Chapter 33

Evaluation of antimicrobial activity of extracts and fractions of species of the genus psychotria

https://doi.org/10.56238/colleinternhealthscienv1-033

Adilso de Freitas Junior

Almir Ribeiro de Carvalho Junior

ABSTRACT

Alkaloids are secondary metabolites, present in plants, which have been exhibiting a series of biological activities, a fact that highlights the importance of research with this theme. The work developed from August/2019 to August/2020, in the laboratories of the IFSC - Campus Criciúma, aimed to isolate, purify and evaluate antimicrobial activities of extracts, fractions and substances isolated from species of *Psychotria* genus.

The extracts were obtained by maceration of the species leaves in methanol. The fractions were obtained by liquid-liquid extraction and the isolation processes by chromatographic methods. The identification of the tested metabolite was performed by NMR of ^{1}H, ^{13}C and high resolution mass spectrometry.

The antimicrobial tests were made with the alkaloid 5-carboxy-strictosidine, extracted from *Psychotria nuda*, against the following microorganisms: *Escherichia coli*, *Staphylococcus epidermidis and Pseudomonas aeruginosa*. Sample did not show any activity when compared to the positive control.

1 INTRODUCTION

Human history is marked by the constant battle waged against several species of bacteria, potential causes of infections and diseases. An example of human success was the discovery of penicillins (1928), produced by fungi of the genus Penicillium, attributed to the British researcher Alexander Fleming [1]. As a response to therapeutic alternatives, microorganisms have developed resistance to antibiotics, which, in the health area, is seen as the greatest challenge to be faced in the coming decades [2].

According to the World Health Organization (WHO), the resistance of bacteria to antibiotics has been growing alarmingly around the world, with the emergence and spread of new resistance mechanisms, developed by microorganisms. This has made it more difficult to treat diseases such as pneumonia and tuberculosis that was responsible, in 2017, for the death of 1.3 million people worldwide [3].

The lack of alternatives for some treatments of infections and the constant development of resistance to existing therapies highlight the need for the search for new substances with antimicrobial action, which can occur via the isolation of secondary metabolites present in plants. [4]

In recent years, substances from natural or modified products have been undergoing clinical trials, mainly as anticancer and antimicrobial agents [4]. Among the classes of substances of natural origin with antimicrobial properties, the alkaloids [5], present in certain genera, such as Psychotria, stand out.

The phytoalexins are substances synthesized when the plant gets in contact with some external agent, generally a parasitic microorganism [6]. In the group of phytoalexins or also secondary metabolites, there are numerous compounds of diverse chemical nature, encompassing the entire class named alkaloids that have a protective action in plants to UV radiation due to the aromatic nucleus [7]. These secondary metabolites are produced as a defense mechanism, possessing a characteristic bitter taste, and are stored in cell organelles [8].

Alkaloids as their name suggests, is "that which resembles alkali" [9] due to the presence of the amine function in their structure.

Since their structural variety is so great, a definition has emerged that alkaloid would be a cyclic organic substance containing nitrogen in a negative oxidation state and whose distribution is limited among living organisms [10].

Making structural analyses, during decades of study it has been concluded that the alkaloids are found mixed among various parts of the plants (mainly in the stem and leaves), because, these metabolites are poorly water-soluble in their natural form, but the salts formed upon reaction with acids are generally water-soluble [8].

Species of the genus Psychotria (Rubiaceae) are recognized for the production of alkaloids, mainly indolic alkaloids that have shown a number of biological activities, such as analgesic, cytotoxic, anti-inflammatory, antimicrobial, and others [11].

Species of the genus Psychotria, such as P. nuda (Cham. & Schltdl.), are native to dense ombrophilous forests that, although threatened, can be found in coastal areas such as Rio de Janeiro, Santa Catarina extending to Rio Grande do Sul, and other more isolated regions such as Minas Gerais [12].

