Cyclotides from *Pombalia*, *Noisettia* and *Viola* Genus: Discovery and Inhibitors of Cellular Migration

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Introduction

Violaceae is a rich source of cyclotides and are ubiquitous among all species of this plant family [1]. This protein group is characterized by a peculiar cyclic structure, collectively known as cyclic cystine motif (CCK) conferring them with remarkable stability. These properties together with a number of bioactivities have attracted enormous attention from both the scientific community and pharmaceutical industry for using cyclotides in drug development [2]. In this context, this work describes the identification of cyclotides in fractions of *Pombalia calceolaria*, *Viola cerasifolia*, *Viola submdmiata* and *Noisettia orchidflora*, as well as their potential anticancer properties.

Results and Discussion

Fig. 1. a) *V. submdmiata*. b) *V. cerasifolia*.
c) *N. orchidflora*. d) *P. calceolaria*.

The work presented here contributes to our understanding about the diversity and distribution of cyclotides in the *Pombalia calceolaria* (leaves, stems, seeds and roots), *Viola cerasifolia* (leaves, stems and roots), *Viola submdmiata* (leaves, stems and roots) and *Noisettia orchidflora* (roots) sourced from Forest Atlantic (Rio de Janeiro, Brazil). Although an array of pharmaceutical relevant bioactivities has been ascribed to cyclotides, their key purpose in plants is supposed to be as antibiotic agents to fend off plant pests and pathogens [3].

In order to check the possible presence and differences in the expression of cyclotide in these four plants and your tissues, approximately 60g of each plant part was ground prior to solvent extraction, yielding thirteen crude extracts. For *V. cerasifolia* were obtained three extracts named 1L (leaves), 1C (stems) and 1R (roots). For *V. submdmiata*, 2L (leaves), 2C (stems) and 2R (roots), *N. orchidflora*, 3L (leaves), 3C (stems) and 3R (roots) and *P. calceolaria* 4L (leaves), 4C (stems), 4S (seeds) and 4R (roots). The analysis by RP-HPLC and MALDI-TOF MS showed that these extracts contained a diversity of cyclotides identified bases on their mass, cysteine content and hydrophobicity. The cysteine content was in accordance with established diagnostic methodology whereupon after reduction and carbamidomethylation reaction it is possible to observe mass increase of 348 Da indicating the presence of three intramolecular disulfide bonds in the corresponding proteins. Thus, the extracts showed a wide diversity of peptides with masses in the range of 2900-3600 Da as analysed by MALDI-TOF mass spectrometry (MS), typically the range of masses expected for cyclotides. In general, the expression of the peptides appeared to vary with the plant tissues, according to the Table 1.

Preparative RP-HPLC chromatography of crude extract allowed isolating four bracelet cyclotides, (m/z 3143; 3157; 3166) from roots of *P. calceolaria* and (m/z 3145) from *N. orchidflora*. Cyclotide sequencing was performed by reduction, alkylation and enzymatic digest with endoproteinases Glu-C, trypsin, and chymotrypsin and followed by MS/MS analysis.

Table 1. Peptide masses obtained by MALDI for *V.cerasifolia* 1L (leaves), 1C (stems) and 1R (roots), *V. subdmiata*, 2L (leaves), 2C (stems) and 2R (roots), *N. orchidflora*, 3L (leaves), 3C (stems) and 3R (roots) and *P. calceolaria* 4L (leaves), 4C (stems), 4S (seeds) and 4R (roots).

1F	1G	1R	2F	2G	2R	3F	3G	3R	4F	4G	4R	4S
3108.182		3108.198		2841.653				3145.320	3070.288		3072.172	
3126.218	3124.474	3125.215		2847.027					3085.197		3084.188	
3136.257	3133.181	3135.201		2855.288		3171.399			3093.166		3093.162	
3150.227	3149.162	3148.194		2858.170					3095.106		3096.178	
3153.218		3151.209		2863.715			3178.579	3181.036	3105.174			3105.285
3166.225	3164.226	3166.215	2877.052	2877.088	2878.246	3193.396	3193.904		3109.097		3109.135	3107.981
	3180.225	3180.187	2879.052						3112.156		3112.174	
3191.261			2891.66	2891.119	2891.349			3207.354				3123.954
3195.729			2893.067	2894.014	2894.197		3209.291	3210.964		3125.102	3125.249	
3208.689			2905.079	2905.142	2906.254				3128.516		3128.254	
3214.244			2907.103						3133.607			
3223.274			2909.025	2908.033			3222.666	3223.127	3140.013			
3241.284			2915.056		2915.221			3244.616				
3249.295			2923.054	2923.092						3142.227	3143.263	
3269.744			2929.052		2929.228			3249.168	3150.228		3149.202	
3281.292			2938.066	2938.088			3273.285		3157.000		3157.232	
3288.951			2943.052	2943.289					3166.198	3166.185	3166.212	3165.291
				3070.245		3282.430	3282.753	3282.615			3174.233	
3292.760				3114.229						3180.198	3181.200	
3296.756				3139.202				3295.273	3190.257	3190.291	3190.208	
3312.300	3310.269	3311.254			3148.122			3313.015	3205.247	3205.219	3205.035	
3346.349				3171.294			3341.959			3208.222		
3356.784					3180.342						3225.223	
3369.815				3195.138				3344.560	3303.276		3229.212	
3411.346				3302.272							3328.229	

A bioassay was conducted based on cellular migration, a feature related to the metastasis process, which is involved in 90% of all cancer deaths. Thus, for qualitative evaluation of directional cell migration, MDA-MB-231 cells were seeded at a density of 1×10^5 cells/well in 24-well culture plates (TPPTM) and allowed to reach a confluent monolayer. Then, a scratch was inflicted in the wells using a sterile 100 μ L pipette tip. The medium was removed and a fresh medium without FBS was added to clean the debris. Sequentially, cells were incubated for 22h at 37°C with supplemented medium (10% FBS) and concentration of extracts (200 μ g/mL) and colchicine at 10 mM has been used as a control. When measuring the wound closure, pictures of the wells were taken under a 4X objective (Optiphas) at 0 and 22h. The images were analyzed using

Fig. 2. Percentage of cell migration inhibition and cytotoxicity assays with MDA-MB 213 human breast tumor cells.

Image J software and two independent experiments were conducted in triplicate. All fractions demonstrated high inhibition of cellular migration between 80 to 100%, according to Figure 2. The fractions were evaluated for their cytotoxicity against MDA-MB-231 and DU-145 cancer cell lines and the percentage of unviable cells was determined in relation to the control wells. All fractions exhibited high potency as in the migration assays and cytotoxicity.

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