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Original Research Article

Optimization of cyclotide like peptide extraction methods and characterization of these peptides from *Viola tricolor*

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ABSTRACT

Cyclotides are antimicrobial peptides and play significant role as bio preservative in the food industry. Since foodborne diseases are a universal issue, in the present study the different methods for partial purification of cyclotide like peptides from *Viola tricolor* were compared and the antimicrobial activities of these peptides were investigated on *Staphylococcus aureus Escherichia coli*, which are important in foodborne diseases. Cyclotide like peptides from the aerial parts of the plant were extracted and it was partially purified with three methods including reverse phase chromatography, two phase system with reverse phase chromatography and ammonium sulfate precipitation. The samples were analyzed by SDS-PAGE, Tricine–PAGE, and reverse phase HPLC. It was found that the first method has lower MIC (Minimum Inhibitory Concentration) and is the best method.

***Keywords*:** Cyclotides, Peptides, *Viola tricolor*

INTRODUCTION

Plant antimicrobial compounds are investigated for their functional bio preservative properties and the plant antimicrobial peptides pAMPs) are one of them. The pAMPs consist of cyclotides, snakins, hevein, and knottin-like peptides, lipid transfer proteins (LTPs), peptides from hydrolysates, plant defensins, myrosinase-binding proteins (MBPs), and thionin [1]. Cyclotides are the largest family of plant peptides and are typically comprised of 28 to 37 amino acids, which are ribosomally synthesized. The N-terminal of the original chain of cyclotide peptides is attached to the C-terminal and makes a structural motif called CCK (Cyclic Cistine Knot). These features have the unique structure and remarkable stability of cyclopeptides against heat, chemicals, and enzymes. They have a wide variety of biological activities such as anti HIV, antimicrobial, anti pests, anti-fungal, and anti-tumor. Cyclotides are appropriate for drug design and food preservatives [2-6].

In the 1970s, the first cyclotide of *Oldenlandia affinis* (Rubiaceae), African plant leaves was found and called kalata B1 [7], and then the other cyclotides were isolated from other plants with different methods [8]. Nowadays, more than 150 cyclotides of approximately 30 plants of Violaceae and Rubiaceae families have been isolated [9, 10]. *Violat ricolor* is a member of the Violaceae plant family and is utilized in traditional medicine in order to soothe coughs and relieve fever, besides its antitoxic nature [6]. Three cyclotides containing cytotoxic activity have been reported based on a study on the cytotoxicity of the plant extraction [11]. In the present research, we compared the *Viola tricolor* (Fig. 1) cyclotides partial purification methods. Ultimately, the antimicrobial effects of cyclotide solution were measured on gram-positive (*S. aureus*) and gram-negative (*E. coli*) bacteria.

Figure 1. *Viola tricolor*

MATERIALS AND METHODS

Cyclotides extraction

Viola tricolor was collected from Damavand in Iran and dried at 50 ^{o}C. About 25 g of aerial parts of the plant were powdered. The extraction was done by dichloromethane/methanol (1:1) overnight at 21 ^{o} C in a shaker incubator. The solution was passed through Whatman paper filter. Then the water was added to this solution (1/2 whole volume). Two phases were separated

and then methanol was evaporated and the obtained solution was freeze-dried. The powder was dissolved in water; acetic acid was added to the extract (2% whole volumes), passed through the polyamide filter and dried. Further purification was performed by three different methods.

At the first method (reverse phase chromatography) the powder was dissolved in the ammonium bicarbonate buffer (pH 8.05) and immediately passed through SPE-C18 column, and the column was washed with ethanol 20%, 50% and 80%. Finally separated fractions were collected, dried and analyzed.

