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ORIGINAL ARTICLE

An experimental design approach to obtain canthinone alkaloid-enriched extracts from *Simaba* aff. *paraensis*

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Abstract The species of the genus *Simaba* (Simaroubaceae) are found in almost all Brazil and they are used by local population for the treatment of ulcers and malaria. The genus is characterized by the presence of quassinoids and canthinone alkaloids. There is a great interest in canthinones due to important biological activities associated with this class of alkaloids. In this study, methods were developed for obtaining enriched fractions of canthinone alkaloids from *Simaba* aff. *paraensis* using experimental factorial design analyzed by gas chromatography. Three alkaloids were detected: canthin-6-one, 4,5-dimethoxycanthin-6-one and the major 9-methoxycanthin-6-one. Within the experimental domain, factorial designs 2^2 helped establish the minimum amount of solvent and minimum time necessary to obtain extracts enriched in canthinone alkaloids from *S.* aff. *paraensis* barks by two extraction methods. These results represent a reduction in costs for obtaining canthinone alkaloids described for the first time in *S.* aff. *paraensis*.

1. Introduction

Species of *Simaba* genus (Simaroubaceae family, Simarouboidea subfamily, Simaroubea tribe, Simaroubinae subtribe) are found in almost all regions of Brazil and they are used by the population for the treatment of ulcers and malaria (Barbosa et al., 2011). Among the various substances already identified in these species, the most important are

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quassinoids and alkaloids of canthinone type, both with important biological activities (Muhammad et al., 2004; Almeida et al., 2011; Barbosa et al., 2011; Alves et al., 2014; Devkota et al., 2014).

Canthinone alkaloids are a subclass of β-carboline alkaloids with an additional D ring. They are often found in plant extracts from Simaroubaceae and Rutaceae families (Devkota et al., 2014). The alkaloid canthin-6-one showed activity against *Plasmodium falciparum* (chloroquine resistant and sensitive strains) (Cebrián-Torrejón et al., 2011) and *Trypanosoma cruzi* (Ferreira et al., 2011), besides ulcerogenic activity in ethanol-induced gastric ulcer in mice (Almeida et al., 2011) and cytotoxic activity against many cancer cell lines (Dejos et al., 2014). Thus, it is evident the great potential of canthin-6-one and its derivatives in the treatment of neglected diseases.

Considering the urgency to obtain new targets against different diseases, it becomes necessary to study extractive methods that increase the amount of these substances for subsequent evaluation of biological activity. It is noteworthy that phytochemical studies involve extractive processes which conditions are often determined randomly, leading to poor extraction, overspending of plant material, solvents, energy and time. Statistical experimental design could be a good approach for the development of rational extractive methods. The factorial design can indicate the best extraction condition with minimum laboratory effort, accounting for less cost and time. Furthermore, it allows evaluating many variables at the same time and their interaction effects (Lundstedt et al., 1998). However, in a quick search on Scifinder® database, a survey on the number of original papers published between 2000 and 2017 indicated 35,945 references containing the concept "plant extraction", but only 290 of them containing the two concepts "factorial design" and 'plant extraction" closely associated with one another. It evidences that this tool is still little used in the area of phytochemical studies and it should be more explored since it may represent cost savings and less environmental damage.

In this context, the aim of this work was to optimize methods to extract alkaloids from *Simaba* aff. *paraensis*, using as an auxiliary tool the statistical design of experiments.

2. Material and methods

2.1. Plant samples

Bark samples of *S.* aff. *paraensis* were collected in Antimary State Forest (Bujari, Acre, Brazil). Voucher specimen is deposited at the Herbarium of "Universidade de São Paulo", with the identification number SPF 199278. Plant material was dried in oven with air circulation at 35 °C, for 24 h.

2.2. Chemicals

Dichloromethane and methanol (HPLC grade) were purchased from Tedia Company Inc. (USA). Ammonium hydroxide and HCl (pro analysis) were supplied by Vetec (Brazil). Ultrapure water was obtained with a MilliQ system (Merck).

