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NATURAL DRUGS

IDENTIFICATION CHALLENGES IN EXAMINATION OF COMMERCIAL PLANT MATERIAL OF *PSYCHOTRIA VIRIDIS*

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Abstract: *Psychotria viridis* (*chacruna*) is a hallucinogenic plant with psychoactive properties associated with the presence of *N*,*N*-dimethyltryptamine (DMT). This species is primarily known as an ingredient of the beverage Ayahuasca, but dry leaves are also smoked by recreational users. The plant is controlled in Poland and France and its proper identification poses many challenges due to the fact that genus *Psychotria* is relatively large and there are other species that are easily confused with *chacruna*. The aim of the present work was to develop an effective authentication procedure for the dried and shredded leaves of *P. viridis,* to be used in comparison of chemical and botanical characteristics of its commercial products. Dried leaves of *P. viridis* originating from Brazil, Peru and Hawaii were purchased from Internet providers. For DMT identification, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) methods have been elaborated, validated and applied. In order to clarify the existing differences among samples, chemometric methods have been used. Botanical features and the gas chromatography tandem mass spectrometry (GC-MS) chromatograms have been analyzed using hierarchical cluster analysis (HCA). Our studies revealed significant variety among plant material marketed as *P. viridis*. Grouping of samples based on their micromorphology features and GC-MS results did not correspond well with the presence of DMT. Based on our results an indisputable identification of dried specimens as *P. viridis* is very problematic. It is necessary to postulate changes in legislation regarding regulation of *P. viridis* and replace it with DMT as controlled substance.

Keywords: *Psychotria viridis*, identification, chromatography, micromorphology, cluster analysis

Psychotria viridis Ruiz & Pavon from the Rubiaceae family is known as a popular hallucinogenic plant derived from the Amazon lowlands (1). Leaves of *P. viridis* known also as *chacruna* contain *N*,*N*-dimethyltryptamine (DMT), an indole alkaloid responsible for the psychoactive properties of the plant. The action of DMT resembles to some extent LSD, however, the caused sympathomimetic effects are more intense and onset and duration of effects are much shorter (2). As LSD, DMT belongs to the group of serotonergic hallucinogens activating several 5-HT receptors. Although the mechanism of action of DMT is not yet fully understood, its hallucinogenic effects are attributed to its agonist activity to 5-HT_{2A} and 5-HT_{2C} receptors (3).

DMT is not active orally due to oxidative deamination by monoamine oxidase A (MAO) to indole-3-acetic acid which is not able to cross the blood-brain barrier and produce hallucinogenic effects (4). In the Amazon region, leaves of *P. viridis* are used in combination with stems of *Banisteriopsis caapi* in the form of a drink called Ayahuasca (5). The alkaloids included in *B. caapi* block MAO and thus allow for the hallucinogenic effect after drinking Ayahuasca beverage. The dry leaves of *P. viridis* are also smoked by recreational users and can be used for extraction of DMT (6).

P. viridis is controlled in France and Poland (7, 8). In the other countries not the plant, but its main active compound DMT is illegal. According to US law live plants and seeds can be legally bought and sold (9).

Identification of plant material is difficult due to the fact that the genus *Psychotria* comprises nearly

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2000 species (10, 11), moreover, some other genera in South American flora are being confused with *chacruna*. The problem with proper plant identification results also from the divergent information about the contents of DMT. The list of *Psychotria* spp. containing DMT varies depending on the literature source. There are reports that some *Psychotria* species supposedly containing DMT are actually lacking it (12-14).

Nevertheless, *P. viridis* is always recognized as being the one that possesses hallucinogenic properties associated with DMT presence.

The aim of the present work was to develop an effective authentication procedure for the dried and shredded leaves of *P. viridis*. We based our method on DMT detection and microscopic confirmation of identity of the plant material. In the search for diagnostic features of *P. viridi*s we used micromorphological examination of live plant material. To confirm the presence of DMT in the samples, the low cost chromatographic methods commonly used in police laboratories were developed: thin layer chromatography as a first screening tool and high performance liquid chromatography method when the highest sensitivity is required.

The known methods of DMT identification in *P. viridis* and in Ayahuasca involve use of gas chromatography tandem mass spectrometry (GC-MS) (15, 16), capillary electrophoresis (CE) (17), liquid chromatography tandem mass spectrometry (LC-MS) (18, 19) and nuclear magnetic resonance spectroscopy (NMR) (20). Currently popular, barcoding methods, can be a good solution for the species identification of powdered plant material. However, they are still in the process of development and characteristic DNA sequences have to be found. This is challenging in case of *P. viridis* as the genus comprises a large number of species.

