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Assessment of environmental condition and drying process of the plants on the concentration of alkaloids and cytotoxicity of traditional Ayahuasca Tea

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Abstract

Introduction: Ayahuasca is a traditional psychoactive tea of Amazonian indigenous, used medicinal and spiritual purposes. Wide variation in the concentration of *N,N*-dimethyltryptamine (DMT), Harmaline (HRL), Harmine (HRM) and Tetrahydroharmine (THH) alkaloids in Ayahuasca has been reported worldwide.

Objective: To evaluate the causes of variations in alkaloids concentrations of Ayahuasca prepared with fresh and dehydrated plants from different environments and determine the best drying method to plants according to alkaloids content and cytotoxicity of Ayahuasca tea.

Material and methods: The environment interference on the alkaloids of the two species was evaluated in samples of Ayahuasca tea prepared with fresh plants. The most suitable drying process to the two species was evaluated in sample Ayahuasca tea prepared with plants submitted to drying under the sun conditions and five different temperatures in forced circulation oven. The concentration of the alkaloids determined by high performance liquid chromatography with UV-vis detector with diode array detection (HPLC-DAD). The *in vitro* cytotoxicity of Ayahuasca was evaluated in human keratinocytes cells (HaCaT) by colorimetric assay.

Results: Environmental characteristics, preparation process and temperature of plants drying interfered on DMT, HRL, HRM and THH concentrations of Ayahuasca. No effect cytotoxicity was detected with relationship to psychoactive alkaloids in samples of Ayahuasca tea prepared with fresh or dried plants.

Conclusion: Concentration of DMT, HRL, HRM and THH alkaloids in Ayahuasca are influenced by plants environmental. The most suitable drying process was obtained in forced circulation oven at 43 and 45°C to *P. viridis* leaves and *B. caapi* stems respectively. The Ayahuasca prepared with fresh or dry plants no showed cytotoxicity in human keratinocytes cells.

Keywords: Traditional Ayahuasca; Alkaloids; Dry Plants; *Psychotria; Banisteriopsis*; Cytotoxicity.

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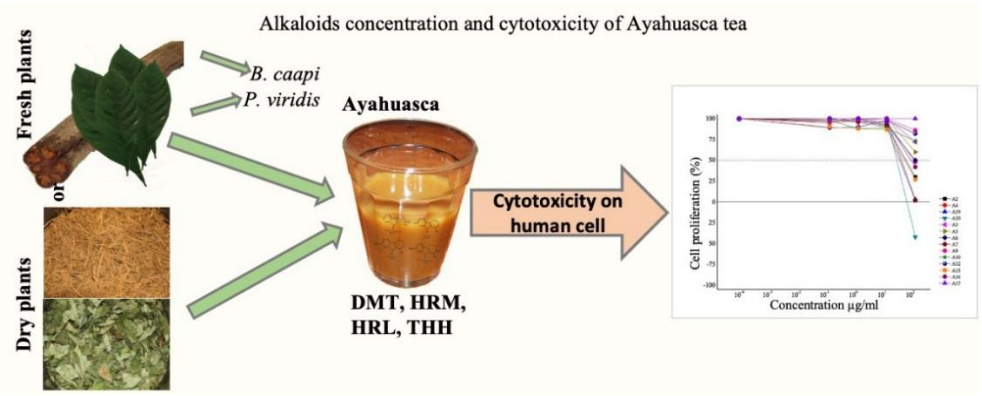
Graphical abstract



1. Introduction

Ayahuasca tea is a decoction prepared with leaves of *P. viridis* shrub popularly known as "rainha", "chacrona" or "chacruna" and stems of *B. caapi* commonly known as "mariri", "Ayahuasca", "caapi", "jagube" [1–3]. The decoction of the plants together is the way of preparing traditional Ayahuasca. This tea has been used in traditional indigenous medicine of the Amazonian peoples for healing and also in spiritual rituals since pre-Columbian times [4–6]. In several countries, such as Colombia, Peru and Brazil it is the fundamental element of indigenous cultures [7, 8]. The preparation is highlighted for the psychoactive potential triggered by the synergism of the compounds *N,N*-dimethyltryptamine alkaloids (DMT) present in *Psychotria viridis* leaves together with the *ß*-carbolinic group compounds: harmine (HRM), harmaline (HRL), and tetrahydroharmine (THH) (Figure. 1) found in the stems of *Banisteriopsis caapi* [9], and are considered by tea consumers as a result of consciousness expansion.