At the second method (two phase system and reverse phase chromatography), butanol and water (1:1) were added to the powder. The solution was shaken, and the butanol phase and aqueous phase separated. This step was repeated three times and in each time butanol phases were collected and the solvent evaporated. As above, the powder was dissolved in the ammonium bicarbonate buffer (pH 8.05) and immediately passed through SPE-C18 column. Then the column was washed with ethanol 20%, 50% and 80%. Finally the resulting fractions were collected, dried and analyzed. The third method (ammonium sulfate precipitation) the resulting powder was dissolved in water and precipitated by ammonium sulfate (saturation concentration 90%). The suspension was prepared and centrifuged for 20 min at 4 ^{o} C. The obtained precipitation was isolated and resolved. Then it was separated using dialysis bag (2000 MWCO).

Bradford assay

Bradford assay is a colorimetric method for determination of protein and peptide concentration.

Bradford solution preparation

10 mg of Coomassie Briliant Blue G250 was dissolved in 5 ml of ethanol 95%. Then 10 ml of phosphoric acid 85% was added and mixed thoroughly. Final volume was reached to 100 ml with water. The solution was filtered using filter paper.

Standard protein solution

To determine the protein concentration, the standard graph was drawn. 1 mg of BSA (Bovine Serum Albumin) was dissolved in water slowly. Then different concentrations of BSA were prepared in 6 tubes. 1 ml of Bradford solution was added to 20 µl of them and samples then mixed. Absorption was repoted at 595 nm [12].

SDS-PAGE

The discontinuous polyacrylamide gel electrophoresis (15%) containing SDS was used to investigate extracted proteins in according to

Laemmeli method (120V) and stained with Coomassie Brilant Blue R250 [13].

Tricine-PAGE

Tricine-PAGE was performed in order to determine the exact weight of the peptides. It contains running buffer, cathode buffer and anode buffer. It has three different parts consists of condensing gel (in this part proteins are condensed), spacing gel and separating gel, in this part proteins are separated based on their molecular weights. Separating gel: 3% C (the percentage of bisacrylamide), 16.5% T (the percentage of acrylamide and bisacrylamide), spacer gel: 3% C, 10% T, stacking gel: 3% C, 4% T. Electerophoresis was done at constant voltage 30V for about 1h. Voltage was raised up to 100V. At the first, the gel was fixed in solution containing 50 % methanol and 10 % acetic acid for 1 h. Then it was stained with solution containing 0.025 % Serva blue G in 10 % acetic acid for 2 h, finally it was destained with solution containing 10 % acetic acid for 2 h [14].

Reverse Phase- HPLC (RP- HPLC)

Reverse phase high performance liquid chromatography (RP-HPLC) was used to separate organic molecules based on their partitioning between stationary phase and the mobile phase [21]. However, proteins and peptides as organic molecules behave differently. They were absorbed to the stationary phase through hydrophobic forces and eluted by organic solvent. The elution of peptides from reverse phase supports was done by reducing the polarity of washing solution. HPLC analysis was performed using KNAUER (Germany) system and C18 column (Length × ID: 250 × 4.6 mm with precolumn) (Table. 1).

Two different phases were used to wash column. Mobile phase A consists of 0.05% TFA and mobile phase B consists of acetonitrile (ACN) (% 90) and trifluoroacetic acid (0.05 %). At the first, the column was washed with the 95 % gradient of solvent A and 5 % gradient of solvent B. Then the process of washing was continued by different percent of solvent A and solvent B (according to Table 1). Due to the hydrophobic surface, cyclotides was eluted in a late retention time.

Determination of antibacterial activity

Antibacterial activity was determined by Micro-broth dilution method according to CLSI [15]. At first, 20 µl of fluid nutrient broth culture was added to all the wells except the first well. Then 10 µl of each extraction was added to the first well. 5 µl of sample from the first well was transferred to the next well and the dilution

Table. 1. Column washing with a mobile phase

Run number	Time (min)	*Solvent A (%)	**Solvent B (%)	Flow (ml/min)
1	0	95	5	1
2	5	95	5	1
3	15	75	25	1
4	40	0	100	1
5	50	0	100	1
6	50.2	95	5	1
7	55	95	5	1

*Solution A: Trifluoroacetic acid (TFA) (% 0.05) in water
**Solution B: Acetonitrile (ACN) (% 90) + Trifluoroacetic acid (0.05%) in water

was done. The three wells at the end of the plate were used as culture medium without bacteria, culture medium with bacteria and culture medium with sample. Then two different kinds of bacteria included *Escherichia coli* and *Staphylococcus aureus* with 0.5 Mcfarland concentration diluted to 0.01 were added. The plates were placed in incubator at 37 ^{o}C for about 24 to 58 h.