Due to the biological importance of these metabolites and their occurrence in the genus Psychotria, the objective of this research was to evaluate the antimicrobial activities of fractions and substances isolated from the species P. nuda, P. leiocarpa, P. vellosiana and P. suterella, against the microorganisms Staphylococcus epidermidis, Escherichia coli and Pseudomonas aeruginosa.

Figure 01- Plants of the species (A) P. vellosiana (Benth), (B) P. nuda (Cham. & Schltdl.), (C) P. leiocarpa (Cham. & Schltdl.) and (D) P. suterella (Müll.Arg.).

Source: a) LOPES (2016); b) MAÇANEIRO (2020); c) BAGATINI (2018); d) SILVA (2013).

2 ACTIVITIES DEVELOPED

2.1 OBTAINING THE EXTRACTS

The extracts were obtained through an extraction technique known as maceration, which consists in cold extraction without agitation. For this, the leaves of the plants

P. leiocarpa, P. nuda, P. suterella and P. vellosiana, were separated, ground and placed in an appropriate recipient with methanol (extraction solvent). This procedure occurred three times for each sample, in order to promote maximum extraction of metabolites. The subsequent steps were filtration and concentration of the extracts at a rotary evaporator, whose masses are shown in Table 1.

Table 01- Masses of extracts obtained

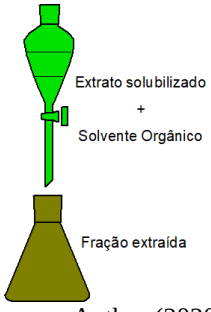
Extract	ID No.	Plant part	Mass(g)
P. nuda (I)	1	Sheets	46,5g
P. leiocarpa	2	Sheets	0,926g
P. suterella	3	Stem	6,0455g
P. nuda (II)	4	Sheets	27,9205g
P. vellosiana	5	Sheets	13,4984g

*P. nuda I and P. nuda II, are both extracts from different recurrent plants of the species P. nuda.
Source: Author (2020)

2.2 EXTRACTION LIQUID : LIQUID

The extracts were solubilized in a solution of MeOH:H2O (1:3) and passed through liquid x liquid extractions in a settling funnel depicted in Fig. 2.

Figure 02- Liquid x liquid extraction system



Source: Author (2020)

The extract solubilized in MeOH was placed in the separation funnel together with the organic solvents listed in Table 2.

Table 02- Solvents used for extraction

Solvent	Polarity	Goal
Hexane	Apolar	Separate the polar fractions from the apolar ones.
Ethyl Acetate and/or Dichloromethane	Polar intermediate	Separating the medially polar fractions
n-Butanol	Polar	Separate the more polar fractions

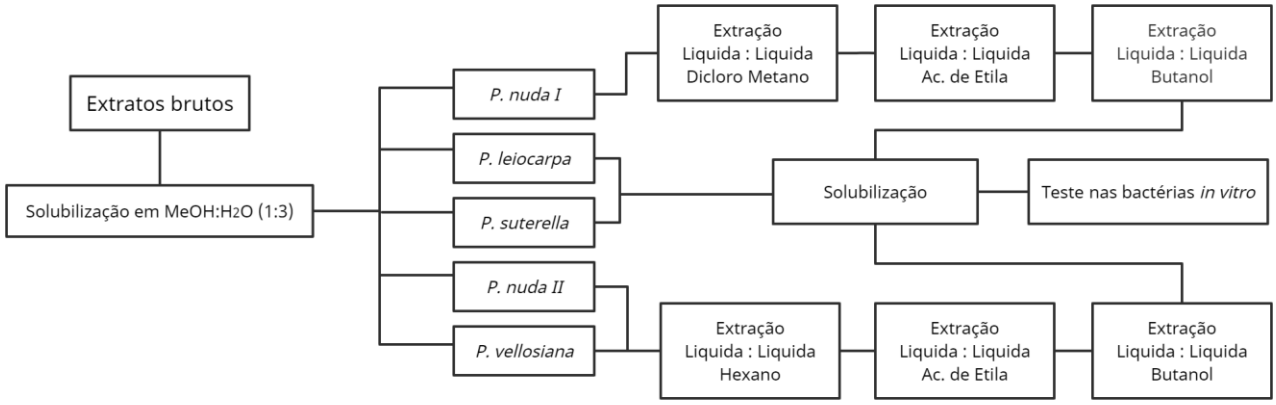
Source: Author (2020)

The extraction was done from the less polar solvent to the more polar one, in a 1:1 ratio with the sample volume, in order to extract the entire polar fraction where the alkaloid is in higher concentration.