At the same time, there is a lack of fast and simple methods like thin layer chromatography (TLC) or high performance liquid chromatography (HPLC), for identification of DMT in *P. viridis*. One of the difficulties in HPLC analysis of DMT in plant samples may be attributed to relatively strong basic character of the compound. Utilizing basic properties of DMT the ion pair chromatography method has been developed, as well as fast and cost effective method of thin layer chromatography. Both methods had been validated and successfully applied in the analysis of all seven *chacruna* samples.

Our studies revealed significant variety among plant material marketed as *P. viridis*. In order to clarify the existing differences among samples, pharmacognostic examination and chemometric methods have been used. Botanical features and the chromatograms obtained with GC-MS method for methanol, ethyl acetate, dichloromethane, chloroform and hexane extracts have been analyzed with hierarchical cluster analysis (HCA).

EXPERIMENTAL

Plant materials

The examined products were purchased from the Internet through the http://www.maya-ethnobotanicals.com/ website. The shredded leaves of *P. viridis* originated from Brasil (samples 1 and 2), Hawai (sample 3) and Peru (samples 4-7) according to the provider's declaration.

Reagents and materials

Chloral hydrate was purchased from Carl Roth GmbH (Germany), Sudan III from Merck (Germany), isopropanol pure p.a. from Chempur (Poland) and glycerol pure p.a. from Standard (Poland). Sudan III reagent was prepared according to United States Pharmacopeia (USP).

Methanol, chloroform, ethyl acetate, dichloromethane and hexane pure p.a. were purchased from POCH (Poland), triethylamine pure p.a. from Sigma Aldrich (Belgium), anisaldehyde from Sigma Aldrich (Germany), 99.9% acetic acid from POCH (Poland), and 95% sulfuric acid pure p.a. from Chempur (Poland). Anisaldehyde solution was prepared according to European Pharmacopoeia.

HPLC reagents such as acetonitrile (MeCN) HPLC grade S were purchased from Rathburn Chemicals Ltd. (UK), sodium hexanesulfonate pure p.a. from Sigma Aldrich (Switzerland), and 85% orthophosphoric acid from Merck (Germany). Deionized water was obtained with deionizating filter system Water PRO PS from Labconco Corp. (Kansas City, USA). Membrane filters Chromafil Pet-45/25 from Macherey-Nagel GmbH & Co.KG (Germany).

N,*N*-Dimethyltryptamine (DMT) standard with the purity 99.4% was purchased from Lipomed AG (Switzerland) lot 00013890-9019.

Apparatus

Light microscope Biolar C Ti hal with digital video optikam Pro5 from PZO (Poland) and Optica Vision software; automatic TLC Sampler from Camag (Switzerland); Video-scanner Desaga with a software for video documentation ProVidoc (Germany); HPLC Ultima 300 System Dionex, with UV-VIS detector, 2 pumps, autosampler and Chromeleon Datasystem; GC-MS Agilent GC7890A+ 5975C VLMSD instrument from Agilent Technologies (USA) were used.

For the statistical analysis "Statistica 10" software from StatSoft (USA) was applied.

Methods

Sample and standard preparation for the chromatographic analysis

Powdered dried plant material (0.5 g) was extracted with 5.0 mL of methanol in an ultrasound bath for 30 min and filtered through membrane filters. The standard solution was prepared as a 0.5 mg/mL ethanol solution of DMT. The solution for the method validation in specificity parameter was also prepared by mixing 1 mL of extract from the Hawaii sample and 1 mL of the standard solution of DMT (mix). For GC-MS analysis the same sample preparation procedure was applied and different extraction solvents were used: methanol (MeOH), ethyl acetate (EA), dichloromethane (DCM), chloroform (TCM) and hexane (H).

TLC method

TLC silica gel plates G from Merck KGaA (Germany) were used for analysis. Mobile phase was chloroform/methanol/triethylamine (60 : 5 : 6, v/v/v). Samples solution (10 µL), 10 µL of DMT standard solution and 5 µL of prepared mixture (mix) were applied as bands. Chromatograms were developed to 4/5 of the plate height, then dried in air, treated with the anisaldehyde solution, heated in 105°C, examined in daylight and photographed with a video-scanner.