RESULTS

In order to eliminate nonpolar materials, the extraction was done by a two phase system in which nonpolar materials were entered into the dicholoromethan phase and removed. Then the solution was passed through a polyamide filter to remove tannin and phenol. Hydrogenic interaction between proton donor groups of polyphenol and carbonyl oxygen of the amide group belonging to polyamides, and solution containing cyclotides passed through the filter. Then the three methods were applied to separate:

Method 1: Reverse-phase chromatography

Reverse-phase chromatography was used for the separation of polar and nonpolar components (Figure 2).

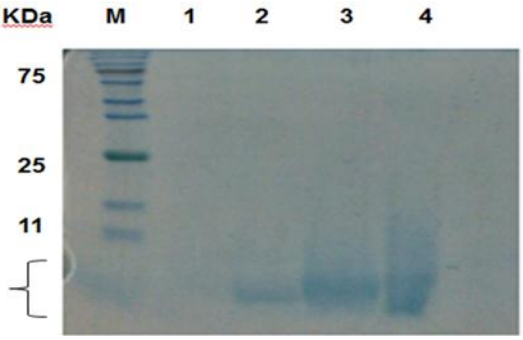


Figure 2. Method1. M: molecular weight marker, 1: SPE-C18 column output (washing by ethanol 20 %), 2 and 3: SPE-C18 column Output (washing by ethanol 50 %), 4: SPE-C18 column Output (washing by ethanol 80 %). polyacrylamide gel (15 %), stained by coomassie blue, constant voltage 120 V.

Method 2: Two-phase system and reverse phase chromatography

The two-phase system method was adopted for *Viola odorata* cyclotides extraction by Zarrabi *et al*. and finally, the separation was performed by SPE-C18 column (Figure 3). In this protocol we combined the methods used by Zarrabi and Hashempour [17, 20].

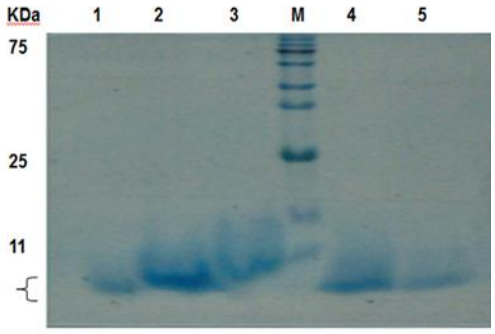


Figure 3. Method 2. M: molecular weight marker, 1: The SPE-C18 column Output (washing by ethanol 20 %), 2: SPE-C18 column Output (washing by ethanol 50 %), 3: SPE-C18 column Output (washing by ethanol 80 %). Method 3. 4: peptides precipitated by 90 % of ammonium sulfate saturation (dialysis), 5: peptides precipitated by ammonium sulfate saturation of 90 % (not dialysis). Polyacrylamide gel (15 %), stained by Coomassie blue, constant voltage 120 V.

Method 3: Precipitation by ammonium sulfate

The extraction containing a lot of components such as protein. To separate protein moiety from the other materials; proteins were precipitated using 90 % of ammonium sulfate saturation (Fig. 3). Finally, all the three methods were analyzed by SDS-PAGE (Figure2,3), Tricine-PAGE (Figure 4), and RP-HPLC.