2.3. Alkaloid isolation and identification

Maceration was exhaustively achieved on finely triturated barks (500 g) at room temperature in dichloromethane containing 2% ammonium hydroxide (6 × 2 L/day), with 1 h of ultrasonic bath daily. After 6 days of extraction and the evaporation of solvents under reduced pressure, 2.7 g the crude extract was obtained, yielding 0.54%. This extract was submitted to preparative thin-layer chromatograph for the isolation of its major alkaloid. Aliquots containing 15 mg of the crude extract were applied to aluminum sheets covered by the stationary phase RP-18 and eluted with methanol/water (7:3) to furnish 3.5 mg of 9-methoxycanthin-6-one. Chromatograms were revealed by UV at 365 nm.

NMR measurements were performed at 400 MHz for ^{1}H and 100 MHz for ^{13}C on a Bruker Advance 400 spectrometer from $CDCl_3$ solutions using TMS as internal standard.

The direct infusion ESIHRMS analysis was recorded on a Bruker compact Q Tof high-resolution mass spectrometer (HRMS) controlled by Compass data analysis 4.2. Bruker compact Q Tof run at mode ESI positive, 4500 capillary voltage, −100 V set end-plate offset, 2000 V charging voltage, nebuliser at 4.0 bar, dry heater at 250 °C, dry gas at 9.0 L/min and a mass scan range of m/z 100–1000 for MS.

2.4. Preliminary extractions

In this step, the following liquid extractors were tested: (a) hydrochloric acid, 5% (v/v), (b) 2% of ammonium hydroxide in dichloromethane (v/v), (c) dichloromethane, (d) ammonium hydroxide, 2% (v/v). Each extraction was performed in triplicate with 2 g of plant material and 20 mL of the solution (a-d) for 1 h in an ultrasonic bath, protected from light.

2.5. Experimental design

The methods (a) hydrochloric acid, 5% (v/v), and (b) 2% of ammonium hydroxide in dichloromethane (v/v), described in item *2.3*, were chosen as starting points to develop the process for obtaining extracts enriched in canthinone alkaloids. For each solvent, a 2^2 full factorial design was used to study two variables in seven runs with three replicates of the central point. Variables and domain were: solvent volume (V): 20–30 mL; and extraction time in ultrasound bath (t): 10–50 min. In each run, the pH was adjusted to 10 with ammonium hydroxide and thus partitioned with 2 × 10 mL of dichloromethane. Response variables were yield (%), percentual of alkaloids canthin-6-one, 9-methoxycanthin-6-one and 4,5-dimethoxycanthin-6-one. For the analysis of variance (ANOVA), the significance level was set at p-level = 0.05. Statistical analyses were performed using Statistica 6.0 (Statsoft Inc., Tulsa, OK, USA).

2.6. GC-MS analyses

All extracts were analyzed and identified by gas chromatography (Agilent 6890 N) coupled to a quadripolar mass spectrometer (Agilent 5973 N), with ionization by electronic impact (70 eV). The apparatus was fitted with a DB-5MS column (internal diameter: 0.25 mm, length: 30 m, film thickness: 0.25 μm). Carrier gas was helium at flow of 0.5 mL/min. The injector port temperature was 290 °C, splitless mode. The transfer line temperature was 280 °C, ion-source-heating was 230 °C and the scan-range was 40–700 m/z. The GC oven program was as follows: 70–315 °C at 5 °C/min and a final hold time of 11 min. Interpretation and identification of the fragmentation mass spectrum was carried out by comparison with the Wiley NBS mass spectrum database and literature data (Ohmoto et al., 1976; Chen et al., 2009; Cordell et al, 1978; Readel et al., 2003). Results were expressed as relative percentage of peak area in chromatogram.

3. Results and discussion

*3.1. Alkaloids in **S. aff. paraensis** and isolation of 9-methoxycanthin-6-one*