HPLC method

HPLC analysis was performed on Luna C18(2) 250 × 4.6 mm, 5 µm column from Phenomenex (USA). The mobile phases were: A: 10 mM solution of sodium hexanesulfonate monohydrate adjusted to pH 2 with orthophosporic acid, B: acetonitrile. The analysis was performed in gradient: A : B 80 : 20 (2 min), 65 : 35 (20 min), 20 : 80 (26 min), 80 : 20 (30 min). The detection wavelength was λ 254 nm, injection volume 10 µL, flow rate 1 mL/min, time of analysis 30 min. DMT standard solution, methanolic extracts from the *Psychotria viridis* samples mixture of equal parts of extract solution from Hawaiian *Psychotria* and standard solution (mix) and blank were injected.

Microscopic examination

Specimens prepared from the leaf surface and powdered material were examined. Specimens were boiled and observed in chloral hydrate solution, and after the reaction with Sudan III reagent.

GC-MS method

GC-MS was performed with a 5% phenylmethylpolysiloxane capillary column HP-5MS (30 m × 0.25 mm i.d.; film thickness, 0.25 µm; Agilent J&W Scientific (USA). The temperature program consisted of the initial temperature of 100°C held for 0.5 min, followed by a linear ramp up to 300°C at 12°C/min. The inlet temperature was set at 230°C, and the injection was set in the split 1 : 80 mode. The carrier gas was high purity helium at a flow rate

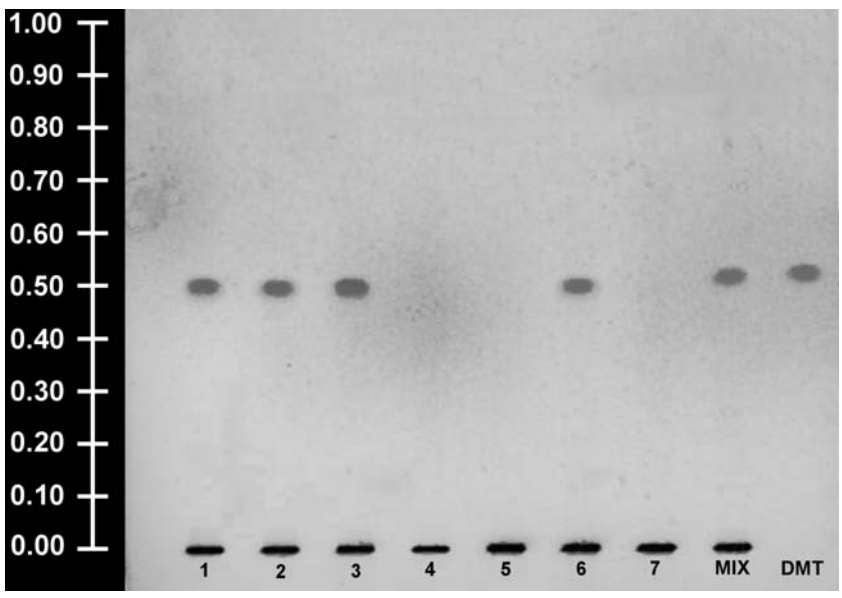

Figure 1. TLC chromatogram of DMT standard solution, sample solution (1-7), a mixture of equal volumes of the standard and sample number 1

Table 1. Micromorphological features of *P. viridis* observed in the examined plant material.

Feature number	Feature description	1	2	3	4	5	6	7
Origin		**Brazil**	**Brazil**	**Hawaii**	**Peru**	**Peru**	**Peru**	**Peru**
I	Color	reddish	reddish	reddish	grey	grey	green	grey
II	Polygonal cells of the adaxial epidermis (surface view)	yes	yes	yes	yes	yes	yes	yes
III	Striated cuticule on the upper epidermis	yes	yes	yes	yes	no	yes	yes
IV	Stomata on the adaxial surface	no	no	no	no	no	no	no
V	Crystals of calcium oxalate in the upper epidermis: styloids, small prisms and no bundled raphides	yes	yes	yes	no	no	rarely	yes
VI	One-layer palisade parenchyma	yes	yes	yes	yes	yes	yes	yes
VII	Bundles of large raphides	yes	yes	yes	yes	yes	yes	yes
VIII	Crystals of calcium oxalate in parenchyma: styloids and single crystals	yes	yes	yes	no	no	rarely	yes
IX	Fat in cells	yes	yes	yes	yes	yes	yes	yes
X	Polygonal cells of the abaxial epidermis (surface view)	yes	yes	yes	yes	yes	yes	yes
XI	*Rubiaceus* stomata in lower epidermis	yes	yes	yes	yes	yes	yes	yes
XII	One-celled, non-glandular trichomes on the abaxial epidermis	yes	yes	yes	rarely	yes	yes	yes

