Abstract: The Tabernaemontana genus, belonging to the Apocynaceae family, consists of approximately 110 species. Among these, the species under study, Tabernaemontana catharinensis is popularly known in Brazil as "leiteiro de vaca". This species is especially rich in indole alkaloids, which are compounds with promising biological potential, such as anticholinesterase and antioxidant activities. The objective of the study was isolate, purify, and identify indole alkaloids from the root bark of T. catharinensis. The plant material was dried at room temperature, ground, and subjected to methanolic extraction. The extract was partitioned with dichloromethane/water. The phytochemical study was performed by column chromatography and analytical thin layer chromatography. The structures were established on the basis of spectroscopic methods, including 1D and 2D NMR. The phytochemical study of the T. catharinensis species led to identification of four alkaloids, 12-methoxy-Nb-methylvoachalone (1), coronaridine (2), 5,6-dioxoibogamine (3), and 19(S)-heyneanine (4). Alkaloids (3) and (4) are being reported for the first time in the species.

Keywords: Indolic alkaloids. Tabernaemontana catharinensis. Apocynaceae.

Resumo: O gênero Tabernaemontana, pertencente à família Apocynaceae, é constituído de aproximadamente 110 espécies. Dentre estas, a espécie em estudo, Tabernaemontana catharinensis é conhecida popularmente no Brasil, como “leiteiro de vaca”. Essa espécie é especialmente rica em alcaloides indólicos, que são substâncias que possuem potenciais biológicos promissores, como a ação anticolinesterásica e antioxidante. O objetivo foi isolar, purificar e identificar alcaloides indólicos das cascas da raízes de Tabernaemontana catharinensis. O material vegetal foi seco à temperatura ambiente, moído e submetido à extração metanólica. O estudo fitoquímico foi realizado por cromatografia em coluna e cromatografia de camada delgada analítica. O estudo da espécie T.catharinensis levou à identificação de quatro alcaloides: coronaridina (1), 12-metoxi-Nb-metilvoachalotina (2), 5,6-dioxoibogamina (3) e 19(S)-heyneanina (4). A determinação estrutural dos compostos foi feita utilizando Ressonância Magnética Nuclear unidimensional e bidimensional. Os alcaloídes (3) e (4) estão sendo relatados pela primeira vez na espécie.

Palavras-chave: Alcaloídes indolícos. Tabernaemontana catharinensis. Apocynaceae.
INTRODUCTION

The Tabernaemontana genus contains approximately 110 species, 27 of which are Brazilian (Morales, 2009). The genus is especially rich in monoterpenic indole alkaloids, which are useful chemical markers of the genus and have great value for the classification of the species, in addition to demonstrating considerable variety of carbonic skeletons and diverse biological activities.

The Tabernaemontana catharinensis species, popularly known as “leiteiro de vaca”, occurs in Argentina, Bolivia, Brazil, and Paraguay. This species is also known as T. affinis, T. australis, Peschiera australis, T. hilariana, T. hybrida, T. acummiata, T. salicifoliae, and Peschiera albidiiflora (Reis, 2018).

In folk medicine, this species is used as an antidote for snake bites, to relieve toothache, and as a dewormer. Alkaloids and extracts containing alkaloids of this species showed several biological activities: anti-inflammatory (Camponogara et al. 2019), analgesic (Brum et al. 2019), antitumor (Rosales et al., 2019), antioxidant (Pergher et al. 2019), antimicrobial and antileishmanial (Pereira et al., 2005; Reis, 2018), and trypanocidal (Pereira et al., 1999).

In the present work, we report the investigation of the methanolic extract from the root bark of T. catharinensis, which allowed us to characterize four already known monoterpenic indole alkaloids, however, two of these are being reported here for the first time in the species.

OBJECTIVES

Isolate, using classical chromatographic methods, and identify, using spectrometric methods, indole alkaloids from the root bark of T. catharinensis, family Apocynaceae.

METHODS

General experimental procedures

The plant material was powdered using a TECNAL hammer mill.

The extract was concentrated under reduced pressure on a rotary evaporator, FISATOM 802.

Analyses using the analytical tool thin layer chromatography (TLC) were performed with silica gel 60 F254 MERCK. The compounds were visualized by irradiation with an ultraviolet lamp, with wavelengths of 254 nm and 365 nm and/or with chromogenic developers (Dragendorff reagent and 2% vanillin solution in concentrated sulfuric acid), followed by heating.