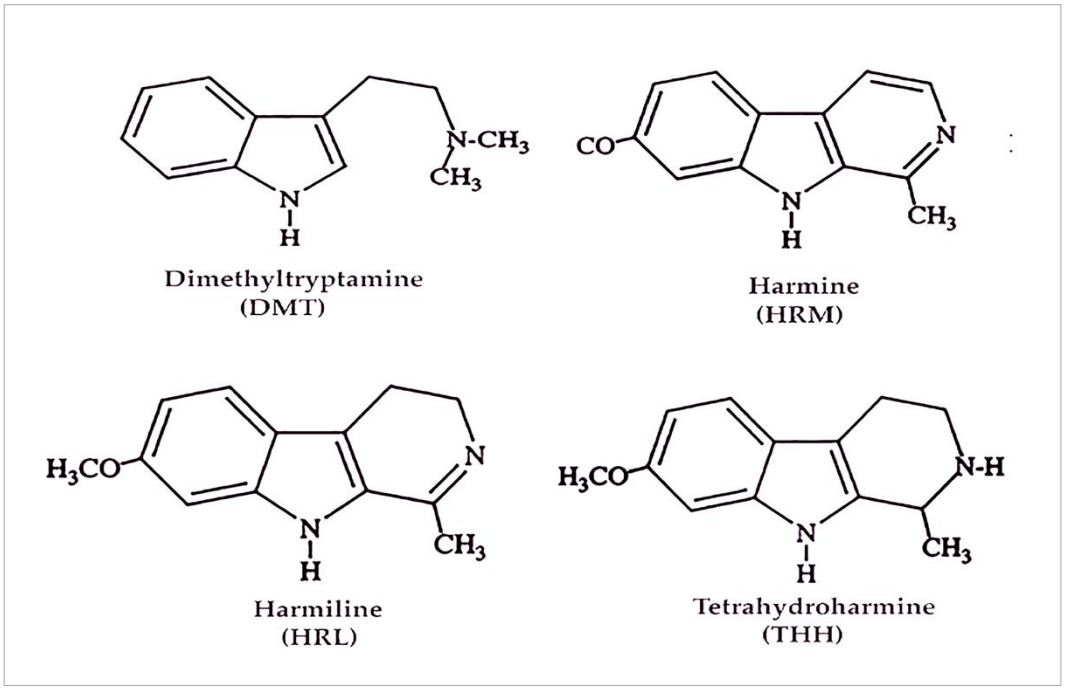


Figure 1 Chemical structures of the psychoactive alkaloids of Ayahuasca tea. *N,N*-dimethyltryptamine (DMT), and *ß*-carbolines harmine (HRM), harmaline (HRL) and tetrahydroharmine (THH).

The incorporation of this beverage for healing purposes and religious support in urban contemporary society emerged from indigenous and *caboclas* populations of tropical forests [10]. Studies of the bioactive compounds of Ayahuasca tea [1, 11–13] and of the plants that are used for the preparation [14, 15] were prompted by increased religious and therapeutic use of Ayahuasca in the United States and several other countries in Europe, but also by the worldwide trend for psychedelic therapy research. The entheogenic effect of Ayahuasca was attributed to a direct

relationship with DMT content, that when taken orally is inactivated by visceral monoamine oxidase (MAO). This effect is reversed by the action of *ß*-carbolines, which are highly active reversible MAO inhibitors, protecting DMT from deamination by MAO, enabling the beverage's oral use [5].

Concentration variations of DMT, HRM, HRL and THH alkaloids were found in Ayahuasca samples consumed in European countries, and in some cases, synthetic product were also detected [16]. Ayahuasca tea consumed in these countries, is known to be generally produced in Amazonian countries and shipped abroad. Silveira [17] quantified the alkaloids in Ayahuasca samples stored throughout seven days at high temperature, mimicking the mail transportation conditions, not finding any significant decrease of DMT, whereas meaningful variations of *ß*-carbolines alkaloids were detected, with detection of THH decrease of up to 67.9%. Other studies have also detected concentrations variations of these alkaloids in Ayahuasca samples of the same source [11]. In samples prepared by traditional ritualistic procedures, which consists in the decoction of *P. viridis* leaves together with the stems of *B. caapi*, alkaloid variation reached a tenfold magnitude [13]. For other hand, great variability of *B. caapi,* with at least 30 different varieties described and cataloged, which have a direct relationship with different levels of alkaloids found in Ayahuasca preparations, aside from various plant proportion used by the different groups [5, 18] was also reported.

Regarding origin, plant-environment relationship interferes with the secondary metabolism process, acting as a driving force for the biosynthesis of phytochemicals in plants [19, 20]. This approach was reported by Miranda et al. [15] for *P. viridis*, the species that contributes with DMT, but in this study the authors did not report the alkaloid content in the tea. Another factor that influences secondary plant metabolism is the genetic diversity within the species, well described for the species used in the Ayahuasca beverage and for other medicinal species [18, 21]. To ensure a safe use of Ayahuasca in countries outside the geographical area of naturally occurring plants (Europe and North America), a strategy would be to provide inputs for the preparation of Ayahuasca *in loco* at these destinations. To achieve a quality product that provides the expected effect an alternative is the plant dehydration inside parameters that maintain phytochemical quality ensured. The drying process generates a stable product that could be easily shipped, with a reduced volume compared to the fresh plant, furthermore, the plant would be available during the whole year long [22]. Despite being considered one of the most usual and fundamental processes for preserving medicinal plants, drying can exacerbate loss of bioactive compounds depending on the temperature applied [23].

The interference of both the environment and drying condition in the concentrations of bioactive compounds from medicinal plants is well documented [24, 25]. Despite the increased consumption of Ayahuasca in various extra-Amazonian countries, as well as their described pharmacological and therapeutic potential, yet data establishing the contribution of the environmental effect on the variation of alkaloid content in *B. caapi and P. viridis* are still to be determined. Nevertheless, there no studies that access these plants drying temperature parameters.

The literature data together with the breakthrough use of this tea points out the need to establish quality control parameters for the use of Ayahuasca beverages. The first point refers to the alkaloids variations in the tea found worldwide, with another issue referring to strategies to ensure that no adulteration has been introduced to Ayahuasca beverages consumed in other countries outside the Amazon regions. Ensuring the effect of Ayahuasca traditionally used by amazon peoples.

To address the causes of variation in alkaloids content in Ayahuasca tea, and determine the most suitable drying process to plants used in the preparation this tea, herein we evaluated of the concentration of DMT, HRM, HRL and THH (Figure.1) from plants collected in two different ecosystems in the Amazon region, and we test the influence of different temperatures and dry method on the content of these alkaloids in the final product. Furthermore, the cytotoxicity of Ayahuasca tea prepared with fresh and dried plants was tested in human keratinocyte cells.

2. Material and methods

2.1. Collection areas and plant material

Wild *B. caapi,* and cultivated *P. viridis* samples from two distinct ecosystems were collected in the Brazilian Amazon, located 360 km apart from each other. One of the locations was *Campinarana* (CAMP), stationed south of Roraima State (00°57'03" N and 59°54'39" O) and the other was *Terra Firme* (TF), located in the North of the State of Amazonas (02°02'04" S; 60°01' O). Both sites share the same broad climate type, considered as humid equatorial *Af*, according to Koppen [26], without clear stabilization seasons and marked by annual rain seasonally [27]. Each ecosystem in this study was characterized according to data previously described in the literature [15, 28, 29] and by field observations.