2.3 PURIFICATION OF EXTRACTS

The purification of the extracts is represented objectively in Fig. 3, identifying the procedures adopted for the separation of each of the samples.

Figure 03- Flowchart of the adopted purification process



Source: Author (2021)

Part of the extracts of P. nuda (II) (27.9205g) and P. Vellosiana (13.4984g) were solubilized in 160 mL of a methanol and water solution (1:3) in an ultrasonic bath. These solutions were poured into settling funnels and submitted to extraction with hexane, ethyl acetate and n-butanol (increasing order of polarity), obtaining the fractions in the respective solvents.

Table 03- Masses obtained by extracting and drying P. nuda II (4) and P. vellosiana (5).

Extract	**Heavy mass (g)**
4	0,1987 g
5	0,361 g

Source: Author (2020)

For the P. leiocarpa and P. suterella extracts, due to their low masses, they were weighed and taken directly to the solubilization process represented in 2.5.

Table 04- Weighed masses of P. leiocarpa (2) and P. suterella (3) for further solubilization.

Extract	**Heavy mass (g)**
2	0,1004 g
3	0,2066 g

Source: Author (2020)

P. nuda (I) leaves (426 g), after drying and grinding, were subjected to extraction with methanol at room temperature, yielding 46.5 g of crude extract. This extract was poured into a MeOH:H2O solution (1:3) and subjected to liquid-liquid extraction using dichloromethane, ethyl acetate and n-butanol (increasing order of polarity).

The fraction in n-butanol (1.7 g) was purified using column chromatography (CC) with silica gel as stationary phase and CH2Cl2 and CH2Cl2:MeOH solutions (up to 20 %) as mobile phases, obtaining 12 new fractions. Fraction 5 (200 mg) was purified by CC, using Sephadex LH-20 as stationary phase and MeOH as mobile phase, obtaining 32 mg of a brown solid, which was chosen to be the first to be tested on microorganisms.

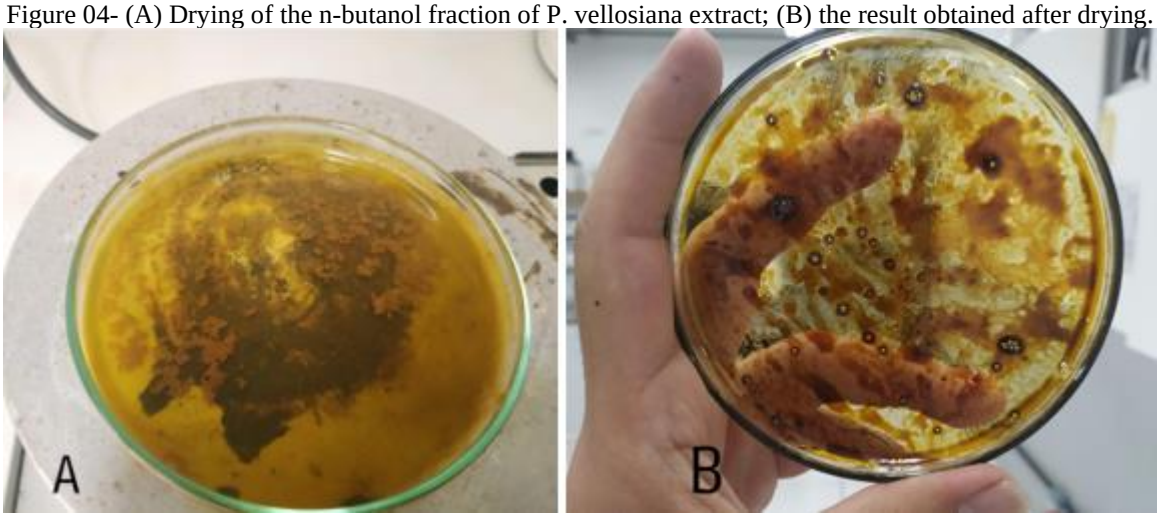
After the tests, this solid was submitted to Nuclear Magnetic Resonance (NMR) analysis of1 H and13 C and mass spectrometry for further identification of the alkaloid present in it.