The column chromatographic separations were performed on silica gel MERK DARMSTADT 60 (0.063-0.200 mm).

The purity criteria adopted were the visualization of a single spot in TLC, using different eluent systems.

The solvent mixtures were expressed in % v/v.

Nuclear Magnetic Resonance (NMR) spectra were recorded on a BRUKER spectrometer, model DPX-500, operating at a frequency of 500 MHz for 1H and 125 MHz for 13C. The solvent used for all spectral analyses was CDCl3.

Plant Material

The vegetal material made up of the root bark from Tabernaemontana catharinensis was collected in the surroundings of the municipality of Bom Jesus do Itabapoana, RJ. The material was subsequently classified and identified by Professora Luiza S. Kinoshita from the State University of Campinas (UNICAMP). The exsiccation was deposited at the UNICAMP Herbarium with voucher specimen UEC117862.

Extraction and isolation

The root bark was subjected to successive extractions at room temperature, using methanol as the solvent, giving rise to the crude extract (42.4g). This was subsequently partitioned in dichloromethane/water, obtaining the organic phase (20.6g) and the aqueous phase (19.6g).

A portion of the organic phase (18.5g) was subjected to silica gel column chromatography, using dichloromethane/methanol as eluent in increasing polarity concentrations. The fractions were combined by analytical thin layer chromatography, obtaining 14 fractions in total, where fraction 13 gave rise to compound 1 (369.6mg).

Fraction 8 (1.8g) was rechromatographed on a silica gel column, using dichloromethane/methanol in increasing polarity concentrations as eluent and, after the fractions were combined, provided 8 new fractions in total, where fraction 8.2 resulted in compound 2 (7.6mg). The 8.3 fraction (813.2 mg) was also rechromatographed on a silica gel column, using dichloromethane/ethyl acetate as an eluent in
increasing polarity concentrations, which, after the fractions were combined, provided 16 new fractions, where the 16.6 fraction gave rise to compound 3 (4.50g) and fraction 16.9 gave rise to compound 4 (46.1mg).

RESULTS AND DISCUSSION

In the current research, we report the investigation of the methanolic extract of the root bark from T. catharinensis, which led to the characterization of the four known monoterpenic indole alkaloids: 12-methoxy-Nb-methylvoachalone (1), coronaridine (2), 5,6-dioxoibogamine, (3) and 19-(S)-heyneanine (4), which were identified with spectral data from the literature for 1H and 13C NMR spectra (Figueiredo et al, 2010; Gonçalves, 2011; Souza et al, 2010). It should be emphasized that only the spectral data of alkaloids 3 and 4 will be discussed, since they are being reported for the first time in this species. The structures of all identified compounds are shown in Figure 1.

Alkaloid 3 was isolated as a yellow oil, showing a positive test for Dragendorff. Analysis of the 13C NMR spectrum (Table 1, Figure 3 - Annex) enabled recognition of the presence of nineteen carbon atoms, one methyl carbon, four methyleneic carbons (all sp3, and one linked to a heteroatom), eight methyline carbons (four aromatic sp2, and four sp3, one linked to a heteroatom), and six quaternary carbons (all sp2, two of which are carbonyl)

The spectrum of 1H NMR (Table 1, Figure 4, Figure 5, and Figure 6 - Annex) and 1H-1H-COSY (Table 1, Figure 7 - Annex) of alkaloid 3 show four signals related to aromatic hydrogens at H 8.17 (1H, m, H-9); 7.22 (1H, m, H-10), 7.22 (1H, m, H-11), and 7.52 (1H, m, H-12) characteristic of an indole nucleus, indicating that ring A is free of substituents (Lounasmaa and Tolvanen, 1986), confirmed through heteronuclear 1JCH correlations of the methyl and carbon atoms presented in the HSQC spectrum (Table 1, Figure 8 - Annex) between CH-9 (C 169.72) and H-9 (H 8.17), CH-10 (C 121.00) and H-10 (H 7.22), CH-11 (C 123.86) and H-11 (H 7.22), and CH-12 (C 112.32) and H-12 (H 7.75) together with the presence of a broad singlet signal at H 11.05 referring to a hydrogen of an HN group (Azoug et al., 1995).

In alkaloid 3 there is the absence of a carbomethoxy group linked to C-16, common in the skeleton for monoterpenic indole alkaloids (Zenk, 1980).