For all analyzes, the plant material was collected from individuals randomly chosen within each area, five individuals of wild *B. caapi* and 20 *P. viridis* trees cultivated in an agroforest, between 6 and 10 am in January/2016, August/2016, November/2017 and February/2019 were collected.

Species names were verified in http://www.theplantlist.org and the material identified under Voucher INPA Nº. 282931, 282932, 282933, 282934, 282935, deposited in the Herbarium of National Institute of Amazonian Research.

2.2. Pre-processing of plants and preparation of Ayahuasca

After each collection, the leaves and stems samples were washed in running water. Thereafter, *B. caapi* stems were pre-processed by smashing the shoots with wooden sticks for the separation of fibers. During pre-processing of the plant material collected in Janeiro/2016, August/2016 and collection half in November/2017, the leaves and stems were weighed separately, and then divided the samples of each species into two batches of equal weights. The first batch was used to prepare Ayahuasca with fresh plants, whereas the second batch was subjected to drying at temperatures of 60 ℃, 50℃ and 40℃ before tea preparation, and resulting tea was analyzed to determine the DMT, HRM, HRL, and THH alkaloid content.

The other samples that were gathered in November/2017 (collection half) and February/2019 being pre-processed as previously described, however *B. caapi* stems and *P. viridis* leaves were subjected to different drying conditions according to the material of each species used in Ayahuasca preparation: a) stems at 45 ℃ and leaves 40 ℃; (b) stems and leaves in the sun and; (c) stems at 45 ℃ and leaves at 43℃. After drying, this material was used to prepare Ayahuasca beverage and determine alkaloid content.

For drying purposes, a forced circulation oven with air renewal and controlled temperature (TE 394/3 Tecnal) was used. A mesh trays was used for drying plant material in the sun. Drying was monitored by weighing samples using a precision weighing-scale at 12 h intervals and then reduced to 6 h when the material reached 50% of the initial weight, maintaining this interval until constant weight. After complete drying, the plant materials were packed in plastic bags, protected from light and stored in boxes until the time of use.

The maximum moisture content of the plants was measured according to Silveira [30] The *B. caapi* stems and *P. viridis* leaves samples were submerged in water for saturation until reaching constant weight, considering this the maximum fresh weight (FW). To determine dry weight (DW), the samples were dried in forced circulation oven with air renewal (TE 394/3 Tecnal) at 90°C for stems and 70°C for leaves. Weighing was carried out at a 24h interval, being considered dry weight when the difference between two consecutive weightings was less than 1%. Maximum moisture content (MC) was calculated as MC (%) = (FW - DW)/FW x 100.

Ayahuasca, either with fresh or dehydrated plants, was prepared following the ritual adopted by the *Centro Espírita Beneficente União do Vegetal* (CBUDV) with define proportions of *P. viridis* leaves and *B. caapi* stems and water volume, whereas fire pressure and cooking time remained constant in all preparations for analysis. The average yield was 5-7 L of tea for each batch. Ayahuasca was then stored in a plastic container while still warm (about 60°C), without air inside the bottle to minimize the process of fermentation. Thereafter the samples were left to cool at room temperature and subsequently stored at 5°C.

2.3. High Performance Liquid Chromatography Analysis with UV-vis Detector with Diodes Arrangement (HPLC-DAD)

Sample preparation

The liquid samples of Ayahuasca were concentrated under vacuum (FISATON®) until attaining approximately 10% of the initial volume. They were then stored in appropriate containers and frozen at -80°C and further freeze-dried at 4 atm pressure for 72 hours. The samples were stored, protected from light, at 5°C. Aliquots (10±1 mg) of each Ayahuasca sample were weighed, transferred into a volumetric flask (10mL) and dissolved in HPLC grade methanol under vigorous stirring (vortex) using ultrasound (10 min), adjusting the final volume to 10 mL (1 mg/mL stock solution). At the time of analysis, an aliquot (200 µL) of each stock solution was mixed with HPLC grade methanol (800 µL) in a 2 mL conical polypropylene tube under vortexing (1,400 rpm, 10 min) and centrifuged (12,000 rpm, 5 min). Thereafter an aliquot (200 µl) from each supernatant was transferred to a glass auto sampler bottle containing 800 µL of methanol and homogenized by vortex for further analysis.

2.4. Preparation of Standard Solutions

The harmine (HRM) and harmaline (HRL) standards were purchased from Sigma-Aldrich (Steinheim, Germany), and Tetrahydroharmine (THH) from Cayman Chemical, whereas *N, N* -dimethyltryptamine (DMT) was synthesized by the tryptamine dimethylation method [11, 31]. For each analyte a calibration curve containing five points was plotted, ranging from 100 to 1000 µg/mL, obtained through linear regression (the area under the curve versus concentration), considering $R^2 \geq 0.98$.

2.5. Analysis conditions by High Performance Liquid Chromatography Analysis with UV-vis with Diode Array Detection (HPLC-DAD)

Lanaro [32] described the method used. The experiment was carried out using a HPLC Prominence System (Shimadzu, Kyoto, Japan). An Atlantis T3 column (150 x 3.0mm, 3µm) equipped with an Atlantis T3 protection column (30 x10 mm, 5 µm) (Waters Corporation, Milford, MA, USA) maintained at 35°C. The mobile phase was a solution of phosphoric acid in ultrapure water (10 mmol/L, pH adjusted to 3.0 with triethylamine, A) and HPLC grade acetonitrile (B). Gradient elution, with a flow rate of 1 mL/min, started with 40% A and 60% B maintained for 1 min and then a gradual change to 5% A and 95% B over the next 13 min., with this final proportion being maintained for 5 min. The column was rebalanced to 60% B over 0.5 min and maintained at that concentrations for 3 min (total gradient time = 21 min). The temperature of the auto sampler was not controlled, and the injection volume was 10 µL. The diode array detector at 35 ° C was adjusted to acquire spectra from 195 to 600 nm. For quantification, the chromatograms were obtained at 279 nm (DMT), 291 nm (tetrahydroharmine), 320 nm (harmine) and 375 nm (harmaline).