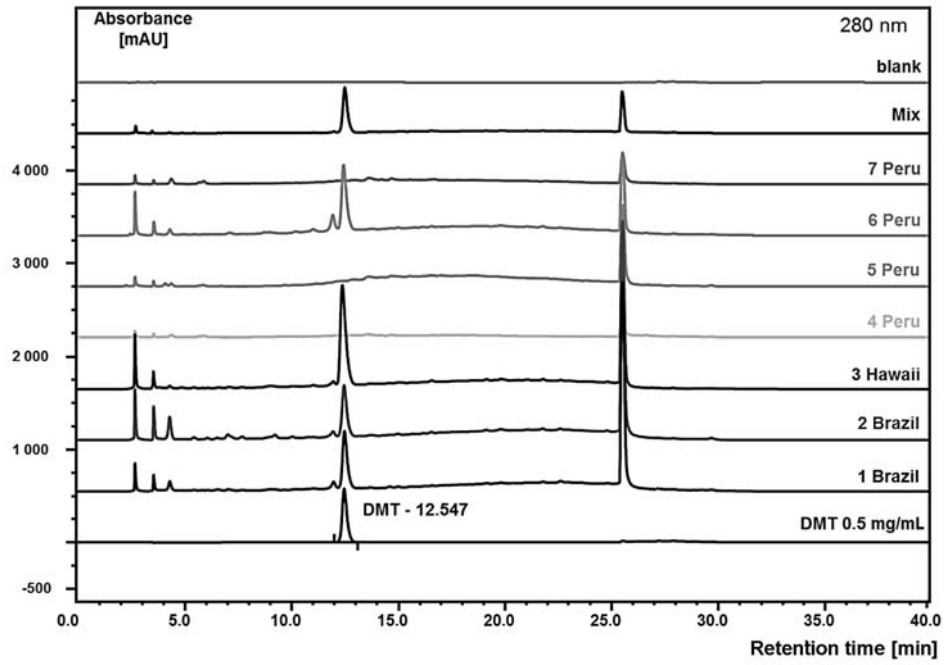

Figure 2. HPLC chromatogram of DMT standard solution, sample solution (1-7), a mixture of equal volumes of the standard and sample number 1 solutions and methanol as blank

