Identification and characterization of cyclotides from Brazilian *Psychotria* species

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Psychotria genus belong to tribe Psychotrieae (Rubiaceae) and comprises approximately 2,000 species[1]; some of them are reported as cyclotide-containing plant species[2,3]. Cyclotides are characterized by a head-to-tail cyclized backbone usually composed of 28-37 amino acids, and a unique knotted disulfide topology involving six conserved cysteine residues termed the cyclic cystine-knot (CCK) motif[4,5]. The CCK motif is responsible for their exceptional resistance to chemical, enzymatic and thermal degradation[6]. This topology confers them remarkable stability and wide range of biological and/or therapeutic applications, between them, anti-HIV, anthelmintic, insecticidal, molluscicidal, antimicrobial, uterotonic, cytotoxic, hemolytic, trypsin inhibitory, and immunosuppressive activities. This work deals with the identification and characterization of cyclotides accumulated in the Brazilian plant species: *Psychotria vellosiana* (stems and leaves) and *Psychotria leiocarpa* (aerial parts).

Dried and pulverized stems and leaves from *P. vellosiana* and aerial parts from *P. leiocarpa* were extracted with CH₃OH-H₂O (6:4, v/v) at room temperature 4 times over a period of 24 h. The extract was partitioned with CH₂Cl₂-CH₃OH-H₂O (1:1:1, v/v/v) (4 times) and the aqueous phases were separated and concentrated on a rotary evaporator prior to freeze drying, yielding what is further referred to as aqueous extract. The aqueous extracts were dissolved in CH₃CN-H₂O (1:9; v/v) and immediately used for solid-phase extraction (SPE). C₁₈ SPE cartridge (Strata-Phenomenex C₁₈ 55 µm, 70 , 500 mg) were activated with CH₃OH and subsequently equilibrated with aqueous 0.1% trifluoroacetic acid. After application of the extracts, the cartridge was washed with mixtures of buffer A (0.1% aqueous trifluoroacetic acid) in B (90% acetonitrile, 0.08% trifluoroacetic acid) in the proportions 8:2 (v/v) and 2:8 (v/v), respectively. The fractions eluted in buffer B 80%, named stems C₁₈_80% PV, leaves C₁₈_80% PV and C₁₈_80% PL showed the presence of masses in the range of 2900-3700 Da as analyzed by MALDI-TOF mass spectrometry (MS). To determine whether these fractions contained peptides with disulfide bonds, they were treated with the reducing agent 10 mM dithiothreitol and alkylation agent 100 mM iodoacetamide, and the mass increase of 348 Da were observed, suggesting that three disulfide bonds had been reduced.

The C₁₈_80% PL fraction was analyzed using LC-MS (HRESIMS) and was possible to find seven cyclotides; two of them are already described in the literature.

Figure 1: Putative cyclotides identified by LC-MS in C_{18} _80% PL from *Psychotria leiocarpa*. **A)** Base peak chromatography. **B)** Table showing each mass related with its retention time. C_{18} Kromasil column (250×4.6 mm, $5 \mu\text{m}$; flow rate: 1 mL per min), $300 \text{ \AA}$, linear gradient of CH_3CN (25-65% B in 40 min), and UV 220 nm. Solvents: Buffer A (H_2O /0.1% HCOOH) and Buffer B (90% CH_3CN /0.1% HCOOH). *The masses shown are monoisotopic.

The leaves and stems C_{18} _80% PV fractions were analyzed using LC-MS (HRESIMS) and were possible to find some cyclotides to both tissues.

Figure 2: Putative cyclotides identified by LC-MS in leaves (red line) and stems (green line) C_{18} _80% PV fractions from *Psychotria leiocarpa*. Highlighted in gray, the region eluting putative cyclotides. The masses are represented in black when are common to both tissues, in green to stem-specific and in red to leave-specific.

From stems C_{18} _80% PV were isolated the möbius cyclotides, with m/z 2889 and 2905. The cyclotides sequencing were performed by reduction, alkylation and enzymatic digest with endoproteinas Glu-C and trypsin and followed by MS/MS analysis. The spectra were carefully examined and the sequences were proposed, based on the presence of both b - and y -series of ions (N - and C -terminal fragments). The Ile and Leu are assigned on basis of homology.

Figure 3: Cyclotides isolated from *P. vellosiana*. *m/z* of 2889 and 2905, respectively.

Here, some cyclotides are identify to *P. leiocarpa* and *P. vellosiana*. From this last species, were possible to isolated and to sequence two novel möbius cyclotides.