2.6. *in-vitro* cytotoxicity assessment

Cell lineage

For assessing the anti-proliferative activity, a human non-tumor lineage (HaCaT, immortal keratinocytes) was used, which was provided by the Faculty of Dentistry of the University of Campinas, São Paulo, Brazil. The cell line was grown in T75 flasks (Corning) in complete medium (RPMI-1640, supplemented with 5% fetal bovine serum and 1% penicillin /streptomycin) at 37 °C under humid atmosphere supplemented with 5% CO_2. These conditions were used both for the maintenance of the cells and for the experiments. The cells were used between passages 5 and 12.

Assessment of cytotoxicity

Briefly, the HaCaT cells were seeded in 96-well plates (T1 plates, between 3 to 6 × 104 cells/mL, 100 µL/well), incubated for 24 h, treated with Ayahuasca sample (0.15, 1.5, 15 and 150 µg/mL) and doxorubicin (0.015, 0.15, 1.5 and 15 µg/mL, 100 µL/mL in triplicate), and then incubated for 48 h at 37 °C in 5% CO_2. A second plate, denominated T0, was prepared to infer the absorbance value of untreated cells at the moment of sample addition. Untreated (T0 plate) and treated (T1 plates) cells were fixed with 50% trichloroacetic acid and stained with sulforhodamine dye (0.4% in acetic acid 1%). Absorbance was recorded at 540 nm using a microplate reader (Molecular Devices®, VersaMax model). Using the absorbance values, the cell growth (%), at each sample concentration, was calculated considering at 100% of cell growth the difference between the absorbances of treated cells after 48 h incubation (T1) and at the sample addition moment (T0). The curve cell growth vs. sample concentration was plotted and GI_{50} (concentration required for 50% growth inhibition) was calculated by sigmoidal regression using the Origin 8.0 software (Origin Lab Corporation, Northampton, MA, USA). These analyzes were performed at the Laboratory of Phytochemistry, Pharmacology and Experimental Toxicology (LAFTEX) at the Faculty of Pharmaceutical Sciences at UNICAMP.

3. Results and discussion

This work allowed to verify the influence of the environment and the drying process of *Banisteriopsis caapi* stems and *Psychotria viridis* leaves in the concentration of four psychotropic alkaloids present in Ayahuasca tea, prepared according to traditional ritual. Considering the great interest in the use of Ayahuasca for religious purposes and for treating various neurological and psychiatric conditions, studies of these variables provide important contributions to establish the necessary parameters to ensure reproducibility of the psychotropic and pharmacological effects [4, 15, 33–37].

The standardizing all Ayahuasca tea preparations used in this study regarding the proportion of the plants, initial water volume, fire intensity, final yield and the samples storage at -5°C before the freeze-drying process allowed to minimize the variability in the results of content of alkaloids caused by tea preparation, already reported by Mckenna [5] and in stability studies of samples monitored by Liquid Chromatography coupled to Mass Spectrometry (LC-ESI-MS/MS) showed that DMT and HRM alkaloids were stable over a period of 12 months when stored under refrigerated conditions,

and the content of THH significantly dropped within a four-month interval, with different profiles among the evaluated samples [17].

During the study, the TF ecosystem exhibited an average annual rainfall of 2.585 mm and soil water retention capacity greater than the CAMP ecosystem who presented an average annual rainfall rate of 1.847 mm, which is approximately 30% less than TF [38]. CAMP had still a longer summer period, which is common in the region and has already been confirmed in other studies [15, 39], the soil is poor in minerals and there is high leaching caused by the water intensity of rainfall [30, 40]. Considering the plant-environment relationship interferes with secondary metabolites produced by plants [19, 20, 24, 41, 42], these factors together make the plants from CAMP ecosystem to suffer a period of water stress, which does not occur with the TF plants.

The evaluate of variation of the gross and relative levels of DMT, HRM, HRL and THH in Ayahuasca tea prepared with plants collected in Campinarana (CAMP) and Terra-firme (TF) evidence that parameters such as the place and time of collection, as well as the effect of the drying process of the plants for this study cause different results on the concentration of these four alkaloids in the Ayahuasca tea. Estrella-Parra et al. [4] showed that concentration variation has effect on the synergic activity among of these four alkaloids and may affects psychotropic effect of Ayahuasca tea.

Considering the content of alkaloids in Ayahuasca samples prepared with fresh plants collected in the winter (Table 1, samples A3 and A4), the concentration of DMT, HRL and THH were 54.22, 50.09 and 42.09%, respectively; being higher in TF plants, whereas HRM concentration was similar for both ecosystems. However, when the plants were collected during the summer (Table 1, Samples A2 and A13), an increase in DMT concentration (44.34%); decrease in the concentrations of HRL and HRM (46.83 and 48.04%, respectively) with maintenance of THH concentration when comparing TF and CAMP was observed. Furthermore, when comparing two different years of harvesting in the same season (summer) (Table 1, samples A5 and A13 from CAMP and A2 and A7 from TF), samples A13 and A5 showed an increase of 49.6% in DMT content and 5.6% for THH, with a decrease in HRL levels of 19.68% and for HRM of 20.58% compared with samples A2 and A7 of TF. Whereas a 24% decrease in DMT and 11.84% THH in samples A2 and A7 with a concomitant increase to 35.47% and 14.93% for HRL and HRM content was detected, showing how climatic inter-annual variations in water availability in the soil directly influence the content of alkaloids (Table 2). The significant decrease in soil water availability from January to November in the CAMP ecosystem was reflected in the samples with a decrease of 24% in DMT and 14.93% in HRM, whereas for TF samples, where the soil water availability variation was positive, an increase of 49.6% was observed for DMT and 5.6% for THH.

