SUPERCRITICAL EXTRACTION OF INDOLE ALKALOIDS FROM *Tabernaemontana catharinensis*: AN EVALUATION OF THE COSOLVENT ON THE EXTRACT COMPOSITIONS

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Tabernaemontana catharinensis is a tree rich in indole alkaloids, such as coronaridine and voacangine that have antileishmanial activity. SFE from *T.catharinensis* using CO_2 + 5.1% (m/m) of cosolvent (ethanol, isopropyl alcohol, methanol, water and their mixtures) was studied. The global yields and the compositions of extracts were determined. The data were taken at 250 bar, 45ºC, and total solvent flow rate of 6.1×10^{-5} kg/s.The crude extracts were fractionated to obtain the alkaloid-rich fractions. These fractions were analyzed by Thin-layer Chromatography. Gas-chromatography/Flame Ionization Detector was used to quantify the voacangine. The average global yield was approximately constant (2.4±0.1%) for the alcoholic cosolvents; the global yield was significantly larger (15±1%) for the cosolvent water and its mixture alcoholic mixtures. Nonetheless, the content of alkaloids in the extracts was strongly affected by the cosolvent, the methanol showed to be the more effective cosolvent.

Keywords: Indole alkaloids, *Tabernaemontana catharinensis*, supercritical extraction, cosolvent

INTRODUCTION

Tabernaemontana catharinensis (syn. *Peschiera catharinensis* A.DC.) is a tree of the Apocynaceae family rich in indole alkaloids. In general, the alkaloids present in Tabernaemontana genus have a large pharmacological application. The crude extracts and alkaloids isolated from *T.catharinensis* have been demonstrated antitumoral, anti-inflammatory, analgesic and tripanocidal activity [1], [2], [3]. Among the compounds isolated from the bark of this tree special attention is given to the indole alkaloids coronaridine and voacangine, which have antileishmanial activity [4]. Leishmaniasis is an endemic infectious disease considered worldwide an important public health problem [5]. Up to now, no drug has demonstrated to be entirely satisfactory to cure of this infection. In addition, the drugs that had been used in the disease treatment are toxics, very expensive or both [4].

The use of supercritical CO_2 plus cosolvent to extracts alkaloids has been studied [6], [7], [8]. The purpose this work was to analyze the composition of *T.catharinensis* extracts obtained by SFE with CO_2 using different cosolvents.

MATERIALS AND METHODS

Raw materials preparation and characterization

Thin branches of *T.catharinensis* were colleted in May 2002 (Campinas, São Paulo, Brazil). The material was dried at ambient conditions in the absence of light and subsequently triturated in a hammer mill (Treu & Cia. Ltda, Model granular, Rio de Janeiro, Brazil).

Afterwards, the raw material was conditioned under vacuum in plastic bags (0.5 kg) and stored in a domestic freezer (Metalfrio, double action, São Paulo, Brazil) at temperature below -15ºC.

Experimental SFE procedure

The experiments were conducted using a SFE unit containing an extraction cell of approximately 221×10^{-6} m^3 (length of 37.5×10^{-2} m and inside diameter of 2.74×10^{-2} m) and maximum pressure of 400bar described by Pasquel et al [9]. The bed was formed inside the extraction cell using $(7.14\pm0.01)\times10^{-3}$ kg of *T.catharinensis* and $(340\pm1)\times10^{-3}$ kg of glass spheres (Mesh 10), and, then, adapted into the SFE unit. The thermostatic bathes were turned on and the unit was allowed to reach the operating temperature set at 45ºC, based on the results of Pereira [8]. The thermostatic bath controlling the CO_2 inlet flow to the pump was set at -10ºC. After thermal equilibration, the system was slowly pressurized up to the operating pressure (250 bar); then, the valves at the extractor's outlet were opened and the extraction process began. The intermittent processe was used [8]. Samples were collected in glass flasks (100×10^{-6} m^3). The experiments were run for two hours, then, the CO_2+cosolvent flow stopped and the system final depressurization (45 minutes). The following cosolvents were used: methanol (MeOH: Merck, P.A., lot K30177809), ethanol (EtOH: Merck, P.A., lot K29614683), isopropyl alcohol (IsoC3: Merck, P.A., lot K29992334), water. Having finished the extraction, the glass flasks were put in the vacuum furnace (Napco- modelo 5831, EUA), without heating to remove the alcoholic cosolvent, or in a liofilizator (FTS Systems, Stone Ridge, New York, USA) to remove the aqueous cosolvent, remaining there by 24 hours. The global yield (X_0) was calculated as the ratio of the total mass of extract by the initial mass of *T. catharinensis.*

Analysis of the SFE extracts

The identification and characterization of the indole alkaloids required the following procedure: fractionation of the crude extract (SFE extract) to obtain the indole alkaloidal fraction [4], which, was then analyzed by Thin-layer chromatography followed by Gas-Chromatography with Flame Ionization Detector (GC/FID) [10].

