



## The Study of Antimicrobial Activities of Partially Purified Cyclotide Content and Crude Extracts from *Viola tricolor*

Zahra Khoshkam <sup>1</sup>, Mahboobeh Zarrabi <sup>1\*</sup>, Zargham Sepehrizade <sup>2</sup>,  
Maryam Keshavarzi <sup>3</sup>

<sup>1</sup> Department of Biotechnology, Faculty of Biological Sciences, Alzahra University, Tehran, Iran.

<sup>2</sup> Department of Biotechnology, Faculty of Pharmacy, Tehran University, Tehran, Iran.

<sup>3</sup> Department of Plant Sciences, Faculty of Biological Sciences, Alzahra University, Tehran, Iran.

### ARTICLE INFO

#### Article type:

Original Article

#### Article history:

Received: 12 Dec 2015

Revised: 22 Jan 2016

Accepted: 14 Feb 2016

#### Keywords:

*Viola tricolor*,  
Cyclotide, extraction,  
Antibacterial effect

### ABSTRACT

**Background:** Searching for the potent natural antibiotics will improve the treatment of most infectious diseases. Cyclotides are potent active peptides derived from some plant families like Violaceae. *Viola tricolor* (the pansy) has been found to have numerous cyclotides. Nowadays cyclotides attract more attention for their antibacterial activities. The current research studied the antimicrobial properties of semi-purified cyclotides from *V. tricolor*.

**Methods:** Extraction and purification of cyclotides from Iranian *Viola tricolor* were performed by fractionation and solid phase extraction methods, and their antimicrobial effects were studied against several bacterial strains using diffusion assays. Also, attendance of cyclotide in the extract was verified by Tricine-SDS page and spectroscopic methods.

**Results:** Antimicrobial effects of semi-purified cyclotides and crude extracts resulted in the antibacterial activity potential of *V. tricolor* totally extracted samples against gram negative bacteria, *E.coli* and *P. aeruginosa*. However, there is need for optimizing the assay method and the culture media.

**Conclusion:** *Viola tricolor* as a remedy represents the antibacterial potential, which may not be unrelated to its cyclotide content although the effectiveness of cyclotides may also differ because of their synergism, natural structure and bioactivities, the amount of purified content, and the way they were assayed.

- Please cite this paper as: Khoshkam Z, Zarrabi M, Sepehrizade Z, Keshavarzi M. The Study of Antimicrobial Activities of Partially Purified Cyclotide Content and Crude Extracts from *Viola tricolor*. *J Med Bacteriol*. 2016; 5 (1, 2): pp.29-35.

## Introduction

Treatment of most infectious diseases using different synthetic and natural antibiotics should be better than the past centuries. However in spite of these advances, newer problems such as nosocomial infections have come and this is the main reason for more interest of the researchers for finding more and better drugs (1). Cyclotides are fascinating circular proteins ranging from 28 to 37 amino acid residues that are naturally expressed in plants (2). They are believed to have an important role in the plants, defense system by their insecticidal effects (3); they have also been shown to be cytotoxic (4), anti-HIV (5), antimicrobial (6), and hemolytic agents (7). All of these mini-peptides share a unique head-to-tail circular knotted topology of three disulfide bridges, called "cystine knot" topology resulting in their significant thermal, chemical and enzymatic resistance (2, 8). Cyclotides are present in many plants from the Violaceae, Rubiaceae, Fabaceae and Cucurbitaceae families (9). *Viola tricolor* (the pansy) is widely cultivated as an ornamental plant. Previous investigations have determined several special cyclotides like tricyclon A & B, vitri A-F, Cycloviolacin O12 & O2, kalata S, varv (A, D, E, F, H, Hm & He) from *V. tricolor* till now (10-12). Many plants of the Violaceae family have been used in traditional remedies, and *V. tricolor* have been used in folk medicine externally and internally as an adjuvant in treating various skin conditions such as eczema, impetigo, acne and pruritus also internally as an auxiliary agent to promote metabolism (13). Most of medicinal properties of *V. tricolor* have been previously reported in association with the contribution of nonpeptidic compounds (14, 15). Antimicrobial activity of this herb for several crude extracts with different polarity has been evaluated before, which suggests a synergistic interaction between the compounds of *V. tricolor* herb, showing its low to significant activity against most of the tested microorganisms (15).

This study aimed the extraction and semi-purification of cyclotides from Iranian *Viola tricolor*, and then examination of their antimicrobial effects against several bacterial strains. The presence and quality of the partially purified cyclotides were evaluated by Tricine-SDS-PAGE and Bradford methods.