Previous analysis of several Ayahuasca samples obtained during five years from different religious groups revealed wide variation in the alkaloid profile [43]. Moreover, Ayahuasca plants showed marked alkaloid concentration variations when collected in different seasons at the same site [32]. These studies based their results on the different forms of preparation, the sources and the quantity/proportion of the plants used in Ayahuasca preparation. Variations found in this study are smaller than those found by Callaway [44] and Lanaro [32] (Table 3). Considering that herein standardized samples were used from the plant collection site to the preparation of Ayahuasca tea from a single religious source, the alkaloids variation levels found in this study are directly related to the season of collection and to the characteristics of the environment where the plants were obtained. These results show that to standardizing the proportions of plants used in the preparation of Ayahuasca, in an attempt to increase consumption safety and control the concentration of entheogenic compounds must necessarily consider the place and time of harvest [24].

The concentrations of HRM, HRL, and THH alkaloids detected showed that the conversion of HRM into HRL and from this to THH during decoction, as suggested by Callaway [44], in our samples occurred in a lower scale as detected for HRM and HRL content in the tea samples evaluated (Table 1). Less influence of decoction process on the variation in the content of alkaloids, reinforces the importance of standardizing the proportion/quantity of plants used in the preparation of Ayahuasca to avoid exacerbated variations in the tea alkaloid concentrations.

Regarding the ratios of Ayahuasca alkaloids prepared with fresh plants collected in the winter DMT/HRL, DMT/HRM, and DMT/THH were higher in plants from TF ecosystem, being 1.39, 0.35, and 0.21 respectively. Likewise, in the summer period, the highest ratios were found for the samples prepared with plants from the TF ecosystem, being 2.66 for DMT/HRL, 0.46 for DMT/HRM, and 0.28 for DMT/THH, when compared with the samples prepared with plants from the CAMP (Table 4).

Table 1 Concentration of alkaloids (mg/L) and cytotoxic effect (GI_{50} µg/ml) observed for Ayahuasca prepared with fresh plants and after drying.

Collect				Drying		Alkaloids (mg/L)				
Month/Year	**Site**	**Season**	**Sample**	**No**	**Yes**	**DMT**	**HRL**	**HRM**	**THH**	**GI_{50}**
Aug/16	TF	S	A2	x		73.74	27.72	161.34	263.94	80.5 ± 5.1
			A14		60/60	37.3	15.7	181.9	79.4	n.t.
	Camp	W	A3	x		40.74	31.8	250.8	242.94	>150
			A12		60/60	31.62	14.52	110.76	100.62	>150
Jan/16	TF	W	A4	x		88.86	63.72	256.5	419.58	141.6 ± 13.9
			A10		50/50	39.72	30.66	198.0	230.88	150
	Camp	S	A13	x		41.04	52.14	310.56	274.2	n.t.
			A6		50/50	40.44	23.34	129.48	134.52	150
Nov/17	TF	S	A7	x		55.98	42.96	189.66	232.68	34.5 ± 7.3
			A8		40/40	65.1	51.54	240.12	256.32	n.t.
			A18		50/50	33.36	4.5	116.28	29.76	n.t.
			A11		40/45	52.0	33.4	238.8	206.6	n.t.
			A16		Sun	37.08	5.46	140.58	40.5	119.5 ± 0.5
	Camp	S	A5	x		81.42	41.88	246.3	290.58	>150
			A9		40/40	62.94	11.28	173.76	97.08	>150
			A17		50/50	55.5	19.2	149.28	96.54	>150
			A15		Sun	72.18	29.52	232.8	103.5	65.7 ± 25.0
Feb/19	TF	W	A19		43/45	108.36	39.78	165.3	127.8	111.9 ± 0.3
	Camp	S	A20		43/45	98.94	29.76	119.1	111.42	25*

TF = Terra Firme; Camp = Campinarana; Season: S = Summer, W = Winter; Samples: Ayahuasca prepared with fresh plants (A2-TF; A3-CAMP; A4-TF, A13-CAMP; A7-TF and A-5 CAMP); Drying : 60/60, 50/50, 40/40, 40/45, 43/45 = temperature (°C) of drying *P. viridis* leaves and *B. caapi* stems, respectively; Sun = leaves and stems dried in the sun; GI_{50} (µg/ml) = concentration of lyophilized tea necessary to inhibit the proliferation of HaCaT lineage cells by 50%; * approximate value depending on experimental values; nt= not test. Alkaloids concentration determined by HPLC-DAD.

Table 2 Climatic variations occurred between the summer of 2016 and 2017 in the CAM P and TF ecosystems

Ecosystem	Date	T (^{0}C)	Rainfall (mm)	Air humidity (%)	Water in the soil (%)
TF	Aug/16	28.07	4.72	72.92	62.94
TF	Nov/17	28.50	3.92	73.27	82.84
CAMP	Jan/16	28.41	0.99	63.08	52.40
CAMP	Nov/17	30.56	0.17	57.10	6.57

TF: Terra firme; CAMP: Campinarana; T: temperature. Given values are monthly averages.

Table 3 Comparison of alkaloid concentration in Ayahuasca samples prepared with and without standardization from different sources. Quantified by HPLC-DAD.