Analysis of the 13C NMR spectrum (Table 1, Figure 3 - Annex) showed the presence of a signal at C 169.72, which is consistent with the chemical displacement of a carbonyl group from a lactam and also the presence of one more signal referring to a carbonyl carbon at C 184.62. The absence of the signals related to the coupling of the two methyleneic groups, 2H-5 to the nitrogen atom with 2H-6, suggests the proposal of an indole nucleus free of substituents, but with the presence of two carbonyl groups linked to the carbon atoms C-5 and C-6, respectively.

The position of the carbonyl linked to the carbon atom C-5, forming a lactam with N-4, was confirmed by the long distance 3JCH correlation presented in the HMBC spectrum (Table 2, Figure 9 - Annex) as a wide doublet signal at H 4.07, referring to H-21.

The presence of a triplet signal with three
hydrogen integration at $\text{H} 0.98$ with $J = 7.3 \text{ Hz}$, relative to a methyl group in the aliphatic part of the molecule, presented in the 1H NMR spectrum (Table 1, Figure 6- Annex), confirms the presence of an ethyl group attached to the C-20 carbon atom. The data set above allowed us to propose the structure of the 5,6-dioxoibogamine alkaloid, previously identified in T. hystrix (Souza et al., 2010). Alkaloid 4 tested positive with Dragendorff reagent. The analysis of the 13C NMR spectrum (Table 2, Figure 10 and Figure 11- Annex) enabled recognition of the presence of twenty-one carbon atoms. The signals at $\text{C} 174.95$ and $\text{C} 52.00$ are consistent with the displacements for carbon atoms of a carbomethoxy group, and the signals at $\text{C} 118.48$ (C-9); 119.51 (C-10), 122.33 (C-11), and 110.48 (C-12) are related to an indole core with an unsubstituted A ring.

A hydroxyethyl group linked to CH-sp3 is recognized by changes in the signals referring to hydrogen atoms linked to carbon atoms CH3-18 (3H; $\text{H} 1.13$; d) and CH-19 (1H; $\text{H} 4.18$; qui) in relation to alkaloid 3 (Table 2, Figure 11 and Figure 12- Annex). The attribution of the chemical shifts of the remaining methyleneic and methyleneic hydrogen atoms in the aliphatic chain is described in Table 2, together with the correlations observed in the HSQC spectrum (Figure 15- Annex).

The set of data above, added to those presented in the spectrum of 1H-1H-COSY (Figure 14 - Annex) allowed us to propose structure 4 for the identified alkaloid. The relative configuration of the carbon atom C-19 (19S) was established based on the analysis of the 1H NMR and 13C NMR spectra, and through comparisons with literature data for heyneanine alkaloids and their epimer 19-(R)-heyneanine (Atta-ur-Rahman et al., 1987; Matos et al., 1976; Lemos et al., 1996; Wenkert et al., 1976).

The hydrogen bond formed between the hydroxyl group, allocated on carbon atom C-19, and nitrogen N-4 forces the hydroxyethyl group to a more rigid conformation (partial structures 4a and 4b, Figure 17), causing differences in chemical displacements of the hydrogen and carbon atoms of CH3-18 and CH-19 of alkaloid 4 and its epimer. The literature records values of $\text{H} 1.11$ (3H-18) and $\text{H} 4.12$ (H-19) for 4 and $\text{H} 1.27$ (3H-18) and 3.92 (H-19) for its epimer (Matos et al., 1976) which corroborate the proposed structure 4 for the isolated alkaloid. In addition, the chemical displacement values of CH2-15 (C 22.9) and CH-21 (C 59.7) carbon atoms (Table 2, Figure 7) recorded in the literature for 4 (Atta-ur-Rahman et al., 1987) are different from those reported for the epimer: C 28.6 (CH2-15), 54.7 (CH-21) (Lemos et al., 1996; Wenkert et al., 1976). These differences are explained by the protective $\gamma$ effect of the methyl group (CH3-18) on carbon CH2-15 in 4 (partial structure 4a) and on CH-21 carbon in the epimer (partial structure 4b - Figure 17).

