectively.

After validation, the quantification of the total terpenes in the fruit powder, in the ethanolic extract and in the dry extract of *P. emarginatus* added by 15% colloidal silicon dioxide was performed and the values obtained were 12.65 µg/mL (9.6%), 10.38 µg/mL (17.08%) and 13.74 µg/mL (16.1%) respectively. The dried extracts added by 20% of colloidal silicon dioxide showed 13.14 µg/mL (16.22 %) and that with 25% of colloidal silicon dioxide showed 11.50 µg/mL (14.37%).

Characterization of the Dried Extract.

The photomicrographs shows that the dry extract (20% Aerosil®) is composed by irregular and spherical particles, rough surface, rough-coated porous silica with wide size distribution. The solids were obtained as fine powders with a particle size ranging between 27 and 81.4 µm. In general, the dried extracts obtained by spray drying resulted in a high proportion of spherical particles[31] and these shape is an important feature for the application of the spray dried to obtain the intermediate pharmaceutical products[29].

In the differential scanning calorimeter (DSC) of the chemical marker (6α,7ß-dihydroxyvouacapan-17ß-oate methyl ester), the first event (endothermic) occurred between the temperatures of 187.09 and 206.07°C, possibly indicating the fusion of the marker. Another thermal event was observed occurring at temperatures above 200º C. The temperature range between 261.92 and 319.57°C degrades the material analyzed. The temperatures above 400° C that showed exothermic peaks can be attributed to the carbonization of the material analyzed. In the analysis of thermogravimetry (TG), the weight loss resulting from the degradation of the analyzed material starts at 194.43° C and when gets close to 350° C. It reaches massive loss of about 96% of the analyzed material. The results for the thermal analysis (DSC and TG) showed that the drying parameters (inlet and outlet temperature) used in the drying process of the ethanolic extract of *P. emarginatus* fruit was safe by preventing the thermal degradation of the chemical markers.

The dry extracts containing 15, 20, and 25% of the colloidal silicon dioxide yield dried were 14.4%, 16.2%, and 15.5%; and the terpenes degradation rate was 11.6%, 10.9% and 21.0%. The results of the preliminary assays showed a better drying performance and product quality with the use of the colloidal silicon dioxide (20%) as drying adjuvant. The colloidal silicon dioxide is a widely used adjuvant in the pharmaceutical industry and has a pronounced feature adsorbent[31]. The yields dried are variable and related to plant characteristics and to the drying process[32]. The percentage of the degradation varies for different extracts depending on the variability of chemical constituents[33].

In the pharmacological assay, was noted that the intraplantar application of capsaicin on hind paw of mice produced intense nociception. For the animals pretreated (60 minutes) p.o with vehicle, the reactivity of nociception was

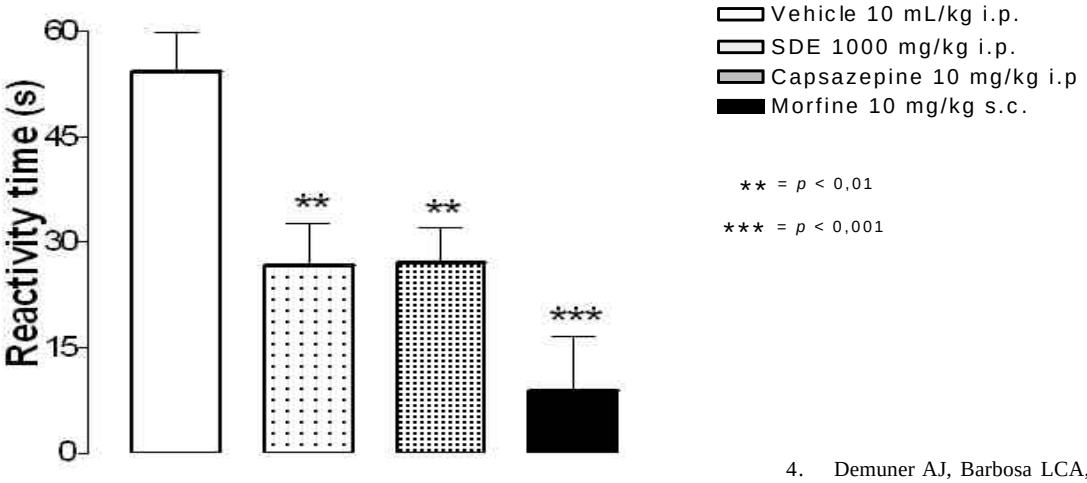

Figure 1. Reactivity time to the intraplantar application of capsaicin (1.6 µg.paw-1) in the hind paw of mice previously treated (30 min) i.p. with control vehicle (10 mL/kg), or standardized dry extract of the *P. emarginatus* (1000 mg/kg), previously treated (30 minutes) i.p. with capsazepine (10 mg/kg), and s.c with morfine (10 mg/kg). Results expressed as mean ± SEM of animals for experimental groups (p = 0.01; *** p = 0.001).**

54.30 + 5.50 s. The pre-treatment with dry extract of the *P. emarginatus* (SDE) reduced the reactivity time and presented the value 26.62 + 5.98 s. The group treated with capsazepine or morphine reduced the reactivity time to pain and it was 27.12 + 5.01 s and 8.75 + 7.78 s, respectively (Figure 1). The observed reduction in the time of reactivity was 50.9% for the SDE, 50% for capsazepine (vanilloid antagonist), and 83.9% for morphine compared to the vehicle.

The obtained results showed that the drying process of the extract of the *P. emarginatus* remained as the antinociceptive activity described for this plant[11]. The capsaicin is the painful substance used to determine if compounds possessing antinociceptive activity act by the vanilloid VR1 receptors and it produces this effect by binding it to a receptor expressed by nociceptive afferent neurons. This is known as vanilloid receptor, as many compounds similar to capsaicin are based on the structure of vanillic acid, which is a typical cation channel activated by ligands. The agonists like capsaicin opens the channel, which is permeable to Na^+, Ca^{2+} and other ions, causing depolarization initiating the action potential[34].

From these results we intend to conduct researches for the development of herbal medicines containing the standardized dried extract of the *P. emarginatus*.

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