Author	Standard	Source (n)	Samples (n)	DMT (mg/L)	HRL (mg/L)	HRM (mg/L)	THH (mg/L)
Callaway (2005) [44]	No	4	20	160-5840	100-900	450-6250	450-5260
Lanaro et. al (2018)	No	1	9	402-2070	28-181	295-2894	850-2053
This study	Yes	1	20	41-108	28-64	161-311	239-420

The ratio of THH/HRM both in the summer and winter seasons was higher and constant for samples of tea prepared with plants from TF ecosystem (Table 4), which is twice the ratio found by Callaway [44], with samples from UDV. However, considering the entheogenic effects of Ayahuasca for religious, health purposes, and the treatment for chemical dependency, to our knowledge, the best ratio of these two alkaloids is still unknown.

This is the first study describing parameters to standardize procedures and proportions of plants (*P. viridis/B. caapi*) to be used in the preparation of Ayahuasca, which is key for comparison of samples from different regions, and thus to assess the environmental effects on alkaloid concentration. Quantification of alkaloids in Ayahuasca samples used in religious rituals, will allow a more accurate assessment of the real psychotropic effect of this beverage. Furthermore, considering that active plant compounds also have relationship with the specie's genetic variability [21, 45], further studies are necessary to understand the role of genetic variability in alkaloid variations in plant tissue

To the present date, the preparations of Ayahuasca have been mainly done using fresh plant material. With the increasing popularity of the entheogenic effect and pharmacological benefits that Ayahuasca provides, applying post-harvesting treatments, as dehydration, may contribute to increase the quality of Ayahuasca used in countries where *P. viridis* and *B. caapi* cultivation is limited [5, 12, 17, 46]

In this context, this work analyzed the effect of the post-harvest drying process on the content of alkaloids in Ayahuasca tea prepared with *B. caapi* and *P.viridis,* dehydrated under the same temperatures (40, 50, 60℃, and at sun). Ayahuasca tea made with plants dried at 60°C, from both TF and CAMP ecosystems, (A14 samples and A12, Table 1) compared to the correspondent beverage prepared with fresh plants demonstrated a decrease of 49, 43, 12, and 69.9% of DMT, HRL, HRM, THH content respectively to TF ecosystem, whereas to samples made with plants of CAMP ecosystem the decrease was of 22.4, 54.7, 56.0, and 59.3% for DMT, HRL, HRM and THH content, respectively. When samples from both TF and CAMP prepared with dehydrated plants at 50℃ (samples A10 and A6, Table 1) are compared with their corresponding samples prepared with fresh plants (samples A4 and A13, Table 1) and with TF plant sample demonstrated 55.4, 51.8, 22.8 and 45.3 (mg/L) for DMT, HRL, HRM, and THH concentrations, respectively. Regarding samples with CAMP plants, the reduction of alkaloid concentration was 1.5, 55.2, 58.3, and 50.9 (mg/L) for DMT, HRL, HRM, and THH, sequentially. These results may have relationship with thermal instability of labile compounds in Ayahuasca tea, which can cause reactions during the drying process [22, 47].

Table 4 Alkaloids ratios observed for Ayahuasca prepared with fresh plants and after drying.

Coleta				Drying		Alkaloids ratio					
Month/Year	**Site**	**Season**	**Sample**	**No**	**Yes**	**DMT/HRL**	**DMT/HRM**	**DMT/THH**	**THH/HRL**	**THH/HRM**	**HRM/HRL**
Aug/16	TF	S	A2	x		2.66	0.46	0.28	9.52	1.64	5.82
			A14		60/60	2.38	0.21	0.47	5.06	0.44	11.59
	Camp	W	A3	x		1.28	0.16	0.17	7.64	0.97	7.89
			A12		60/60	2.18	0.29	0.31	6.93	0.91	7.63
Jan/16	TF	W	A4	x		1.39	0.35	0.21	6.58	1.64	4.03
			A10		50/50	1.3	0.2	0.17	7.53	1.17	6.46
	Camp	S	A13	x		0.79	0.13	0.15	5.26	0.88	5.96
			A6		50/50	1.73	0.31	0.3	5.76	1.04	5.55
Nov/17	TF	S	A7	x		1.3	0.3	0.24	5.42	1.23	4.41
			A8		40/40	1.26	0.27	0.25	4.97	1.07	4.66
			A18		50/50	7.41	0.29	1.12	6.61	0.26	25.84
			A11		40/45	1.56	0.22	0.25	6.19	0.87	7.15
			A16		sol	6.79	0.26	0.92	7.42	0.29	25.75
	Camp	S	A5	x		1.94	0.33	0.28	6.94	1.18	5.88
			A9		40/40	5.58	0.36	0.65	8.61	0.56	15.40
			A17		50/50	2.89	0.37	0.57	5.03	0.65	7.78
			A15		sol	2.45	0.31	0.7	3.51	0.44	7.89
Feb/19	TF	W	A19		43/45	2.72	0.66	0.85	3.21	0.77	4.16
	Camp	S	A20		43/45	3.32	0.83	0.89	3.74	0.94	4.00

TF=Firme; Camp = Campinarana; Season: S = Summer, W = Winter; Samples: Ayahuasca prepared with fresh plants (A2-TF; A3-CAMP; A4-TF, A13-CAMP; A7-TF and A-5 CAMP); Drying : 60/60, 50/50, 40/40, 40/45, 43/45 = temperature (°C) of drying *P. viridis* leaves and *B. caapi* stems, respectively; Sun = leaves and stems dried in the sun; Ratios: concentration *N, N*- dimethyltryptamine (DMT) as a function of Harmaline (HML) concentration, Harmine (HRM), tetrahydroharmine (THH); THH concentration as a function of HRL, HRM and HRM concentration as a function of HRL determined by HPLC-DAD.

Drying temperatures at 50 and 60°C resulted in a high depletion of active compounds in Ayahuasca. Also, a high variation of compound depletion patterns, especially for DMT, HRL, and THH was observed. This observation emphasizes the sensitivity of these compounds to these temperatures, and therefore are unsuitable for drying Ayahuasca plants, since thermal sensitivity and susceptibility to degradation affect the stability of the compound [23]. Independently of the environment of collect, all the leave samples of *P. viridis* darkened and stems of *B. caapi* became brittle after drying at 50 to 60℃ [22], and which is also related to high temperature, since high temperature leads to irreversible and adverse chemical reactions that cause degradation of product quality [47].