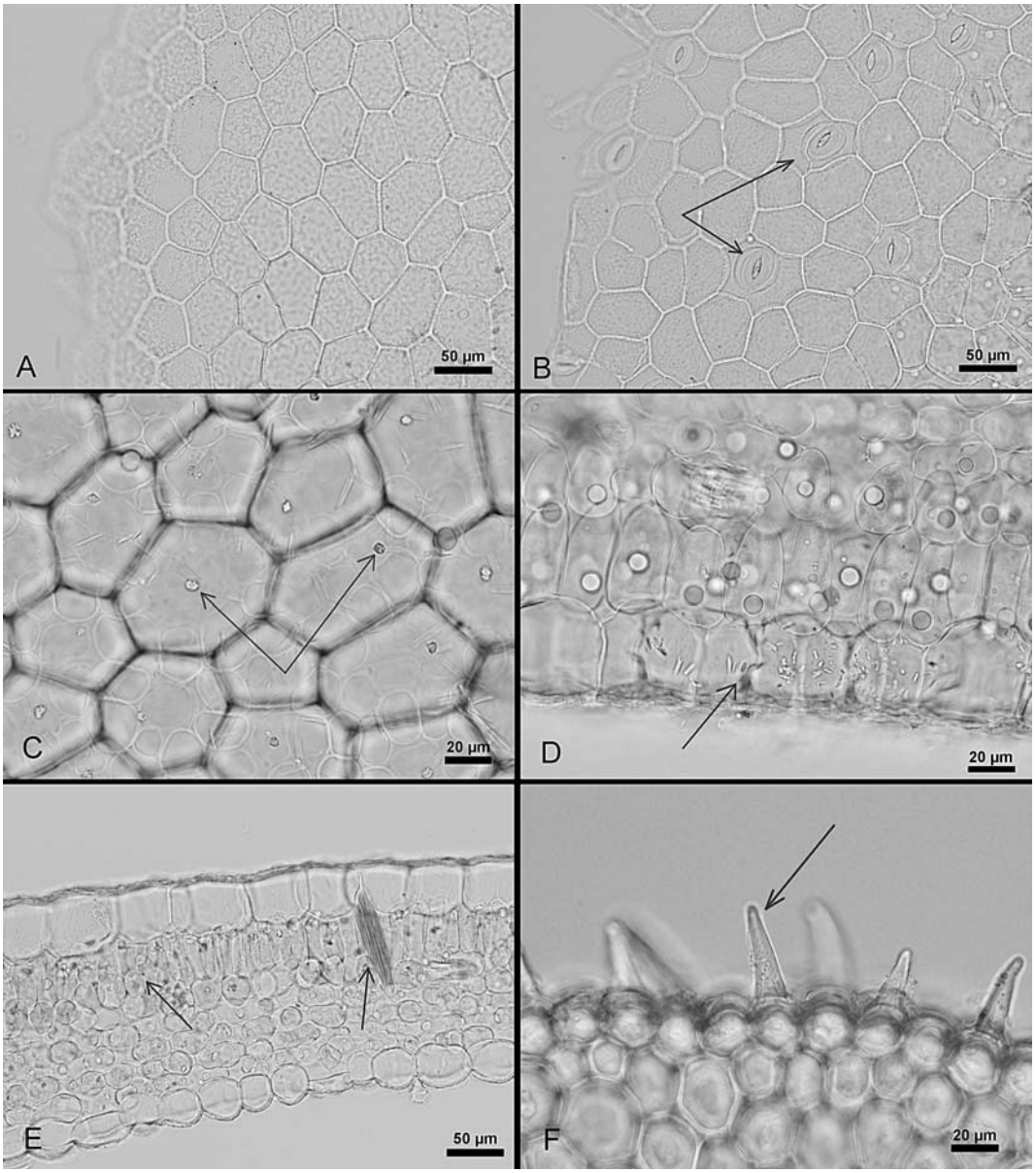

Figure 3. Micromorphological features of *P. viridis*: A – surface view of the upper epidermis: polygonal cells with the striated cuticule; B – surface view of the lower epidermis with paracytic stomata; C – adaxial surface: styloids of calcium oxalate; D – transverse section of the leaf: prisms of calcium oxalate; E – transverse section of the leaf: one-layer palisade parenchyma and bundle of raphides; F – one-celled non-glandular trichomes

of 1.0 mL/min. The MS detector parameters used were: interface temperature, 290°C; ion-source temperature, 230°C; ionization mode, electron ionization (EI); ionization voltage, 70 eV; scan time, 1.0 s/scan; scan range, *m*/*z* 40–550.

Data analysis method

Hierarchical cluster analysis (HCA) was chosen as a statistical tool to interpret botanical and chemical similarity among the plant samples. The hierarchical agglomerative cluster analysis, divides the data into similar groups (clusters). The aim of the grouping is making a stable cluster from objects located in closest neighborhood and to separate them from other clusters.

The application of HCA is justifiable as the objective of the present work is to evaluate the variation among *Psychotria* products and also because the analysis concerns a small number of trials and a small number of variables.

In both cases, botanical and chemical characterization of the samples, the data are discrete. The occurrence or lack of botanical features and the presence or absence of peaks on chromatograms has been analyzed, so there was no need for data normalization.

RESULTS

TLC method

The obtained chromatograms revealed the presence of *N*,*N*-dimethyltryptamine in *P. viridis* samples from Brazil (samples 1 and 2), Hawaii (sample 3) and one sample from Peru (number 6). A violet spot appears with the R_f 0.51 (Fig. 1). In three samples from Peru (samples 4, 5 and 7) DMT spot was not observed.

The identification method was validated using a mixture of equal parts of extract from sample 1 and DMT solution (mix). Limit of detection was established for the concentration of DMT 10 µg/mL The criterion of acceptance was 10% difference in the values of R_f for DMT in the standard and sample solution.

HPLC method

HPLC chromatograms of samples from Brazil (samples 1 and 2), Hawaii (sample 3) and one sample from Peru (sample 6) showed a distinct peak of DMT. The obtained retention times were from 12.46 min to 12.56 min in accordance to standard solution where the DMT retention time was from 12.54 min (Fig. 2). Same as in TLC analysis, in three samples from Peru (samples 4,5 and 7) no DMT was detected.

The areas of the DMT peak in 0.5 mg/mL standard solution was about 155 mAU while for the Brazilian sample solutions: 157 mAU for sample 1 and 140 mAU for sample 2, respectively. Equally, the area under the DMT peak of one Peruvian sample (sample 6) was 186 mAU. The highest concentration of DMT was found in the Hawaiian sample (sample 3) with the peak at 315 mAU. No DMT peaks were detected in samples number 4, 5 and 7.