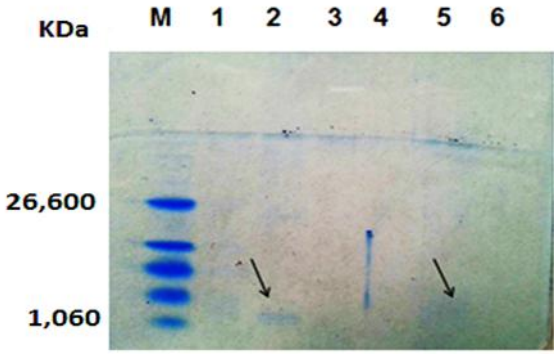


Figure 4. Tricine-PAGE. M: molecular weight marker. Method 2. 1: The SPE-C18 column Output (washing by ethanol 50 %), 3: SPE-C18 column Output (washing by ethanol 80 %). Method1. 2: SPE-C18 column Output (washing by ethanol 80 %). Method3. 5: peptides precipitated by 90 % of ammonium sulfate saturation (not dialysis), 6: peptides precipitated by 90 % of ammonium sulfate saturation (dialysis).

Bradford assay was applied to these methods. SDS-PAGE and Tricine-PAGE were performed in order to determine the weight of the peptides.

Due to the hydrophobic surface on the cyclotides, the RP-HPLC with C18 column was used. Since the C18 column is nonpolar; the nonpolar compounds interact with the column through hydrophobic interactions. Different compounds, based on their polarity, were separated by decreasing the solvent polarity. In the end, the strongest nonpolar compounds were eluted. According to the standard proteins which were extracted by Farhadpour [21], the cyclotides were eluted at 23 to 28 min (Figure 5).

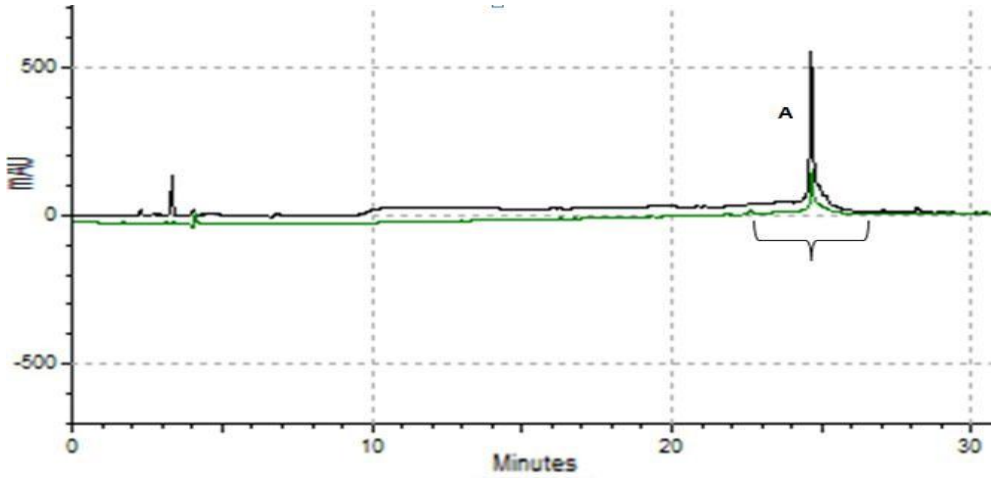
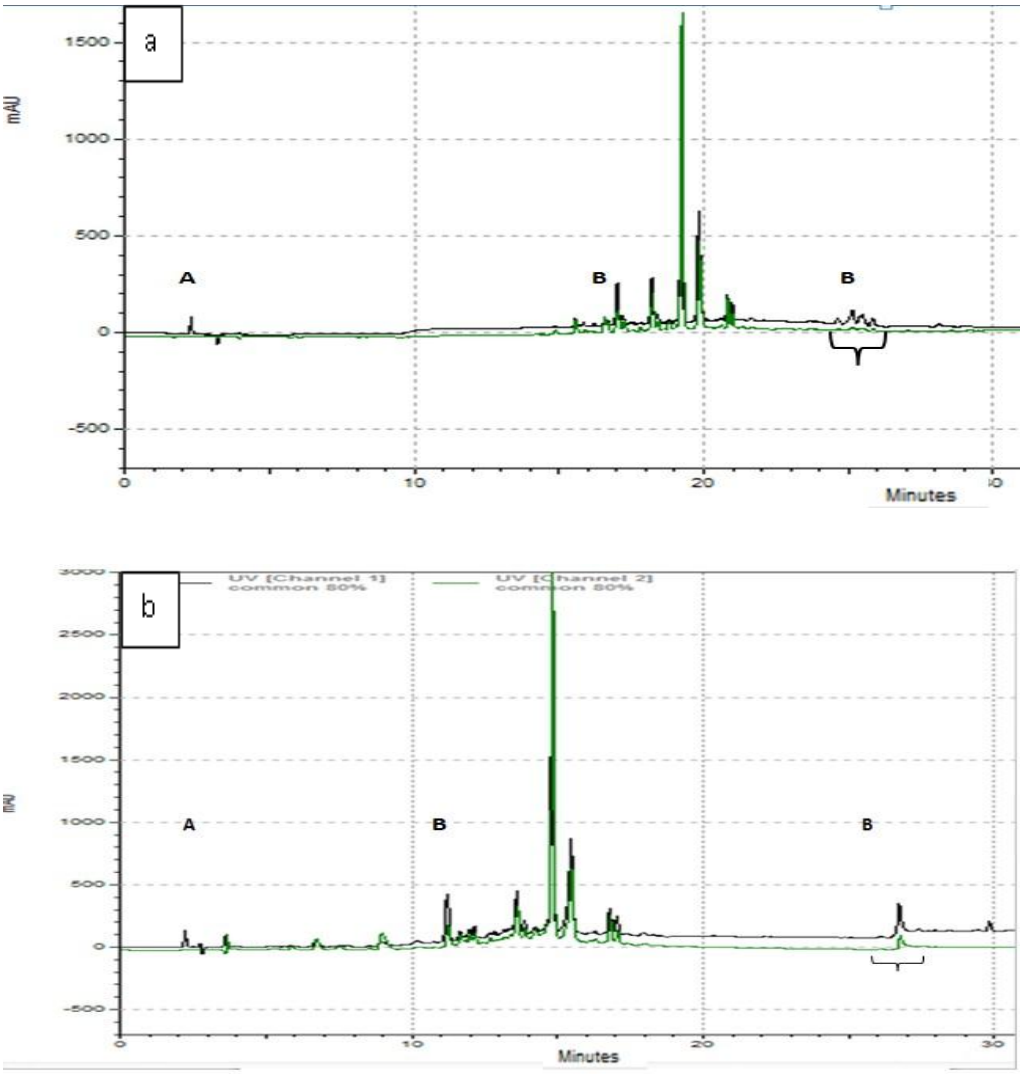


