SFE OF PHARMACOLOGICAL COMPOUNDS FROM *Tabernaemontana catharinensis*: ANALYSIS OF THE ANTIOXIDANT AND ANTIMYCOBACTERIAL ACTIVITIES

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In the present work the pharmacological activities of SFE extracts from *T. catharinensis* were evaluated. The SFE extracts were obtained using supercritical CO_2 plus ethanol as cosolvent (4.6 and 9.2% m/m) at pressures of 250 and 300 bar and temperatures of 35, 45, and 55ºC. The crude extracts were solvent-fractionated (hexanic fraction, alkaloid, and aqueous fractions) and analyzed by Thin-layer chromatography (TLC), and Gas-chromatography/Flame Ionization Detector (GC/ FID). The antioxidant and antimycobacterial activities were determined for the crude extracts and their fractions. The antioxidant activity was measured by the coupled reaction of limonene acid and β-carotene; the antimycobacterial activity against *M. tuberculosis* was measured by Microplate Alamar Blue Assay (MABA) technique. The results show that the minimum antioxidant activity was approximately 55% (after 3 h of reaction) while the maximum was 92% (at the first hour of reaction). The highest antimycobacterial activity was detected in the alkaloidal fraction (MIC = 128 μg/mL) while the aqueous fraction had the lowest antimycobacterial activity (MIC > 512 μg/mL).

Keywords: antioxidant, antimycobacterial, indole alkaloids, SFE, *Tabernaemontana catharinensis*

INTRODUCTION

Members of the Apocynaceae family have been used for centuries in folk medicine; many of the compounds isolated from their extracts are used in new drugs, such as vimblastine, vincristine and reserpine [1]. Among the members of the Apocynacea family is the specie *Tabernaemontana catharinensis* (syn. *Peschiera catharinensis* A.DC.) that has been studied due to its high content of indole alkaloids. The tree occurs in Argentine, Uruguay, Paraguay, and Southern Brazil. Its infusion is used in folk medicine as an antidote for snake bites, to relieve toothache, and, also as a vermifuge to eliminate warts [2]. Pharmacological studies on the crude extracts from *T.catharinensis* have demonstrated its antitumoral, anti-inflammatory, and analgesic activities [2], [3], [4]. The effective actions of these extracts are based on the presence of indole alkaloids. Recently, it was reported that the crude extract and its isolated indole alkaloids exhibited significant tripanocidal and antileishmanial activity [4], [5]. Alkaloids present in *T.pachysiphon* have been showed to be responsible for the antimycobacterial activity in this specie [6]. Investigations with the indole alkaloids voacangine and ibogaine extracted from *T.citrifolia*, determined the

antimycobacterial activities of these compounds against Mycobacterium tuberculosis, Mycobacterium avium, and Mycobacterium kansai [7].

The objectives of the present work were to determine the antioxidant and antimycobacterial activities of extracts of *T.catharinensis* obtained using carbon dioxide plus ethanol, and, also, of the various fractions obtained from the SFE crude extract.

MATERIALS AND METHODS

Raw materials preparation

Thin branches and leaves from *Tabernaemontana catharinensis* were collected by FIOCRUZ (RJ, Brazil) from Guará at the municipality of Campinas (SP, Brazil). The raw material was dried at ambient conditions under the shadow and subsequently triturated. Afterwards, the raw material was conditioned under vacuum in plastic bags and stored in a domestic freezer (Metalfrio, double action, São Paulo, Brazil) at –15ºC. The size distribution of the particles was determined using a mechanical agitator (Abrosinox, model Granutest, Santo Amaro, Brazil) with the rheostat set at 10 during 10 minutes; sieves of meshes (Tyler series) 24, 32 and 48 were used.

SFE experimental procedure

The experimental runs 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 400 bar described by Pasquel et al [8]. The extracts were obtained using supercritical CO_2 plus ethanol as cosolvent (4.6 and 9.2% m/m). The data were taken at 250 and 300 bar, and 35, 45 and 55 ºC, using the metodology described by Pereira [11].

Analysis of the SFE extracts

The identification and characterization of the indole alkaloids required the following procedure: fractionation of the crude extract (SFE extract: EB) to obtain the indole alkaloidal fraction [5] that was analyzed by thin layer chromatography (TLC) followed by gas chromatography with flame ionization detector (GC–FID) [9].