Fractionation of the SFE extract

The SFE extract was dissolved in HCl 5% (fumigating 37%, Merck, P.A.) and washed three times with hexane (Merck, P.A., lot K26803774934), to remove wax and lipidic compounds. The aqueous extract was alkalinized with NH_4OH (25%, Merk, P.A.) and washed three times with chloroform (Merck, P.A., lot K28335045). The organic fraction (AF alkaloidal fraction) was evaporated using a rotatory evaporator (Laborota, model 4001, Viertrieb, Germany), with vacuum control (Heidolph Instruments GMBH, model Rotavac control, Viertrieb, Germany), and bath at 40ºC of the thermostatic.

Thin-layer chromatography (TLC)

The organic fraction (AF) was analyzed by TLC using silica plates (60-PF254, Merck, 20×20 cm, 0.25 mm of height, lot 940378601). The AF was eluted in mixtures using several solvent systems usually used to fractioned indole alkaloids from other alkaloids [10], [11], [12]. The following mixtures were used: (S1) cyclohexane (Merck, P.A., lot K26803774934), chloroform (Ecibra, chromatographic grade, lot 90466), and dichloromethane (Merck, P.A., lot K24900450809), 6:3:1; (S2) chloroform-methanol (Merck, P.A., lot K26224109909), 9:1; (S3) cyclohexane:ethyl acetate (EM Science, chromatographic grade, lot 3903991), 4:1; (S4) chloroform. Due to the unavailability of pure standards, SFE extracts rich in voacangine and

coronaridine obtained by Pereira [8] were used as standard. The plates were revealed in Dragendorff, the specific reagent for visualization of the alkaloids.

Gas-chromatography with Flame Ionization Detector (GC/FID)

The alkaloidal fraction was analyzed in a Gas Chromatographer with a Flame Ionization Detector (GC/FID, Shimadzu, model 17A, Kyoto, Japan), equipped with a capillary column of fused silica DB-5 (30 m×0.25 mm×0.25 μm, J&W Scientific, Folsom, USA). The carrier gas was helium (99.99% purity, White Martins Gases Industriais, 1.7 mL/min). The injector and detector temperatures were 250ºC and 280ºC, respectively. The temperature programming was 100ºC (5 min), 100 – 280ºC, 10ºC/min; 280ºC (10 min). The split ratio was 1/30. Samples of 1μl of extract diluted in ethyl acetate (5×10^{-6} kg of extract diluted in 1×10^{-6} m^3 ethyl acetate; EM Science, chromatographic grade, lot 3903991) were injected. The identification of the substances was based on comparison of chromatograms from extracts with i) literature [4], [10], [11] and ii) standards: coronaridine (79.55%), voacangine (80.99%) and isovoacangine (100%).

RESULTS AND DISCUSSIONS

Global yields: the effects of the cosolvent

The global yields (X_0) were determined in two experiments: the first analyzed only the alcoholic cosolvents (MeOH, EtOH, and IsoC3) and the second the aqueous mixtures of the cosolvents used in the first. Table 1 shows the global yields obtained for each cosolvent. The analysis of variance (ANOVA) for the first experiment, which was done with replication, showed that the global yield was not affected by the type of cosolvent ($p = 0.853$) and had an average value of $(2.4\pm0.1)\times10^{-2}$ kg/kg. For the second experiment the global yields were significantly greater varying from 12.9 to 15.8%, nonetheless, several experimental difficulties were observed: Foam formation that can be attributed to the presence of saponins, which are reported to be present in extracts obtained with water. This has enormously increased the difficulties in the fractionation of the crude extracts.