According to literature, canthinone alkaloids found in *Simaba* genus and other species of Simaroubaceae, Rutaceae and Amaranthaceae families are frequently extracted from the vegetal matrix using alcoholic or hydroalcoholic solutions, with or without the use of alkaline or acid reagents (Ohmoto et al., 1976; Giesbrecht et al., 1980; Mesquita-Saad and Cabral, 1997; Readel et al., 2003; Márquez et al., 2005; Cunha et al., 2008; Khari et al., 2014). Additionally, the identification of the alkaloids in the extracts has been performed preferably by gas chromatography coupled to mass spectrometry (GC/MS) and high resolution mass spectrometry (HRMS). Canthin-6-one, the most representative of the canthinone group, presents as the major fragments (m/z): 220 (base peak and molecular ion), and 192. In the same way, methoxylated derivatives 9-methoxycanthin-6-one, 8-methoxycanthin-6-one, 1-methoxycanthin-6-one, 5-methoxycanthin-6-one and 10-methoxycanthin-6-one have the molecular ion 250 m/z as base peak, with the increase of thirty atomic mass units corresponding to the presence of an additional methoxyl group and main characteristic signs with 235, 222, 221, 220, 208, 207, 192, 180 and 179 (Ohmoto et al., 1976; Chen et al., 2009; Cordell et al, 1978; Readel et al., 2003; WSS).

Initially, this study focused on extractions with various nonpolar and polar solvents (chloroform, ethyl acetate, acetone, ethanol, methanol and water). Methanol and water lead to the best crude yields, but many other substances were extracted in addition to canthinone alkaloids (data not shown). Further partitions with dichloromethane of the crude methanol or aqueous extracts gave fractions with very low alkaloid yields. Thus, the direct extraction of the barks with 2% of ammonium hydroxide in dichloromethane (v/v) was chosen to obtain the crude extract for the isolation of alkaloids. This extract was produced by maceration, kept in ultrasonic bath for 1 h-period daily. Hence this methodology was not used as a starting point for the optimization study, the objective of this step was the exhaustion of the plant material to furnish the main alkaloid. For this, the amount of plant material was large (500 g), the extraction period was too long (6 days) and solvent consumption was high (12 L). The yield of the crude extract was 0.54%. The evaluation of the chromatographic profile of this crude extract obtained by GC-MS indicated that the canthinone alkaloids was canthin-6-one, 9-methoxycanthin-6-one and 4,5-dimethoxycanthin-6-one (Fig. 1). Other minor substances were also present in this extract, such as campesterol, stigmasterol and γ-sitosterol. Likewise, many extracts were also prepared with the leaves of *S.* aff. *paraensis* by the same proceedings used for the barks, and evaluated by TLC and GC-MS analyses, but no alkaloid was detected.

The crude dichloromethane extract from the barks was fractionated by preparative thin-layer chromatography for the isolation of its major alkaloid, 9-methoxycanthin-6-one. The molecular formula was determined to be $C_{15}H_{10}N_2O_2$ on the basis of HR-ESI-MS. The experimental signal of m/z 251.0826 was closed related to the calculated for the molecular formula $C_{15}H_{11}N_2O_2$ (M + 1), m/z 251.0815. In this context, the alkaloid 9-methoxycanthin-6-one was identified as the main constituent of the extract. The structure was confirmed by HRMS, ^{1}H NMR and ^{13}C NMR spectra (Chen et al., 2009; Cordell et al, 1978; WSS, 1995).

The species of *Simaba* genus are characterized by the presence of canthinone alkaloids, quassinoids and triterpenes (Giesbrecht et al., 1980; Arisawa et al., 1987; Muhammad et al., 2004; Barbosa et al., 2011; Almeida et al., 2011). Various species of *Simaba* genus have already been studied, however the species *S.* aff. *paraensis*, the aim of this work, is very little studied. To the best of our knowledge, to date, there is only one publication on the phytochemical study of *S. paraensis*, developed by Santana and Okino (2007). The authors determined the content of carbohydrates, acetyl groups, levulinic acid, 5-hydroxymethyl-2-furfuraldehyde, uronic anidride, insoluble lignin and soluble lignin of 36 Amazon species, including *S. paraensis*. The present study is the first report on the detection and isolation of canthinone alkaloids in *S.* aff. *paraensis*.

Canthinone alkaloids have been found in many species of Simaroubaceae and Rutaceae families. The major alkaloid identified, 9-methoxycanthin-6-one, was also extracted from barks and roots of *Eurycoma longifolia* (Simaroubaceae) (Mahmood et al., 2011; Mitsunaga et al., 1994) and roots of *Eurycoma harmandiana* (Kanchanapoom et al., 2001). Therefore, according to literature, the substances detected in *S.* aff. *paraensis* and reported in the present paper are compatible with those found in other Simaroubaceae species.