The developed HPLC method was validated in a specificity parameter using a blank sample of methanol and a mixture of equal parts of sample number 1 and DMT solution (mix). No interfering with DMT peaks was detected in blank sample. Limit of detection was determined on the base of signal to noise ratio for the concentration of 2 µg/mL. Criteria of acceptance were established as: 5% deviation between standard and sample retention time of DMT, resolution factor for DMT peak more than 1.0 asymmetry factor from 0.8 to 1.6; number of theoretical plates more than 11,000.

Microscopic examination

Microscopic analysis of shredded fresh plant material allowed for the selection of characteristic micromorphological features of *P. viridis* leaves (Fig. 3). The botanical elements and their occurrence in examined samples are presented in the Table 1. Features used for HCA were: I, III, V, VIII, XII.

The data were subjected to Ward's method of linkage after using the Manhattan distance as similarity measure and to single linkage method after using the squared Euclidean distance. The obtained clusters separated samples into three main groups according to the Sneath criterion (21). The first group is represented by samples from Brazil, Hawaii and Peru, samples number 1, 2, 3 and 7. The second group is determined by two Peruvian samples, number 4 and 5. The third cluster consists of one Peruvian sample – number 6. The most significant

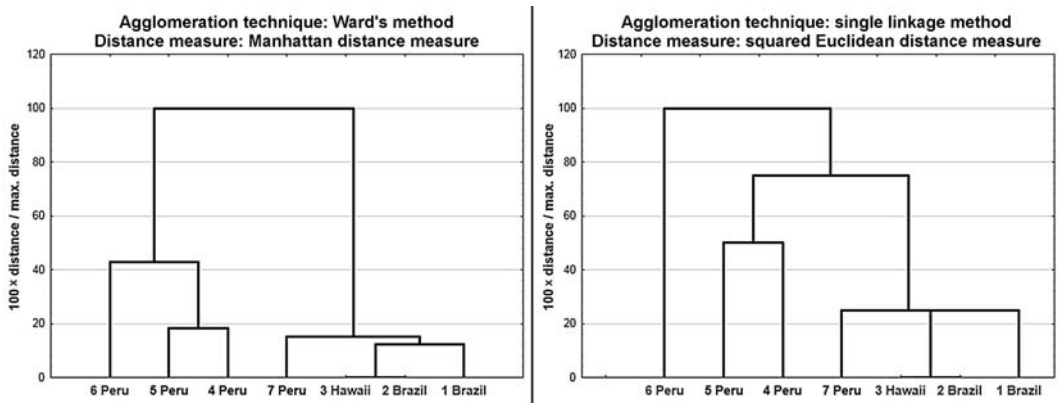

Figure 4. Dendrograms for hierarchical clustering of variables according to botanical features of the examined samples