Water has been used as cosolvent with success in SFE of alkaloids [6], [13], [14], but the same foam formation and other problems was verified by Nossack et al [7] and Schaeffer et al [15]. These difficulties may be related to the phase equilibrium of the systems CO_2/H_2O and CO_2/H_2O/alcohols, which have been studied [16], [17], [18], [19]. According to Bamberger et al [16], the system CO_2/H_2O at the conditions used in this work (250 bar and 45ºC) show a two phase region formed by a CO_2-rich phase and a H_2O-rich phase. For the systems CO_2/H_2O/alcohols liquid-liquid equilibrium regions were detected [18], [19]: Three and four-phases equilibria for the ternary systems CO_2/H_2O/alcohols occur at pressure of 40 to 160 bar and temperatures of 25 to 100ºC, depending of the alcohol in the ternary system [18], [19]. Therefore, at the operating conditions used in the present work, phase separation may have occurred inside the extraction cell, and thus, interfering in the SFE results. Yoda et al [20] have also reported an unusual behavior for the system H_2O+CO_2+stevia leaves at 30ºC and 250 bar. In alcoholic cosolvents and their mixtures strong interaction of the cosolvent and the fractionating solvent mixture was observed. Specific interactions (hydrogen bonding, dipole-dipole, dipole induce dipole, induce dipole-induce dipole) between the cosolvent and one or more solutes can exist influencing the solubility of these solutes in the supercritical fluid, and/or in the fractionating solvent. Several workers have studied the interactions of a solute/cosolvent/matrix complex to correlate and adjust parameters in chemical-physical model [21], [22], [23]. However, to understand this event it is necessary to know all substances present in the crude extracts.

Table 1: Global yields obtained at 45ºC, 250 bar, 6.1 ´ 10^{-5} kg/s, using 5.1% (m/m) of cosolvent

Experiment No. 1*		Experiment No. 2 **	
Cosolvent	$X_0 \times 10^2$ (kg/kg)	Cosolvent	$X_0 \times 10^2$ (kg/kg)
Ethanol (EtOH)	2.48 ± 0.05	H_2O	15.8 ± 0.2
Isopropyl alcohol (IsoC3)	2.5 ± 0.1	H_2O + EtOH (1:1)	15.0 ± 1.2
Methanol (MeOH)	2.4 ± 0.3	H_2O + MeOH (1:1)	15.1 ± 0.1
EtOH + IsoC3 (1:1)	2.3 ± 0.1	H_2O + IsoC3 (1:1)	12.9 ± 0.5
EtOH + MeOH (1:1)	2.5 ± 0.2	**Assays were replicated (2x).	
MeOH + IsoC3 (1:1)	2.4 ± 0.2		

*Assays were replicated (3x).

Voacangine represents about 6.3% of the alkaloidal fraction, and only traces of coronaridine were observed. However, Pereira [8] determined that the content of coronaridine was larger and, in addition, the amount of coronaridine + voacangine represented 15 to 25% of the alkaloidal fraction. The raw material used in this work came from one of the tree used by Pereira [8], but was harvested in autumn of 2002 (May 2002). It has been reported in literature [11], [24] that the maximum amount of alkaloids is reached after the flowering (October-November in the South hemisphere). In spite of the reported information related to the action of coronaridine and voacangine, scarce information is found connected to the biosynthesis of these alkaloids. This is partially due to the complex structure of the indole alkaloids [25]. Thus, at the time the raw material was harvested the biosyntheses of the target alkaloids coronaridine and voacangine was still under development.

Phytochemical profile of the extracts

Table 2 shows the mass of total alkaloids or alkaloidal fraction (AF) and voacangine in the crude extracts (V). In contrast with the global yield, the results shows a significant effect of the type of cosolvent on the content of total alkaloids and of the alkaloid voacangine ($p = 0.023$); the most effective cosolvent was methanol (AF= $58 \pm 1 \times 10^{-4}$ kg/kg). With respect to the mass of voacangine methanol as well as the mixture of methanol and ethanol were effective (2.8 ± 0.1 and $2.5 \pm 0.8 \times 10^{-4}$ kg/kg, respectively), in spite of that the differences observe for the other cosolvent or cosolvent mixtures were not statistically significant ($p = 0.110$). For the assays using isopropyl alcohol in the cosolvent mixture the experimental errors associated with the alkaloidal fraction (AF) were larger than the experimental errors for the other cosolvents or isopropyl alcohol alone. Nonetheless, for all assays the source of experimental errors were the same, and the error discrepancy was observed only during the fractionating step, thus, this may suggests a strong interaction of the cosolvent mixture and the fractionating solvent mixture. In order to elucidate this phenomenon it is necessary to identify and quantify all substances present in the crude extracts, which would require the use of additional analytical methods.