2.4 DRYING

For the solubilization of the alkaloids it was necessary to remove the organic solvent by drying methods. The fractions in ethyl acetate were placed in 600mL beakers in the oven at 40°C for approximately 250 hours, observing daily the volume of solvent in the liquid phase until the beaker was dry.

The fractions in n-butanol were placed in Petri dishes inside the fume hood, over a heating plate with temperature ranging from 40°C to 120°C. Since heating can affect the active ingredient of the metabolite, the temperatures cannot be too high, however, due to the low volatility of butanol, the same had to go through a more intense heating with the help of the exhaust fan to remove the gas vapors formed.

Figure 04- (A) Drying of the n-butanol fraction of P. vellosiana extract; (B) the result obtained after drying.



Source: Author (2020)

2.5 SOLUBILIZATION OF SAMPLES

Based on tests published in the literature and since for the test on microorganisms the samples should be in liquid form, they were solubilized in pure H2O.

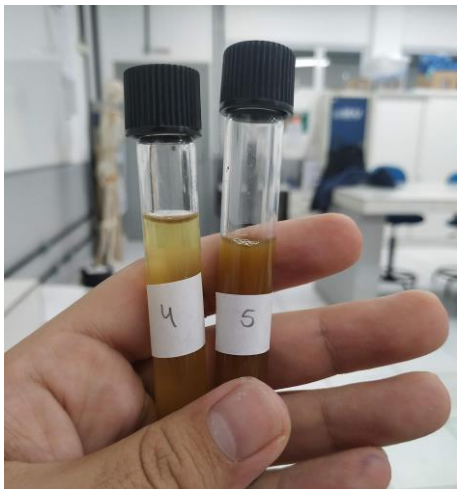
The concentrations of the samples are shown in Table 5.

Table 05- Concentration of the solutions

Extract	Solution volume (mL)	Concentration (g/mL)
1	10mL	0.0002g/mL
2	12 mL	0.0100 g/mL
3	10 mL	0.02066 g/mL
4 (n-butanol 1)	6 mL	0.01595 g/mL
4 (n-butanol 2)	6 mL	0.017166 g/mL
5 (n-butanol 1)	18.5 mL	0.0160 g/mL
5 (n-butanol 2)	6 mL	0.0100 g/mL

*The fractions in butanol were separated in two test tubes with different concentrations.
Source: Author (2020)

Figure 05- Fraction of extracts 4(Butanol 2) and 5(Butanol 2) solubilized in pure H2O.



Source: Author (2020)

2.6 EVALUATION OF ANTIMICROBIAL ACTIVITY

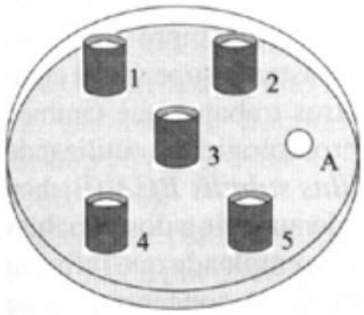
For the microorganism test, it is necessary to pass the previously presented solutions through a nanometric polar filter attached to a syringe, in order to remove any traces of impurities, both organic and inorganic, incorporated during the extractions.