Fractionation of the SFE extracts

The SFE extract (CE) 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 (HE). The aqueous extract was alkalinized with NH_4OH (25%, Merck, P.A.), and washed three times with chloroform (Merck, P.A., lot K28335045), thus, two fractions were obtained: the organic fraction (AF) and aqueous fraction (AqE). The organic fraction or alkaloidal fraction (AF) was evaporated using a rotary evaporator (Laborota, model 4001, Viertrieb, Germany), with vacuum control (Heidolph Instruments GMBH, Viertrieb Germany), bath at 40ºC of the thermostatic. The sequence of the fractionation procedure is shown in Figure 1.

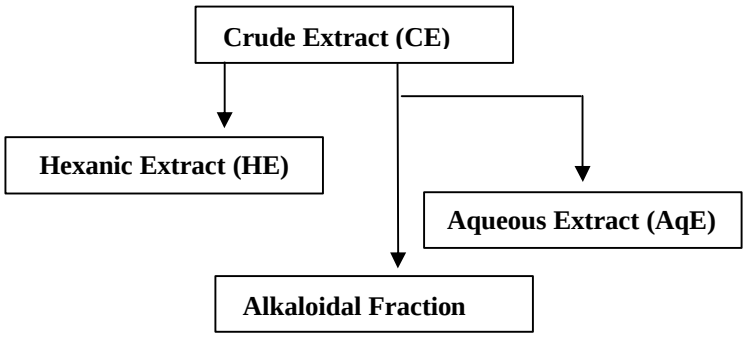

Figure 1: Fractionating scheme for the crude *T.catharinensis* SFE extracts.

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) developed with a mixture 90:10 of choroform (Ecibra, Chromatographic grade, lot 90466l) and methanol (Merck, Chromatographic grade, lot K26224109909). 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 (AF) was analyzed in a Gas-chromatography with Flame Ionization Detector (GC/FID, Shimadzu, model 17A, Kyoto, Japan), equipped with a capillary column of fused silica DB-5 (J&W Scientific; 30 m x 0.25 mm x 0.25 μm, Folsom, USA). The carrier gas was helium at 1.7 mL/min (99.99% purity, White Martins Gases Industriais, Brazil). The injector and detector temperatures were 250ºC and 280ºC, respectively. The temperature programming was 100ºC (5 min), 100 – 280ºC, 10ºC/min; 280 ºC (10 min). The sample 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, lot 3903991, chromatographic grade) were injected. The identification and quantification of the substances was based on comparison of chromatograms from extracts with i) literature data [5], [9], [10] and ii) standards: coronaridine (79.55%), voacangine (80.99%) and isovoacangine (100%).

Antioxidant activity: Coupled oxidation of linolenic acid and b-carotene

The methodology of Hammerschmidt and Pratt [12] was used with the required modifications for the SFE extracts (CE) and their fractions (AF, HE, AqE). The reaction substrate was prepared using 10 mg of β-carotene (99%, Acros, B0070834, Pittsburg, USA), 10 mL of chloroform (99.0%, Ecibra, P.A., lot 13017), 60×10^{-6} kg of linolenic acid (99%, Sigma, U-59A- D4-G) and 200×10^{-6} kg of Tween 80 (Synth, P.A.). This solution was concentrated in a rotary-evaporator (Büchi, CH-9230, Flawil, Germany or Laborota, model 4001, Viertrieb, Germany) at 50 °C and afterwards diluted with 50 mL of bi-distilled water. The reaction was conducted using the following procedure: to 1 mL of substrate was added 2 mL of bi-distilled water and 0.05 mL of the sample diluted in ethanol (99.8%, Merck, P.A, lot 1216046030) (20 mg of extract/1 mL of ethanol). The mixture was set into a water bath (Tecnal, TE 159, Piracicaba, Brazil) at 40°C and the reaction product was monitored using a spectrophotometer (Hitachi, U-3010, Tokyo, Japan) by reading the absorbance at 47 nm after 0, 1, 2 and 3 h reaction.

Antimycobacterial activity

The antimycobacterial activity was determined for the SFE extracts (CE) and their fractions (HE, AF, AqE). The minimum inhibitory concentration of the extracts was measured in Middlebrook 7H9 medium inoculated with *M. tuberculosis* H37Rv - ATCC 27294 using the Microplate Alamar Blue Assay (MABA) technique [13].

RESULTS AND DISCUSSIONS

The Table 1 shows the mass of voacangine (V) and coronaridine (C) present in samples of alkaloidal faction (AF) obtained under differents conditions. At 300 bar, 55ºC, 9.2% (m/m) of cosolvent was detected the largest content of coronaridine and voacangine (11.89×10^{-5} kg/kg in a sample of the AF).

Table 1: Masses of voacangine (V) and coronaridine (C) present in a sample of alkaloidal fraction (AF) with respect to the feed.