Table 2: Masses of the alkaloidal fraction (AF) and voacangine (V) with respect to the feed for assays at 45ºC, 250 bar, 5.1% (m/m) of cosolvent, and 6.1´ 10^{-5} kg/s (Assays were replicated 2x)

Experiment No. 1			Experiment No. 2		
Cosolvent	AF $\times 10^4$ (kg/kg)	V $\times 10^4$ (kg/kg)	Cosolvent	AF $\times 10^4$ (kg/kg)	V $\times 10^4$ (kg/kg)
EtOH	21 ± 4	1.3 ± 0.1	H_2O	100 ± 10	tr
IsoC3	15 ± 1	1.0 ± 0.1	H_2O- EtOH*	66.2	tr
MeOH	58 ± 1	2.8 ± 0.1	H_2O-MeOH*	109.0	tr
EtOH-IsoC3	31 ± 17	2.0 ± 0.5	H_2O-IsoC3*	74.8	tr
EtOH-MeOH	29 ± 2	2.5 ± 0.8	*Assays were not replicated.		
MeOH-IsoC3	31 ± 10	1.8 ± 1.0			

Figure 1 shows the TLC for each organic fraction (AF) obtained from the SFE extracts using alcoholic cosolvents. As can be observed, the plates did not have the layer separation for anyone of the solvent mixtures used to elute the crude extract. For the fractionating solvent mixtures S1, S3, and S4 the layers remained in the bottom of the plate. For S2 the layer eluted up to the top of the plate. In spite of this, alkaloids were detected in all samples used. It is known that the alkaloidal fraction contains alkaloids that may not be detected by GC-FID, but may be detected by TLC, UV-V spectrophotometer, ^{1}H NMR, and ^{13}C NMR [8].

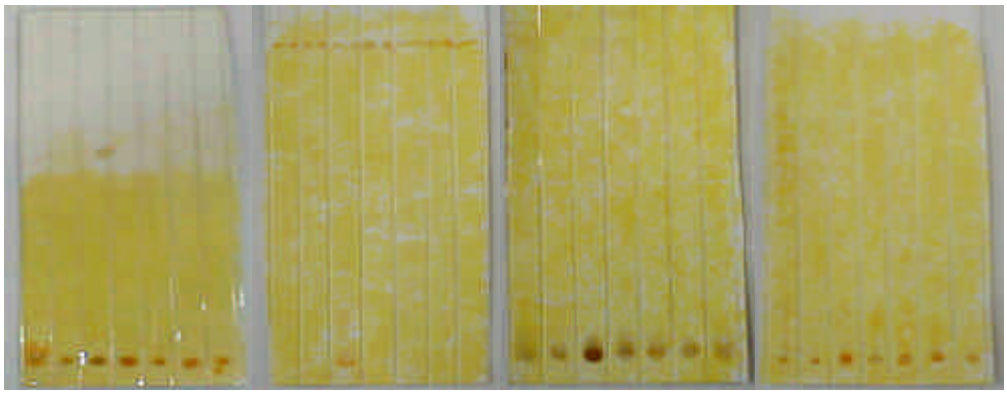

Figure 1. TLC for alkaloidal fractions (AF) obtained from crude SFE extracts using cosolvents: 1- standard sample (alkaloid-rich sample [8]); 2- EtOH; 3- IsoC3; 4- MeOH; 5- EtOH-IsoC3; 6- EtOH-MeOH; 7-MeOH-IsoC3, using the solvent mixtures S1, S2, S3, S4.

CONCLUSIONS

The results have shown that the content of alkaloids in the extracts was strongly affected by the cosolvent; however, the global yield was not affected by the type of alcoholic cosolvent. Considering the amount of the alkaloidal fraction, the methanol was the most effective cosolvent. For the experiments using water as cosolvent the global yields were significantly greater than that obtained using the alcoholic cosolvents; however, foam formation was observed, which increased the difficulties in the fractionation of the crude extracts. The content of voacangine and coronaridine was small compared to literature data [8]; this may be explained considering that the raw material was harvested during the autumn, thus, when the biosynthesis of these alkaloids is still under going.

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