## Material and method

### *Plant material*

The herbs of *Viola tricolor* were collected from Absard region located in east of Tehran Province by our botanist. Total extraction and semi-purification for the aerial parts of *V. tricolor* (leaves & flowers separately) were performed using fractionation protocol (FP), solvent-solvent partitioning (SSP) and solid phase extraction (SPE) methods.

### *Plant total extraction*

About 15 g of powdered plant powdered material leaves and flowers were separately extracted 5 times by 200 ml dichloromethane. The solution was discarded, and the plant residues were dried at room temperature overnight. The dried plant remnant was extracted 3 times with 300 ml of 50% ethanol; then the solution was concentrated to 400 ml and acidified by 2% acetic acid. By using a polyamide column, tannins were removed. The tannin free extract was concentrated and lyophilized. Solvent-solvent partitioning for completion of extraction was performed for about 1 g of previously achieved extract powder solved in 100 ml water, then partitioned 3 times with 100 ml butanol. The resulting water and butanolic phases were collected and concentrated to half the initial volume and then lyophilized (16). Cyclotides mostly share hydrophobic properties; hence, they must enter butanolic phase. However, water phase may also include some important cyclotides.

### *Solid phase extraction*

Semi-purification of cyclotides with hydrophobic properties separated by butanolic phase was achieved by solid phase extraction (SPE) using C18 flash cartridges. 25 mg of the butanolic extract powder, which obtained from previous step was dissolved in ammonium acetate buffer (50 mmol.l<sup>-1</sup>, pH 8) and loaded onto C18 SPE (MACHERY-NAGEL, Germany) cartridge. The cartridge was first activated with methanol and equilibrated with the same buffer (16, 17). For releasing of hydrophobic cyclotides, the column was washed with 4 ml of 20%, 50% and 80% aqueous Ethanol.

### *Bradford assay & Tricine-SDS-PAGE*

The presence and concentration of the peptides in total ethanol extract, its water and butanolic phase, and semi-purified fractions were determined by using Bradford assay (18). In order to determine the purity and molecular weight of the partially purified cyclotides, Tricine-SDS-PAGE was performed using the protocol described by Schagger and Jagwa (19, 20).

### *Bacterial strains, extracts and media*

Examination of the antimicrobial effects of the crude extracts and their both separated water and butanolic phase of leaves (named as EL, WL and BL), and flowers (named as EF, WF, BF) as well as their partially purified cyclotides using C18 SPE column was performed by Radial diffusion assay (6) and also its modified method using MHB based culture media against human pathogenic bacteria, *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923).

### *Radial diffusion assay (RDA) and well diffusion assay*

The modified version of Radial diffusion assay (RDA) was previously used by Lehrer (21) and

Pränting group (6) for some purified cyclotides. In the current study, firstly, we examined the RDA method used by the Pränting group, for this purpose, bacteria were cultured on TSB (Tryptic Soy Broth); after reaching to optimum density they were plated and washed by cold sodium phosphate buffer. Nearly  $4 \times 10^6$  cfu were added to the bottom layer of the media containing 0.03% w/v TSB, 1% w/v LE agarose and 0.02% v/v tween 20. The mixture was added to Petri dish (85 mm). Different amounts of crude samples and semi-purified cyclotides were added to 3 mm gel punch wells. The mixture was incubated at 37°C for 3 hours in order to diffuse the peptides. Then the upper layer of the media (6% w/v TSB, 1% w/v agarose in 10 mM SPB) was added to the plates. The plates were incubated at 37 °C overnight. Due to the some contradictory results of the mentioned antimicrobial assays and better comparison of appropriate media for cyclotides and their crude extracts, we used MHA and modified MHB based culture media ( including MHB, agarose LE 1%, phosphate buffer 0.01 M and 0.02% Tween 20) within the well diffusion assay against the standard bacteria strains. Total protein quantities obtained from Bradford assay and the antibacterial activity results are reported in Table 1.