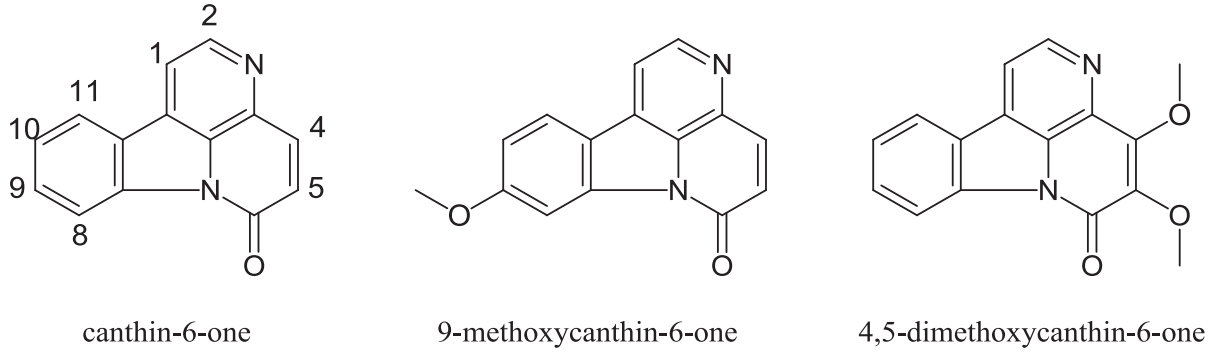

Figure 1 Canthinone alkaloids in *Simaba* aff. *paraensis*.

3.2. Preliminary extractions

During the initial development phase, extracts from the barks of *S.* aff. *paraensis* were obtained for comparison among methods concerning yields and alkaloid content. In this step the following liquid extractors were tested: (a) hydrochloric acid, 5% (v/v), (b) 2% of ammonium hydroxide in dichloromethane (v/v), (c) dichloromethane, (d) ammonium hydroxide, 2% (v/v). After 1 h of extraction in the ultrasound bath and partition with dichloromethane, the final yields were: (a) 0.053% ± 0.006% (b) 0.190% ± 0.025%, (c) 0.192% ± 0.073%, and (d) 0.013% ± 0.003%. The extract with the best yield and the highest content of alkaloids was (b), containing canthin-6-one (10.5%), 9-methoxycanthin-6-one (50.5%), and 4,5-dimethoxycanthin-6-one (1.9%). The extract (a) had the second highest content of alkaloids, with canthin-6-one (8.1%), 9-methoxycanthin-6-one (35.2%), and 4,5-dimethoxycanthin-6-one (1.4%). These two methods were selected for evaluation using factorial designs.

3.3. Development of the extraction process by factorial design

For the development of the extraction processes of canthinone alkaloids from *S.* aff. *paraensis*, the methods (a) and (b) were the starting points: (a) using hydrochloric acid 5% (v/v) as solvent, (b), using 2% of ammonium hydroxide (v/v) in dichloromethane as solvent. The choice was based on the high content of alkaloids in both extracts obtained in the preliminary step. Full factorial designs were performed with 2 factors and 2 levels (2^2), in order to evaluate the relationship between yields and canthinone alkaloid contents. Three central point replicates were accomplished. Two variables were studied: extraction time in ultrasound bath (t) and volume of solvent (V). Final yields and canthinone alkaloid contents in extracts were the response variables. Final yields were determined after pH adjustment to 10 with ammonium hydroxide when necessary, followed by the partition with dichloromethane. The experimental domain and data obtained under the conditions defined by the experimental design are shown in Tables 1 and 2.

The analysis of variance for both sets of data indicated that p-level values for independent variables were higher than 0.05 (Fig. 2). Thus, any volume of solvent in the range of 20–30 mL or extraction time in the range of 10–50 min could be chosen without significant effects on the response variables: yields and total canthinone contents. Hence, for the extraction of 2 g of plant material, the lower limits of the experimental domain, only 10 min in ultrasonic extraction and 20 mL of solvent are enough. Moreover, it shows the robustness of the methodology, since some variations in the experimental domain studied does not adversely affect the response.