The 1H NMR spectrum (Table 2, Figure 12 and Figure 13 - Annex) for alkaloid 4 shows signs of aromatic hydrogens at $\text{H} 7.49$ (1H, d, H-9); 7.12 (1H, t, H-10); 7.19 (1H, t, H-11); and 7.27 (1H, d, H-12) characteristic of an indole nucleus with substituent-free A ring, and a wide singlet at $\text{H} 7.83$ for a hydrogen of an NH group. These data are confirmed by the heteronuclear correlation between the carbon atoms and hydrogens CH-9 (C 118.48)/H-11 (H 7.19), CH-10 (C 119.51)/H-12 (H 7.27), CH-11 (C 122.33)/H-9 (H 7.49) and CH-12 (C 110.48)/H-10 (H 7.12) in the spectrum 2J HMBC (Table 2, Figure 16 - Annex).
In alkaloid 4, the methyl group CH3-18 protects CH2-15 at 5.7 ppm [$\Delta C = 28.6$ (epimer) - 22.9 (4) = 5.7 ppm], with carbon CH-21, which does not have this protection, C 59.8. In the epimer (partial structure 4c) the reverse occurs: the methyl group CH3-18 protects CH-21 at 5.1 ppm [$\Delta C = 59.8$ (4) - 54.7 (epimer) = 5.1 ppm] and, in this case, CH2-15 (C 28.60) does not suffer the protective effect $\gamma$ of the methyl group CH3-18 (Figure 17).

The data confirm the structure of the isolated alkaloid, as well as the stereochemistry of carbon 19, being identified as 19-(S)-heyneanine.

**Compound 1:** 13C RMN – 132.0 (C-2); 101.9 (C-7); 126.9 (C-8); 147.9 (C-12); 127.2 (C-13); 55.2 (C-16); 127.7 (C-20); 172.9 (C-22); 58.1 (CH-3); 64.6 (CH-5); 111.3 (CH-9); 120.5 (CH-10); 104.1 (CH-11); 29.7 (CH-15); 119.6 (CH-19); 18.8 (CH2-6); 28.0 (CH2-14); 62.5 (CH2-17); 64.5 (CH2-21); 11.4 (CH3-18); 31.9 (MeN-1); 48.8 (MeN-4); 54.8 (MeO-12); 52.0 (MeO-22).

1H RMN – 6.11 (m, H-3); 4.70 (d, H-5); 7.01 (t, H-9); 7.00 (d, H-10); 6.67 (d, H-11); 3.08 (s, H-15); 5.35 (d, H-19); 3.69 (m, H-6); 3.04 (m, H-6); 1.67 (m, H-14); 3.66 (m, H-17); 3.46 (d, H-17); 5.16 (d, H-21); 3.87 (d, H-21); 1.56 (d, 3H-18); 3.93 (s, MeN-1); 3.13 (s, MeN-4); 3.91 (s, MeO-12); 3.74 (s, MeO-22).

**Compound 2:** 13C RMN – 136.0 (C-2); 110.0 (C-7); 128.0 (C-8); 135.5 (C-13); 55.0 (C-16); 175.0 (C-22); 118.4 (CH-9); 119.3 (CH-10); 122.0 (CH-11); 110.4 (CH-12); 27.3 (CH-14); 39.1 (CH-20); 57.5 (CH-21); 51.7 (CH-23); 53.2 (CH2-5); 22.1 (CH2-6); 32.1 (CH2-15); 36.5 (CH2-17); 26.5 (CH2-19); 11.7 (CH3-18); 52.6 (MeO-22).

1H RMN – 7.50 (dd, H-9); 7.12 (t, H-10); 7.18 (t, H-11); 7.27 (d, H-12); 1.93 (H-14); 1.38 (H-20); 3.60 (H-21); 2.97 (H-3); 2.84 (H-3); 3.42 (H-5); 3.20 (H-5); 2.30 (H-6); 3.05 (H-6); 1.78 (H-15); 1.17 (H-15); 2.62 (H-17) 1.95 (H-17); 1.61 (H-19); 1.48 (H-19); 0.94 (t, 3H-18); 3.72 (s, MeO-22).

**CONCLUSION**

The phytochemical study of the root bark of the species Tabernaemontana catharinensis led to identification and characterization of four monoterpenic indole alkaloids: 12-methoxy-Nb-methylvoacalone, coronaridine, 5,6-dioxoibogamine, and 19-(S)-heyneanine.

To the best of our knowledge, the 5,6-dioxoibogamine alkaloids and 19-(S)-heyneanine are being reported for the first time in the studied species.

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ANNEX