## **Results**

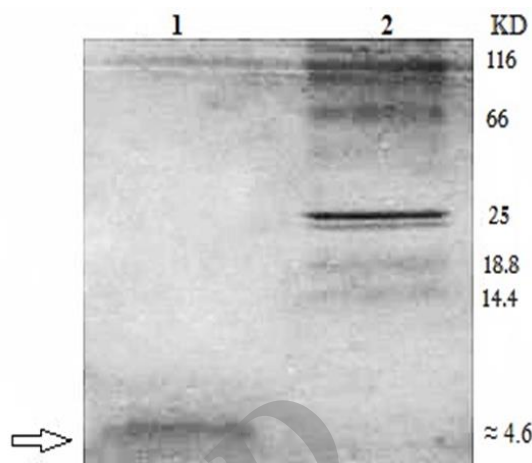
Attendance of partially purified cyclotides in different extracts was determined using the above mentioned methods. Tricine-SDS-PAGE resulted in the presence of peptides with a molecular weight of about 3500 to 4600 Dalton (Figure 1), which is compatible with the small size of Cyclotides. The concentration and purity of peptides in 50% elution of SPE column were more than in 80% elution. However, all crude and fractionated extracts were investigated for their protein quantity and antibacterial activities. The quantities (TPQ) for most, and no protein

**Table 1.** Antibacterial activities and total protein quantities of crude extracts and fractionated ones by RDA and WDA methods.

| microorganism                 | Assay method | Culture media                | extract      | Inhibitory zone diameter (mm) | Substance per well | Total protein quantity |  |
|-------------------------------|--------------|------------------------------|--------------|-------------------------------|--------------------|------------------------|--|
| <i>Escherichia coli</i>       | RDA          | Mentioned in material method | BF-F 50%     | 13                            | 15 µL              | 0.02 mg/ml             |  |
|                               |              |                              | BF-F 80%     |                               |                    |                        |  |
|                               |              |                              | BL-F50%      | NE                            | 15 µL              | 0.005-0.02 mg/ml       |  |
|                               |              |                              | BL-F80%      |                               |                    |                        |  |
|                               | WDA          | MHA                          | EF-crude     | 15                            | 10 mg              | 0.25 %                 |  |
|                               |              |                              | WF-crude     | 8.5                           | 10 mg              | 0.18 %                 |  |
| EL-crude                      |              |                              | 8.5          | 10 mg                         | 0.25 %             |                        |  |
| <i>Pseudomonas aeruginosa</i> | RDA          | In material method           | BF- 50 & 80% | All                           | 15 µL              | 0.005-0.02 mg/ml       |  |
|                               |              |                              | BL-50 & 80%  | NE                            |                    |                        |  |
|                               | WDA          | MHA                          | EL-crude     | 16.5                          | 10 mg              | 0.25 %                 |  |
|                               |              |                              | EF-crude     | 8.5                           | 10 mg              | 0.66 %                 |  |
|                               |              |                              | EL-crude     | 13                            | 10 mg              | 0.25 %                 |  |
|                               |              | MHA                          | BL-crude     | 10                            | 4 mg               | Nd                     |  |
|                               |              |                              | BF-crude     | 17                            | 4 mg               | Nd                     |  |
|                               |              |                              |              |                               |                    |                        |  |
| <i>Staphylococcus aureus</i>  | RDA          | In material method           | BF- 50 & 80% | NE                            | 15 µL              | -                      |  |
|                               |              |                              | BL-F 50%     | 9.5                           | 15 µL              | 0.02 mg/ml             |  |
|                               |              |                              | BL-F 80%     | 9                             | 15 µL              | -                      |  |
|                               | WDA          | MHA                          | BF-crude     | 10                            | 4 mg               | Nd                     |  |
|                               |              |                              | BL-crude     | 8                             | 4 mg               | Nd                     |  |

Abbreviations: RDA, radial diffusion assay; WDA, well diffusion assay; NE, not effective; Nd, non-determined; BF, butanolic extract of flowers; BL, butanolic extract of leaves; EL & EF, ethanol extract of leaves & flowers respectively; WF, water phase from flower extract; fractions of 50 % & 80% eluted from SPE column.

Other non-effective tested samples were not reported.



**Figure 1.** Tricine- SDS- PAGE. lane1: molecular weight of protein marker. Lane2: Solution obtained with 50% aq EtOH. Arrow: Cyclotide.

results showed different amounts of total protein attendance for some fractions washed with C18 column especially 20% elution. Table 1 presents the results of TPQ for some crude extracts and fractions with antimicrobial activity. For special substances like cyclotides mostly sharing hydrophobic charged residues with different polarity, it is better to use appropriate culture media and test method. However, not all cyclotides even in almost pure samples may have antibacterial effects or show their effective potential within the crude extracts better than partially purified samples because of their synergism. Here, we used two culture media for better comparison for crude extracts, especially the crude butanolic ones. For semi-purified samples, only RDA method was used, and via the mentioned method, some of the 50% and 80% semi-purified cyclotide elutions' flowers and leaves had no effective antibacterial potential; however, their including crude butanolic extract presented antibacterial properties using only MHA culture medium. The results could be explained by low amount of cyclotides in the samples or their natural non-effectiveness against the tested bacteria, or even the use of inappropriate assay methods or culture media.