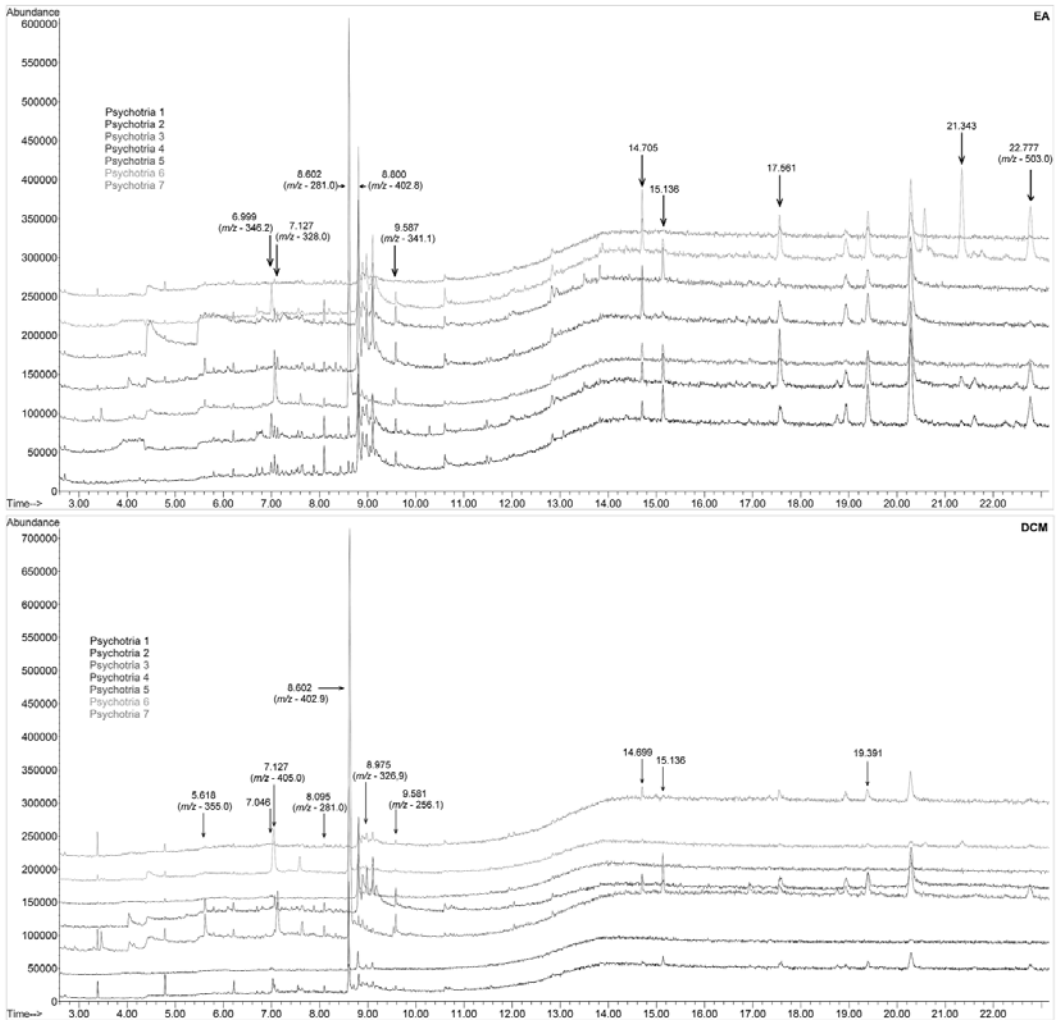

Figure 5. Chromatograms of EA and TCM extracts from dried leaves of *P. viridis*

botanical similarity exists between Brazilian and Hawaiian products. No relation between botanical grouping and DMT content has been obtained. Grouping by botanical variables is presented on dendrograms (Fig. 4).

GC-MS

Five extracts, made with methanol (MeOH), ethyl acetate (EA), dichloromethane (DCM), chloroform (TCM) and hexane (H), from each sample, have been examined with the GC-MS method and five chromatograms of each plant material have been obtained. Every compound was represented by a chromatographic peak and the mass corresponding to this peak. Example chromatograms obtained for EA and TCM extracts are presented in Figure 5.

For further HCA analysis, all retention times assigned to a single mass, were used.

The data were subjected to Ward's method of linkage after using the Manhattan distance as similarity measure. According to the Sneath criterion, three clusters have been separated. The chemical variables classify the samples in three groups. First cluster consists of the plant material from Brazil and Hawaii (samples 1-3), the second from three Peruvian samples (sample 4, 5 and 7). The third cluster is made of one Peruvian sample (number 6). Grouping by chemical variables is presented on dendrogram (Fig. 6).

DISCUSSION AND CONCLUSIONS

Psychotria viridis (*chacruna*) is a hallucinogenic plant, with psychoactive properties associated with the presence of *N*,*N*-dimethyltryptamine. This species is primarily known as one of the two main ingredients of the beverage Ayahuasca, however

dried leaves of *chacruna* are also smoked by recreational users.

In countries where the species is illegal, the legislation requires identifying the plant material that may be a challenge for the police experts. Macroscopic and microscopic investigations are not sufficient as they cannot bring an unequivocal answer whether the examined leaves come from *P. viridis* or not. There are reports that DMT can be present in few species of *Psychotria*, but also in other plants from South and Central America. List of *Psychotria* that contain DMT has not been clearly established yet, but all literature sources confirm the occurrence of DMT in *P. viridis*. Therefore, the detection of this compound in the examined plant material is necessary for the species authentication.

Chromatographic methods have been chosen to determine DMT presence in the investigated plant material. Thin layer chromatography is a simple and cost effective technique which allows for a rapid detection of compounds. High performance liquid chromatography enables for the verification of presence of chemical substances in tested materials with higher accuracy. Both developed methods described in the present work have been validated. Due to obtained high specificity and selectivity, the methods were successfully applied for analyses of *P. viridis* plant material.