To evaluate the antimicrobial activity of the alkaloid against microorganisms

E. coli, S. epidermidis ATCC35984, and P. aeruginosa PA14, the agar diffusion technique was performed. The bacterial strains were previously cultured for 24 h at 37 ± 1 ºC for subsequent preparation of bacterial inocula that were adjusted to an optical density of 0.150 at 600nm (equivalent to 3 × 108 CFU/mL). The pathogenic bacteria were then inoculated uniformly onto Mueller Hinton agar plates, covering the entire surface. On the agar, 3 autoclaved metal cylinders 11 mm in diameter were arranged in which 200µL of aqueous solution of the alkaloid of concentration 200 µg/mL [13] was added. The plates were then incubated at 37 ± 1 °C for 24 hours and the antimicrobial activity was determined from the measurement of the growth inhibition halo [14]. The assays were performed in duplicate and the antimicrobials Rifampicin (8 µg/mL, Sigma-Aldrich, USA) and Gentamicin (8 µg/mL,

Sigma-Aldrich, USA) were used as positive controls for Gram positive and Gram negative bacteria, respectively.

Figure 06- Illustrative image of the agar diffusion technique, the numbers representing the metal cylinders. In the adopted diffusion technique only three cylinders were used.



Source: MARTÍN, (1996).

3 RESULTS ACHIEVED

After the period of action of the fraction with alkaloid on the microorganisms, the characteristics of the culture medium were observed and then the results were tabulated in Table 6, so that it can be observed that the bacteria with a sign (+) remained alive with the action time of 24 hours after contact with the alkaloid and those tabulated with the sign (-) were killed during the same period.

Table 06: Reaction of bacteria in contact with the alkaloid 5-CarboxyStrictosidine isolated from P. nuda I.

Extract	**Concentration**	**Action on bacteria**			
-	-	*E. coli*	*S. epidermidis*	*P. aeruginosa 14*	*P. aeruginosa 27*
1	348 µmol.L^{-1}	+	+	+	+

Source: Author (2020)

After the incubation time, the plates were analyzed and it was observed that the tested substance (aqueous solution, 200 µg.mL-1), in all cases, did not promote the formation of a zone of inhibition, when compared to the substances used as a positive control.

3.1 CHARACTERIZATION OF THE TEST SAMPLE

Based on the MS and NMR analyses (1 H and13 C) of the samples, carried out by UENF, a reading of the results obtained by the tests was made and then compared with the literature in order to justify the incidence of the alkaloid 5-CarboxyStrictosidine in the extract used.

Table 07- Alkaloid found by MS and NMR readings of 1H and C.13

Compounds	**Popular name**	**Molecular Formula**
1	5-Carboxy-Strictosidine	C28H33N2O11

Source: Author (2020)