### Strain sensitivity to *Cyperus rotundus* tuber extract

In the blank disc method, zone of growth inhibition appeared at a minimum inhibitory concentration of 25 mg/ml for all bacterial strains (Table 2).

In broth microdilution methods, the MIC value for all bacterial strains was at a concentration of 0.1 mg/ml (Table 3).

### Discussion

Cyclotides have multiple biological activities, and their anti-microbial activity has been examined in many studies. In the current study, we focused on the extraction and semi-purification of cyclotides from Iranian *Viola tricolor*, and then examination of their antimicrobial effects against several human pathogenic bacteria. Antimicrobial activity of this herb for several crude extracts with different polarity has been evaluated before, suggesting a synergistic interaction between the compounds of *V. tricolor* herb and showing its low to significant activity against most tested of the microorganisms (15). The antibacterial properties of some plant derived cyclotides have been reported in several previous studies. Pránting et al. studied the antibacterial activity of cycloviolacin O<sub>2</sub> and kalata B1 against some gram positive and gram negative bacteria, which resulted in the effectiveness of cyclotide against the gram negative *E. coli* and no activity against the gram positive *S. aureus* (6). Gran et al. also were achieved to the same result by Pránting group; they reported that kalata B1 has no effect against *S. aureus* while it is active against the gram negative bacteria (22). In another study, Tam group examined the antibacterial activities of four synthetic cyclotides against different bacteria strains, which were active against the gram positive bacteria such as *S. aureus* (23); however, they were almost not active against gram negative bacteria. Zarrabi et al. examined the anti-bacterial activity of semi-purified cyclotides from *V.*

*odorata* against *E. coli*, *P. aeruginosa* and *S. aureus*. The results showed that the most susceptible bacterium was *S. aureus* (24). Similarly, Roshan et al. reported the same results (25). However, there are some contradictory results for the antibacterial activities of cyclotides have been studied till now. In the current study, using RDA method, the most susceptible bacterium to the semi-purified peptides extracted from *V. tricolor*'s flowers was *E. coli*, with the inhibitory zone diameter of 13 mm. The crude ethanol and butanolic extracts also showed better activities against the gram negative *E. coli* and *P. aeruginosa* than *S. aureus*. It is possible that the susceptibility between the studied strains as well as the natural activity of all cyclotides could be different. Other explanation could be the synergism effect of the total cyclotides used in some studies.

*Cyperus rotundus* tuber extract has been shown to possess antimicrobial activity (28). While its inhibitory effect against *Streptococcus pyogenes* growth was demonstrated by Mehta et al. (29), the whole plant extract has been shown to be ineffective against strains of *E. coli*, *Pseudomonas aeruginosa* and *Salmonella typhi* (30). A MIC value of 12.5 mg/ml has indeed been determined for certain bacterial strains treated with the rhizomes oil (17), whereas using the disc diffusion method, zone of growth inhibition was revealed in *Pseudomonas aeruginosa* and *Staphylococcus aureus* treated with the ethanolic extract of the plant (31). In a separate study, using the agar disk diffusion method, the ethanolic extract of *Cyperus rotundus* revealed a zone of growth inhibition for *E. coli* and *C. albicans* (32).

### Acknowledgements

We appreciate Iran National Science Foundation (INSF) to the financial support of this project (project 88000118).

### Conflict of interests

None of the authors has any conflicts of interest to disclose and all authors support submission to this journal.

### Financial disclosure

Financial support of this was project provided by Iran National Science Foundation (INSF).