Seven samples of dried *P. viridis* leaves purchased through the Internet were examined botanically and chemically. According to the providers' declaration samples originated from Brazil, Peru and Hawaii. Based on the appearance and micromorphological features similarity among samples from Brazil, Hawaii and one sample from Peru (sample 7) has been found. The same situation applies to the two Peruvian samples. A surprisingly significant distinction between the third sample from Peru (sample 6) and other Peruvian samples was found. Dried leaves were reddish (samples 1 – 3) or grey (samples 4, 5 and 7). Only the plant material of sample 6 had green color. The division into groups based on botanical variables analysis didn't correspond to the division based on the presence of DMT, since DMT was detected in samples number 1-3 and number 6.

These results show that the micromorphological analysis does not allow for a clear species identification. Many micromorphological characteristics depend on the geographical region and environmental conditions. Two Peruvian samples separated on the dendrogram as a middle cluster, did not reveal the presence of DMT. These plant materials probably don't come from *P. viridis*. Another cluster consisted of samples with DMT (Brazilian and Hawaiian) and without DMT (Peruvian sample 7). The third cluster was made only of sample number 6. This plant material even though containing DMT demonstrated a significant botanical dissimilarity.

It is possible that sample 7 belongs to genus *Psychotria* but because of lack of DMT it is not *P. viridis*. Peruvian sample number 6 may come from *P. viridis* but due to the geographical conditions this material is different from the Brazilian and Hawaiian ones. It is also possible that sample number 6 represents another *Psychotria* species with DMT.

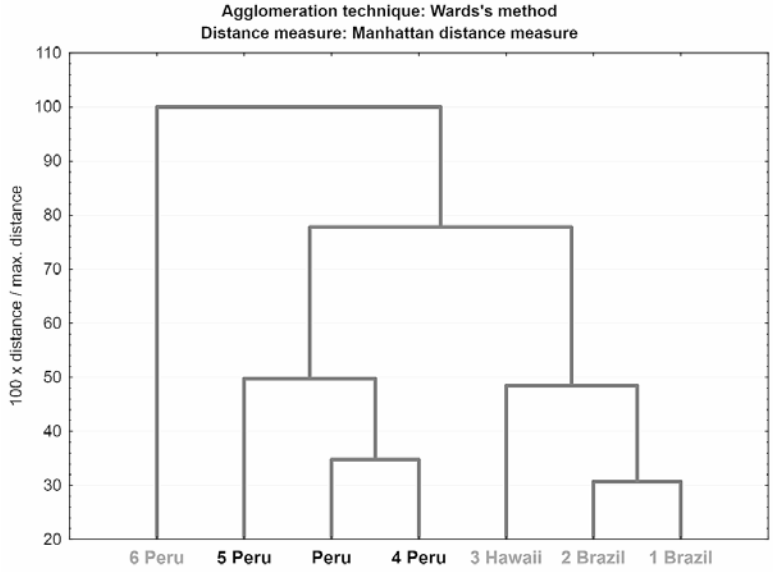

Figure 6. Dendrograms for hierarchical clustering of chemical variables according to the GC-MS results

To exclude the environmental effect and explain if sample number 6 is *P .viridis* or not, the HCA for chromatographic fingerprints was carried out. The aim of the use of solvents with varying degrees of polarity was to extract the greatest amount of compounds according to their chemical nature. As a result of HCA on chemical variables, again three main clusters have been obtained. DMT-free samples are also similar in the context of the total chemical composition and are grouped into one cluster. Therefore, sample 4, 5 and 7 don't come from *P. viridis*.

Moreover, the DMT-containing group of samples is not homogenous. The HCA revealed a significant dissimilarity between sample number 6 and the rest of plant material. Peruvian sample number 6 constitutes a separate cluster on the dendrogram. This sample is different from all the examined samples regarding botanical features and its chemical composition.

A great chemical similarity among leaves from Brazil (sample 1 and 2) and Hawaii (sample 3) was found. This material is probably *chacruna* leaves.

Wrong taxonomical identification of one plant material sample from Peru (sample 7) illustrates a challenge with the proper authentication of *chacruna*. Sample number 7 is botanically similar to the DMT containing ones but definitely is not *P. viridis*. Also grouping by chemical variables links this sample to the Peruvian group that was previously approved as not *P. viridis*.

Our studies revealed significant variety among plant material marketed as *P. viridis*. Grouping of samples based on their micromorphology features and GC-MS results did not correspond well with the presence of DMT. The performed studies have shown that the indisputable identification of fragmented specimen as *P. viridis* is problematic. It is necessary to postulate changes in legislation regarding regulation of *P. viridis* and replace the plant with DMT as controlled substance.

The developed TLC and HPLC methods allow for fast and cost effective detection of DMT in the investigated material.

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