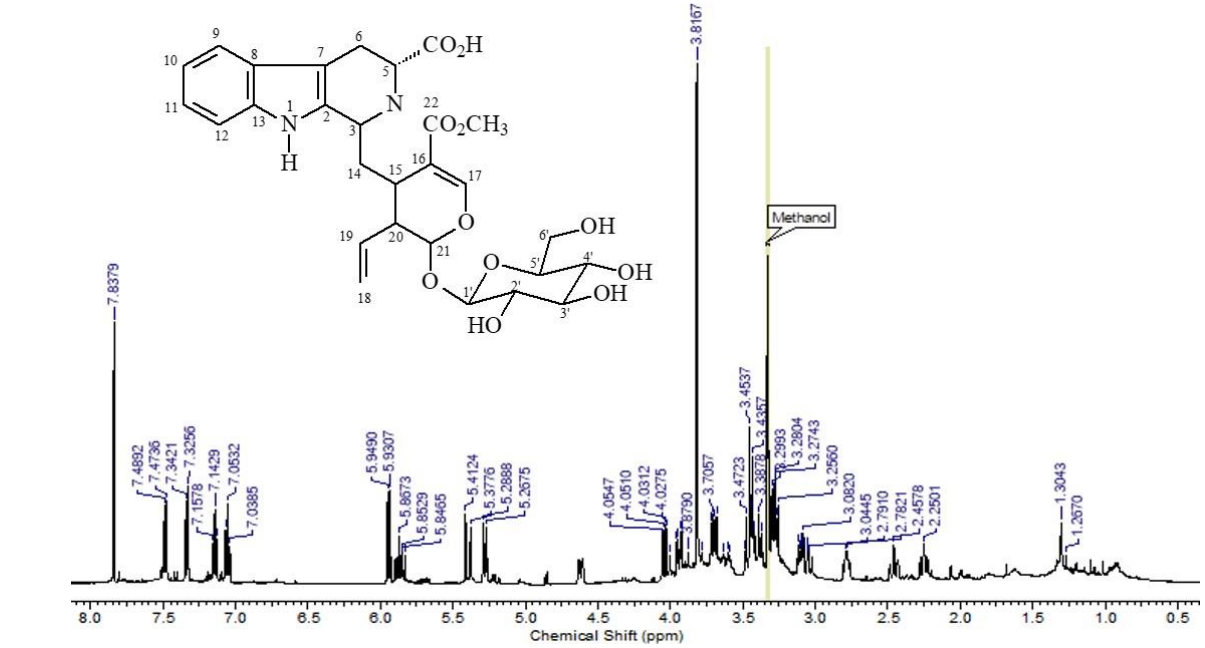
The identification of the tested alkaloid was performed by analysis of the NMR (1 H and13 C, Table 8) and high-resolution mass spectra. In the NMR spectrum of1 H (Figure 6) it is possible to observe characteristic signals of monoterpene indolic alkaloid. The signals of the ortho-substituted aromatic system were observed at dH 7.48 (H-9), 7.05 (H-10), 7.14 (H-11) and 7.33 (H-12). The signal at dH 4.62 was assigned to H-3, neighboring sp carbon2 and nitrogen. The signal integral value of H-5 (1H, 3.94 ppm) suggests substitution at position 5, due to the presence of the caboxyl group. The signal at dH 5.94 (similar to methylenedioxy) and the characteristic sugar signals (spectrum region between 3.24 and 4.85 ppm, H-1' to H-6') confirm the portion originating from secologanine. The NMR data of13 C (Figure 8) corroborated the structural proposal, with highlight to the signal of C-6 at dC 22.7, higher than expected due to the b-deprotection effect caused by the carboxyl group at C-5. These data were compatible with literature data [15].

Table 08- NMR data of1 H and13 C of the sample and comparison with the literature

	5-Carboxi-estrictosidina		Literatura*
C	**δ_C**	**δ_H**	**δ_C**
2	129.0	-	132.2
7	106.9	-	109.0
8	126.1	-	128.0
13	137.1	-	138.4
16	107.2	-	109.9
22	170.4	-	170.9
23	172.2	-	176.5
CH			
3	51.7	4.62 (*d*, 11.7)	53.2
5	58.3	3.94 (*dd*, 12.0, 5.0)	60.1
9	117.7	7.48 (*d*, 7.0)	118.8
10	119.2	7.05 (*t*, 7.0)	120.1
11	122.0	7.14 (*t*, 7.0)	122.6
12	110.9	7.33 (*d*, 7.0)	112.1
15	31.6	3.11 (*m*)	32.4
17	155.8	7.84 (*s*)	156.1
19	133.8	5.87 (*ddd*, 17.4, 10.7, 7.5)	135.2
20	43.8	2.79 (*m*)	45.7
21	96.0	5.94 (*d*, 9.2)	97.6
CH_2			
6	22.7	3.45 (*m*), 3.05 (*m*)	25.2
14	33.3	2.46 (*m*), 2.25 (*m*)	35.6
18	118.4	5.39 (*d*, 17.7); 5.27 (d, 10.7)	119.6
CH_3			
Me-O-22	51.4	3.85 (*s*)	
Glicose			
1'	99.0	4.85 (*d*, 8.0)	100.5
2'	73.2	3.27 (*dd*, 9.1, 8.0)	74.7
3'	77.4	3.43 (*t*, 9.1)	78.0
4'	70.4	3.28 (*t*, 9.1)	71.9
5'	76.5	3.38 (*m*)	78.6
6'	61.7	4.04 (*dd*, 11.8, 1.8); 3.70 (*dd*, 11.8, 7.0)	63.1