Comparing Ayahuasca samples made with plants dried at 40°C (samples A8 and A9, Table1) with their corresponding samples (samples A7 and A5, Table 1), an increase in alkaloid concentrations in Ayahuasca samples with TF plants, being 16.29, 19.97, 26.62, and 10.16% for DMT, HRL, HRM, and THH were detected. Whereas in samples with CAMP plants a reduction of 22.70, 73.07, 29.45, and 66.59 % for DMT, HRL, HRM, and THH was observed.

While drying at 40°C showed unexpected results with reduction and increase of alkaloids content when compared with their corresponding samples, which may have been influenced by the morphological characteristics of the plants, that according to Chua et al. [23] having also influenced the final quality by drying. Samples dried by direct solar heat also showed instability for DMT and *ß*-carbolinic compounds concentration. Furthermore, drying in the sun increases the risk of contamination by exposure of the material by unknown factors, therefore beverages prepared with these inputs could lead to adverse effects [48].

In light of the distinct characteristics of each species that take part in Ayahuasca tea, the plant parts used in the tea preparation, and considering the concentrations found in Ayahuasca prepared with plants dried at 40 and 50°C, samples of Ayahuasca prepared with dried plants under different temperatures for each species were assessed, establishing conditions of (a) 45℃ for stems and 40℃ for leaves and (b) 45℃ for stems and t 43℃ for leaves (Figure 2).

Considering the samples with stems and leaves dehydrated at 40/45 and 43/45°C respectively (Samples A11, A19 and A20, Table 1) the alkaloid concentration of HRL was low but relatively constant. THH/HRM ratios of both samples with CAMP and TF plants in winter and summer were close to 1:1, being constant in most samples (Table 4). For DMT concentration of Ayahuasca samples prepared with leaves dried at 40 to 43°C (sample A11, A19, A120, Table 1), sample A11 with leaves dried at 40°C had a low variation in the DMT content. However, there was also a low DMT content (52.0 mg/L) when compared to samples A19 and A20, prepared with leaves dehydrated at 43°C, that exhibited the greatest DMT concentration (108 and 98,94 mg/L) in both TF and CAMP ecosystems, respectively. This result indicates that DMT could be sensitive to temperatures above 43°C, as occur with trigonelline alkaloid during the coffee roasting process [49].

Ratios of THH/HRM 1:1 and 1:5 were found in report by Callaway [44] and Callaway et al. [43], respectively. These studies indicated that there could be a route to reduce HRM to HRL and from this to THH, however they did not investigate the interference of plant drying or the stability of the ratio between alkaloids.

Drying plants could be an answer to meet the growing demand for Ayahuasca products. Considering the synergistic action of the tea molecules, both the quantitative maintenance of DMT and the ratio between the *ß*-carbolinic compounds appear to be fundamental for the psychotropic effects of Ayahuasca. The highest DMT concentration and greater stability between THH/HRM ration found in Ayahuasca made with plants dried at 43 and 45°C, for *P. viridis* leaves and *B.caapi* stems, respectively allow to indicate that these temperatures in forced circulation oven are suitable for drying these plants, allowing to indicate the use of these drying parameters in the traditional preparation of Ayahuasca.

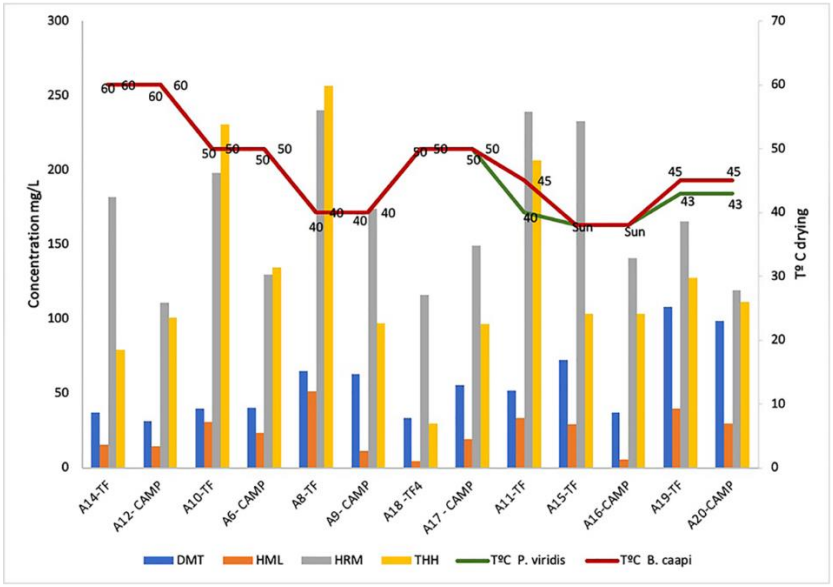


Figure 2 Influence of drying temperature on the concentration (mg/L) of DMT, HRL, HRM and THH in Ayahuasca samples prepared with dehydrated *B. caapi* stems and *P.viridis* leaves.

Finally, the influence of variation in the chemical composition of Ayahuasca tea tested *in vitro* for anti-proliferative activity was evaluated (Table 1, Figure 3). *In vitro* evaluation benefits from culturing cells in a conducive and controlled environment, besides ease and speed of assay execution and relatively low cost [50]. However, the test is a target-oriented assessment. In the model defined by this study (immortal human keratinocytes, HaCaT), *in vitro* evaluation will help to highlight possible effects of Ayahuasca tea on the proliferation of normal tissues such as skin, mucous membranes and bone marrow [51].