The factorial designs 2^2 allowed establishing the suitable amount of solvent and extraction time to avoid waste for the obtaining of extracts enriched in canthinone alkaloids. The best way to evaluate these sets of data is the means comparison. On average, the yield of extraction with hydrochloric acid 5% (v/v), followed by pH adjustment and extraction in dichloromethane (a) was 0.106% ± 0.031%, containing 6-canthin-6-one (13.0%), 9-methoxycanthin-6-one (57.6%) and 4,5-dimethoxycanthin-6-one (1.77%). In comparison with the preliminary method (a), the yield was double than that obtained before the factorial design approach (0.053% ± 0.006%), and canthin-6-one, 9-methoxycanthin-6-one and 4,5-dimethoxycanthin-6-one contents, which were also superior to the initial (8.1%, 35.2% and 1.4%, respectively). Furthermore, for 2 g of plant material, it was possible to reduce the extraction time from 1 h to 10 min, without to increase the amount of solvent and with good yield.

In the evaluation of the extraction method (b) with 5% of ammonium hydroxide in dichloromethane (v/v), the yield was 0.219% ± 0.023%, containing canthin-6-one (9.2%), 9-methoxycanthin-6-one (43.1%), and 4,5-dimethoxycanthin-6-one (1.69%). These results were similar to those obtained in the preliminary extraction with this solvent: 0.190% ± 0.025% yield, containing canthin-6-one (10.5%), 9-methoxycanthin-6-one (50.5%), and 4,5-dimethoxycanthin-6-one (1.9%). This result showed the best advantage of the experimental design applied that was the reduction in extraction time from 1 h to 10 min. However, in this condition, the extract also contained other substances: palmitic acid (4.5%), campesterol (4.5%), stigmasterol (5.1%) and γ-sitosterol (9.6%).

In summary, two extraction methods were developed through a factorial design 2^2 for obtaining fractions enriched in canthinone alkaloids. The experimental design showed robust methods to obtain canthinone-enriched extracts from the barks of *Simaba* aff. *paraensis*. Canthinone-enriched extracts are of extreme relevance due to the biological activity attributed to this class of alkaloids, as well as are a prototype source for the synthesis of therapeutic targets. This first

Table 1 Experimental design to improve extraction conditions in hydrochloric acid, 5% to obtain canthinone alkaloid enriched fractions.

Run	Factors		Responses			
	Time (min)	Volume (mL)	Yield (%)	Canthin-6-one (%)	9-Methoxycanthin-6-one (%)	4,5-Dimethoxy-canthin-6-one (%)
1	10	20	0.127	15.38	73.58	1.99
2	10	30	0.137	12.94	58.91	1.89
3	50	20	0.077	10.11	45.10	1.47
4	50	30	0.097	13.10	57.04	1.87
5	30	25	0.093	11.50	52.48	1.57
6	30	25	0.067	14.87	65.99	2.03
7	30	25	0.147	13.23	50.07	1.58

Table 2 Yield and alkaloid content in the extracts in 2% of ammonium hydroxide in dichloromethane obtained under the conditions defined by the factorial design 2^2.

Run	Factors		Responses			
	Time (min)	Volume (mL)	Yield (%)	Canthin-6-one (%)	9-Methoxycanthin-6-one (%)	4,5-Dimetoxy-canthin-6-one (%)
1	10	20	0.213	8.89	42.85	1.70
2	10	30	0.213	6.23	26.87	1.17
3	50	20	0.267	9.77	47.04	1.83
4	50	30	0.200	10.07	48.56	1.86
5	30	25	0.217	12.01	55.43	2.17
6	30	25	0.197	7.72	36.21	1.32
7	30	25	0.223	9.76	44.64	1.79