### References

1. Livermore DM. The need for new antibiotics. *Clin Microbiol Infect* 2004; 10: 1-9.
2. Craik DJ, Daly NL, Bond T, Waine C. Plant cyclotides: a unique family of cyclic and knotted proteins that defines the cyclic cystine knot structural motif. *J Mol Biol* 1999; **294**: 1327-1336.
3. Jennings C, West J, Waine C, Craik D, and Anderson, M. Biosynthesis and insecticidal properties of plant cyclotides: The cyclic knotted proteins from *Oldenlandia affinis*. *Proc. Natl. Acad. Sci. U.S.A.* 2001; **98**: 10614-10619.
4. Lindholm P, Göransson U, Johansson S, Claeson P, Gullbo J, Larsson R., et al. Cyclotides: A novel type of cytotoxic agents. *Mol. Cancer Ther* 2002; **1**: 365-369.
5. Gustafson KR, Sowder RC, Henderson LE, Parsons IC, Kashman Y, Cardellina JH, et al. Circulins A and B: novel HIV-inhibitory macrocyclic peptides from the tropical tree *Chassalia parvifolia*. *J. Am. Chem. Soc* 1994; **116**: 9337-9338.
6. Pránting M, Lööv C, Burman R, Göransson U, Andersson DI. The cyclotide cycloviolacin O2 from *Viola odorata* has potent bactericidal activity against Gram-negative bacteria. *J Antimicrob Chemother* 2010; **65**: 1964–1971.
7. Craik DJ, Daly NL, Mulvenna J, Plan MR, Trabi M. Discovery, structure and biological activities of the cyclotides. *Curr Protein Pept Sci* 2004; **5**: 297-315.
8. Colgrave ML, Craik DJ. Thermal, chemical, and enzymatic stability of the cyclotide kalata B1: the importance of the cyclic cystine knot. *Biochemistry* 2004; **43**: 5965-5975.
9. Koehbach J, Attah AF, Berger A, Hellinger R, Kutchan TM, et al. Cyclotide discovery in Gentianales revisited-identification and characterization of cyclic cystine-knot peptides and their phylogenetic distribution in Rubiaceae plants. *Biopolymers* 2013; **100**: 438-452.
10. Svargard E, Goransson U, Hocaoglu Z, Gullbo J, Larsson R, Claeson P, et al. Cytotoxic cyclotides from *Viola tricolor*. *J Nat Prod* 2004; **67**: 144-7.
11. Tang J, Wang CK, Pan X, Yan H, Zeng G, Xu W, et al. Isolation and characterization of cytotoxic cyclotides from *Viola tricolor*. *Peptides* 2010; **31**(8): 1434-1440.
12. Gruber CW, Elliott AG, Ireland DC, Delprete PG, Dessein S, Göransson U, et al. Distribution and evolution of circular miniproteins in flowering plants. *The Plant Cell* 2008; **20**(9): 2471-2483.
13. Bisset NG, Wichtl M. Herbal drugs and phytopharmaceuticals. *Medpharm*. 2nd ed. 1994; p. 527.
14. Komorowski T, Mosiniak T, Kryszczuk Z, Rosinski G. Phenolic acids in the local species of *Viola tricolor* L. and *Viola arvensis*. *Herba Pol* 1983; **29**(1): 5-11.
15. Witkowska-Banaszczak E, Bylka W, Matlawska I, Goslinska O, Muszynski Z. Antimicrobial activity of *Viola tricolor* herb. *Fitoterapia* 2005; **76**: 458-61.
16. Claeson P, Göransson U, Johansson S, Luijendijk T, Bohlin L. Fractionation protocol for the isolation of polypeptides

- from plant biomass. *J Nat Prod* 1998; **61**: 77-81.
17. Poth AG, Colgrave ML, Philip R, Kerenga B, Daly NL, et al. Discovery of cyclotides in Fabaceae plant family provides new insight into the cyclization, evolution and distribution of circular proteins. *ACS Chem Bio* 2011; **6**: 345-355.
  18. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 2011; **72**: 248–254.
  19. Schagger H. Tricine-SDS-PAGE. *Nat Protoc* 2006; **1**: 16–23.
  20. Schagger H, Jagow GV. Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 1987; **166**: 368–379.
  21. Lehrer RI, Rosenman M, Harwig SS, Jackson R, Eisenhauer P. Ultrasensitive assays for endogenous antimicrobial polypeptides. *J Immunol Methods* 1991; **137**: 167-173.
  22. Gran L, Sletten K, Skjeldal L. Cyclic peptides from *Oldenlandia affinis* DC: molecular and biological properties. *Chem Biodivers* 2008; **5**: 2014-2022.
  23. Tam JP, Lu YA, Yang JL, Chiu KW. An unusual structural motif of antimicrobial peptides containing end-to-end macrocycle and cystine-knot disulfides. *Proc Natl Acad Sci* 1999; **96**: 8913-8918.
  24. Zarrabi M, Dalirfardouei R, Sephehrizade Z, Kermanshahi RK. Comparison of the antimicrobial effects of semipurified cyclotides from Iranian *Viola odorata* against some of plant and human pathogenic bacteria. *J App Microbiol* 2013; **115**: 367-375.
  25. Roshan A, Zarrabi M, Asgarani E, Kanaani MR. Sequence analysis of gene encoding cyclotides in three species of Violaceae and determination of their anti-microbial activities. *J Biochem Microb Technol* 2014; **2**: 1-7.