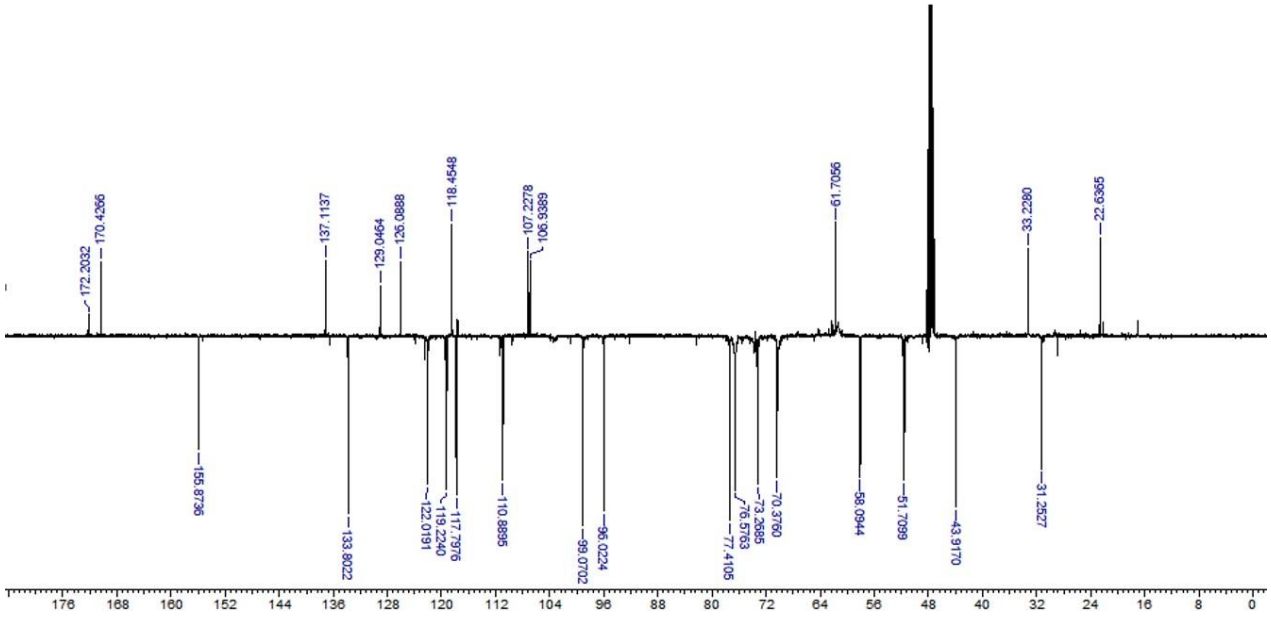
Source: Author (2020)

Figure 07- NMR spectrum1 H of the sample (500 MHz, CD3 OD) and structure of the 5-carboxy-strctosidine alkaloid



Source: Author (2020)

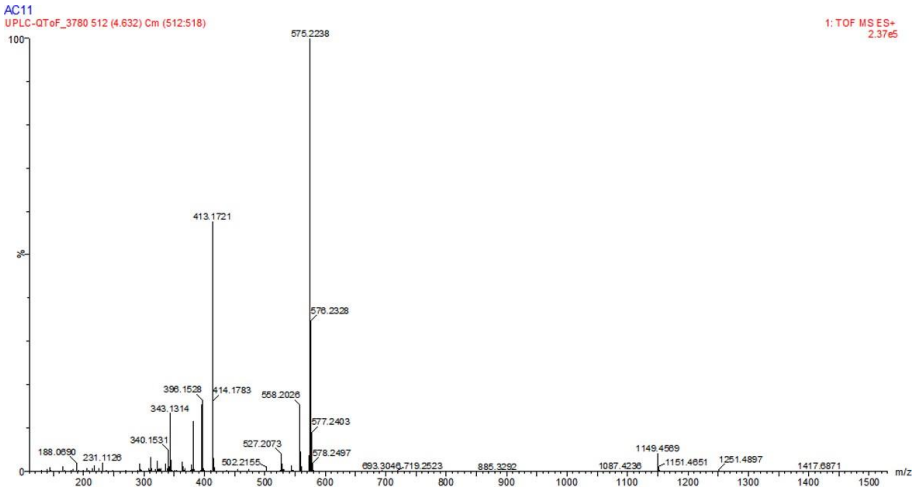
Figure 08- NMR spectrum13 C of the sample (125 MHz, CD3 OD)