P (bar)	T (ºC)	Cosolvent (%, m/m)	(V + C) $\times10^5$ (kg/kg)
250	35	4.6	5.26
250	45	4.6	6.65
300	55	4.6	9.13
300	55	9.2	11.89

The antioxidant activity of all fractions of extracts were verified with coupled oxidation of linolenic acid and β-carotene for crude extracts (CE) and extracts fractionated (HE, AF, AqE), according to Table 2. The antioxidant activities of the extracts HE, CE and AF were superior to activity of β-carotene (control). The hexanic extracts (HE) had greater activity than other fractions (the first hour of reaction had from 89 to 90%, the second hour had from 83 to 88% and the third hour had from 80 to 85%). This fractions content compounds like triterpenes, sterols, fatty acid and triacylglycerols [4], [14] that may be are responsable for this activity. The crude extracts (CE) and alkaloidal fractions (AF) had similar behavior of activity for the extracts obtained at same conditions of extraction. The condition of extraction 4 (300 bar, 55ºC, 9.2 % (m/m) cosolvent) showed more active in the EB, AF and AqE fractions in the three hours of analysis. This condiction had greater content of coronaridine and voacangine in AF fraction (Table 1). The condition of extraction 2 (250 bar, 45ºC and 4.6% (m/m) cosolvent) showed more active in the hexanic extract (HE), in the three hours of analysis. In this experiment the content of active compounds in HE may be obtained in major quantity than in other conditions of process. For all condictions of extraction, the aqueous extracts (AqE) fraction were near activities by β-carotene, the compounds present in this fraction show low active The results showed that the increase of the % cosolvent (from 4.6 to 9.2% m/m, at 300 bar and 55ºC) improved the activity of the AF fraction in the three hours of analysis. This can be due the greater content of alkaloids coronaridine and voacangine obtained in SFE condition that used 9.2% of cosolvent.

Table 3 shows the antimycobacterial activities against M. tuberculosis (MIC) for the various extracts. The fraction AF of *T.catharinensis* had antimycobacterial activities and showed minor values of MIC: from 128 to 256 micrograms/mL. This fractions are rich in alkaloids that may be are compounds activities. The crude extracts (CE) contain these alkaloids but more dilluted and other substances without activity that are restrained in hexamic extract (HE) and aqueous extracts (AqE). The AqE fractions were not active. The

values of the MIC had no differences between the condictions of the procedure for the same kind of fractions (CE, HE, AF, AqE). These results suggest that the differences of coumponds present in various fractions change the activity; therefore the antimycobacterial activity is related with alkaloids presence. In others species of Tabernaemontana, alkaloids had showed antimycobacterial activity against *M.tuberculosis, M.avium* and *M. kansasii* [6], [7].

Table 2: Inhibition of oxidation (%) for the SFE extracts (EB) and their fractions (HE, AF, AqE)

SFE extract	Inhibition of oxidation, %											
	Reaction time, h											
	1	2	3	1	2	3	1	2	3	1	2	3
Control	66	53	46	66	53	46	66	53	46	66	53	46
	250 bar, 4.6% EtOH						300 bar, 55ºC					
	35ºC			45ºC			9.2 % EtOH			4.6% EtOH		
Crude Extract (CE)	85	75	70	87	79	76	88	81	78			
Hexanic extract (HE)	89	83	80	92	88	85	89	84	82			
Alkaloidal Fraction (AF)	87	78	72	87	79	73	88	81	77	82	73	68
Aqueous fraction (AqE)	88	81	77	68	58	53	67	58	53			

Table 3: Antimycobacterial activity MIC (㎎g/mL) for the SFE extracts (EB) and their fractions (HE, AF, AqE)

SFE extracts	Minimum inhibition concentration (MIC), µg/mL								
	250 bar, 4.6% EtOH						300 bar, 55ºC, 9.2 % EtOH		
	35ºC			45ºC					
	M. tb H37Rv	*M. avium*	*M. kansasii*	*M. tb* H37Rv	*M. avium*	*M. kansasii*	*M. tb* H37Rv	*M. avium*	*M. kansasii*
Crude Extract	512	512	> 512	—	—	—	512	> 512	> 512
Hexanic Extract	> 512	> 512	> 512	> 512	> 512	> 512	> 512	> 512	> 512
Alkaloidal Fraction	128	256	256	128	128	256	256	128	256
Aqueous fraction	–	without activity		–	without activity		–	–	–
Alkaloidal Fraction (55ºC)				128	256	256	–	–	–

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