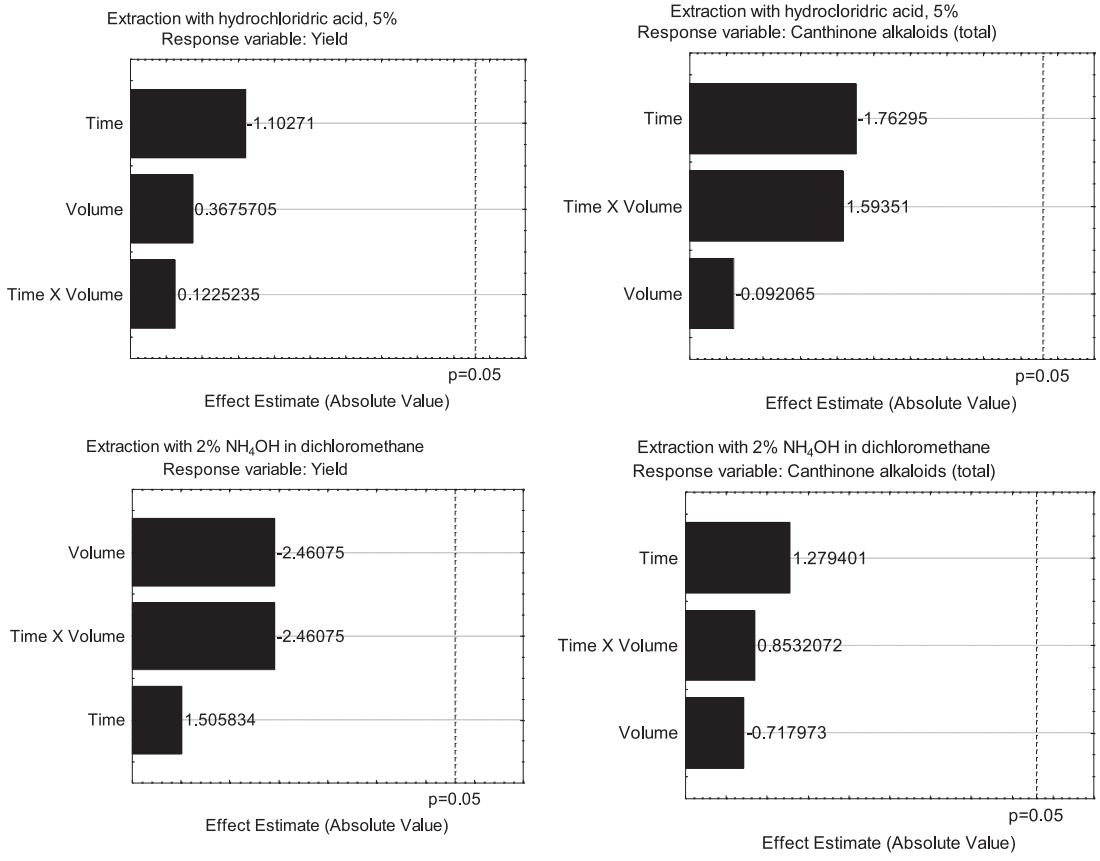

Figure 2 Pareto charts of standardized effects of the factors 'Volume' and 'Time' on the responses 'Yield' and 'canthinone alkaloids (total)' in the two extractions methods evaluated. Values of t-Student tests are shown after respective bars.

phytochemical study on *S.* aff. *paraensis* showed consistent results with those of other Simaroubaceae species. The main alkaloid, 9-methoxycanthin-6-one, was isolated by preparative TLC and identified by spectroscopic methods. One of the methods, which employed hydrocloridric acid, 5% (v/v), and incubation in ultrasound bath, allowed to obtain an extract with 0.106% ± 0.031% yield, containing canthin-6-one (13.0%), 9-methoxycanthin-6-one (57.6%), and 4,5-dimethoxycanthin-6-one (1.77%). The other method employed the extraction with 2% of ammonium hydroxide in dichloromethane (v/v), yielding 0.219% ± 0.023%, containing canthin-6-one (9.2%), 9-methoxycanthin-6-one (43.1%), and 4,5-dimethoxycanthin-6-one (1.69%). Besides alkaloids, other substances were detected only in the 2% of ammonium hydroxide in dichloromethane extract. In this study, the experimental design allowed to evaluate the range of variables volume of solvent and extraction time that not significantly influence in the yields and alkaloids contents. This evidences the potential use of factorial designs in the development of other methodologies to obtain enriched fractions in various classes of substances. The found substances corroborated with the folk use of this species as antimalarial and antiulcer.

Biological investigations of these enriched extracts are in progress to identify antimalarial activity on the extractions developed.

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