Source: Author (2020)

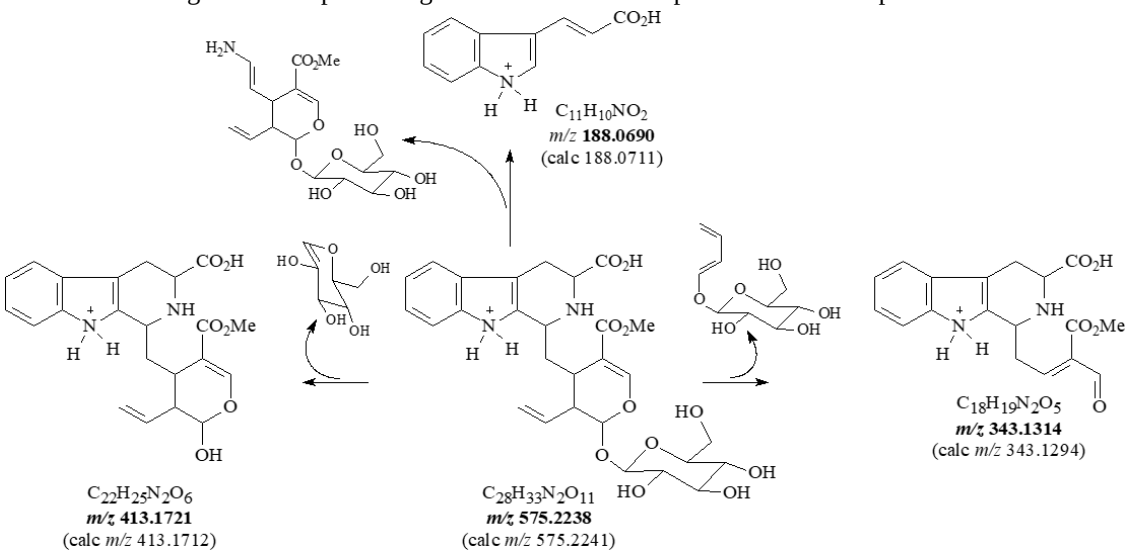
In the mass spectrum of the sample (Figure 9) it was possible to observe the peak of the protonated molecule [M + H]+ at m/z 575.2238 (error = -0.5 ppm), compatible with the NMR data. The signal at m/z 413.1721 ([M + H]+ - 162) confirms the glycosidic portion of the molecule. The formation of the fragment at m/z 343.1314 is characteristic of Retro Diels-Alder reaction (RDA). The fragment with m/z 188.0690, whose formation involves 1,3 hydrogen rearrangements, evidences the presence of the carboxyl at C-5 due to the difference from the typical fragment of an indolic system (m/z = 144). The proposed fragmentations of the mentioned signals are shown in Fig. 10.

Figure 09- High resolution mass spectrum of the sample (positive mode)



Source: Author (2020)

Figure 10- Proposed fragmentation of the main peaks of the mass spectrum



Source: Author (2020)

4 COMPLEMENTARY INFORMATION

The data obtained during the research were presented and published in the 9th Scientific and Technological Integration Symposium of Southern Santa Catarina - SICT-Sul.

5 CONCLUSIONS

With the results previously attested, we were able, by means of MS and NMR readings (1 H and13 C) to isolate and unequivocally identify the alkaloid 5-CarboxyStrictosidine, extracted from P. nuda, but it was not successful in achieving the general objective of having antimicrobial activity.

The separation and purification processes of the other extracts were successful, and future tests will define whether the alkaloids present in them have antimicrobial activity.

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