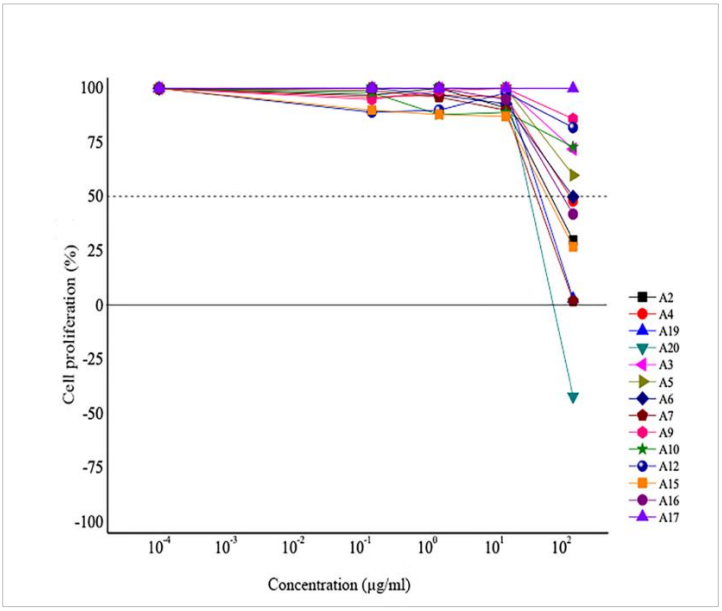


Figure 3 Proliferation of immortal human keratinocytes (HaCaT) after 48 h of exposure to different samples of Ayahuasca tea (see Table 1 for collection and drying conditions for each sample).

The evaluation of anti-proliferative activity after 48h of exposure (Figure 3) followed the protocol developed by the National Institute of Cancer-USA [52]. In this model, cytostatic activity can be expressed as the sample concentration necessary to inhibit cell proliferation by 50% (GI_{50}) [53]. Furthermore, GI_{50} values greater than 30 µg/ml are representative of samples that do not significantly affect cell proliferation [54]. Analyzing the results shown in Table 1 based on these criteria, 90% of all the samples analyzed exhibited GI_{50} above 150 µg/ml, which means no cytotoxicity on the proliferation of normal human cells (HaCaT) (Figure. 3). To Ayahuasca tea prepared with dried plants, only

sample A20, prepared with dried leaves at 43°C and stems at 45°C collected in CAMP during the summer showed light cytotoxic activity when tested on immortal human keratinocytes ($GI_{50} \approx 30$ µg/ ml, HaCaT) (Figure. 3).

Comparing these results of anti-proliferative activity with the levels of DMT, HRL, HRM, and THH, as well as with the relative proportions between them (Table 1), there is no evident correlation between the variations observed for these compounds and the cytostatic effect observed in $GI_{50} \approx 30$ µg/ml for samples A20. Therefore, a more in-depth assessment of the chemical profile of these sample compared to those that did not have a cytotoxic effect are necessary to identify the possible components involved in this light anti-proliferative effect. A systematic review of studies with humans has shown that acute, subacute and long-term use of Ayahuasca have low toxicity [55]. Moreover, assessments of biochemical parameters related to liver damage in serum in 22 volunteers who consumed Ayahuasca twice a month or more for at least one year indicate that chronic consumption of Ayahuasca in a religious context apparently does not affect liver function [56]. Furthermore, both DMT and HRM demonstrated a cyto-protective effect *in vitro* on different cell lines of human neurons submitted to hypoxia [57] or by exposure to 6-hydroxydopamine [58].

4. Conclusion

Variation of alkaloids concentration in Ayahuasca tea has relationship with the place and time of harvest of the species. Rainfall and water capacity of the soil showed to have straight relationship with the alkaloid contents of DMT, HRL, HRM, and THH in Ayahuasca. In the winter period, with higher rainfall, the alkaloids concentration in Ayahuasca prepared with plants from the TF ecosystem increases. Nevertheless, plants from CAMP seemed more adapted to water stress and the tea made with plants from this ecosystem had lower seasonal variation on the alkaloid's concentration.

Data reported herein demonstrated that drying temperature influences the quality of the components detected in the plants used to prepare Ayahuasca. Therefore, the drying parameters of *B. caapi* and *P viridis* species should be standardized based on the morphological characteristics of each species. In this study, the most suitable conditions were achieved in a forced air circulation oven with temperatures of 43°C for leaves and 45°C for stems. This allowed a constant and greater DMT concentrations, as well as maintained constant the quantitative proportion among the ß- carbolines in Ayahuasca prepared with plants from either Campinarana or Terra-Firme ecosystems.

Regarding the in vitro cytotoxicity, the results indicated that variations of alkaloids concentrations in Ayahuasca prepared with dehydrated or fresh plants did not show cytotoxic effects on human keratinocytes with relationship to DMT, HRL, HRM, and THH. Except for sample A20. Therefore, a more in-depth assessment of the chemical profile this sample compared to those that did not show a cytotoxic effect may identify the possible components involved in this slightly anti-proliferative effect. We consider that the cytotoxicity evaluation results found here, considering the anti-proliferative activity as a parameter, can potentially assist in refining screening samples for testing in vivo.

Compliance with ethical standards

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Author contributions

The work is part of the doctoral thesis of OFM who conceived the designed and all stages of research, in addition to the preparation and revision of the manuscript. MA, MAF and ALTGR contributed with concepts, design, analyses to the study and also with review. IMOS and RSM contributed with analyses, preparation and revision of the manuscript, JLC contributed with analyses to the study. All authors discussed, edited and approved the final version.

Disclosure of conflict of interest

None conflicts of interest to declare. The manuscript has not been previously submitted or published in other journal and is not being considered for publication elsewhere.

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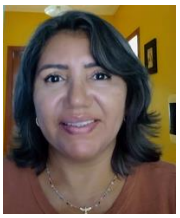
Author's short biography



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