Figure 5. RP-HPLC chromatogram. Standard. A: Cyclotides. According to the standard proteins, cyclotides were eluted at the time of 23-28 min on the charts.

At first, the column was washed by a polar solvent. During this time, the nonpolar compounds attached to the column, and polar compounds came out of the column. The nonpolar compounds with different affinity attached to the column, were then washed and separated by the reduction of washing solvent polarity. Therefore, the nonpolar compounds which strongly attached to the column were eluted sooner. RP-HPLC was performed on both samples and standard; by comparison, between all three methods, the elution time of the peptides was determined. In all three methods according to the chromatogram, cyclotides like peptides were eluted at 23 to 28 min, and these compounds were considered as a solution containing cyclotides like peptides (Figure 6).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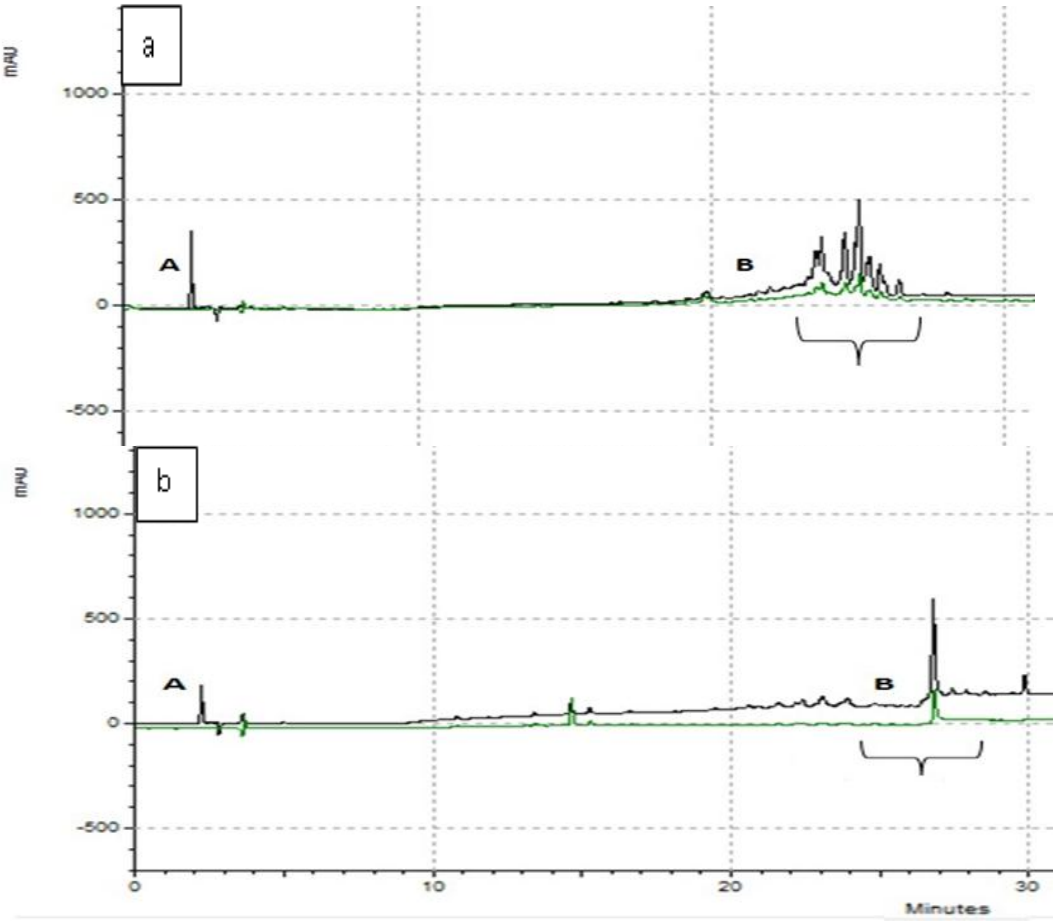
Method 1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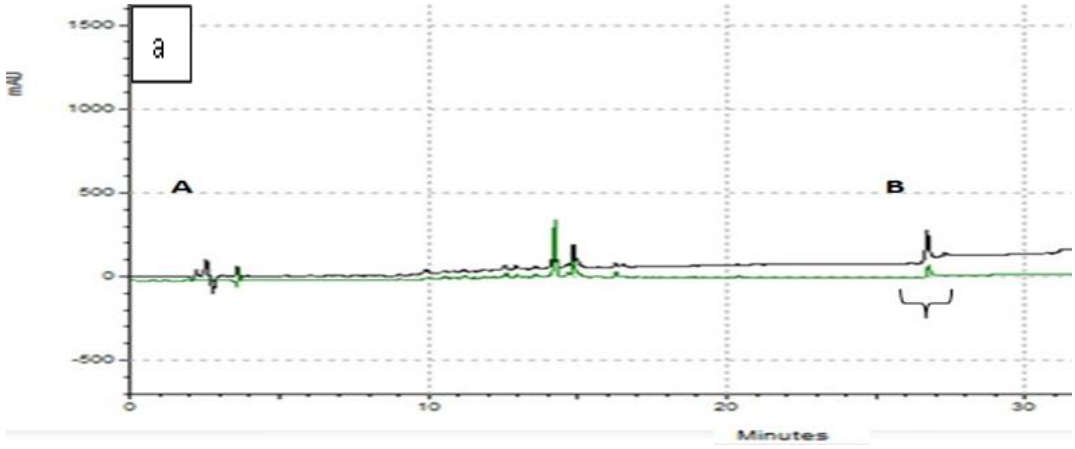
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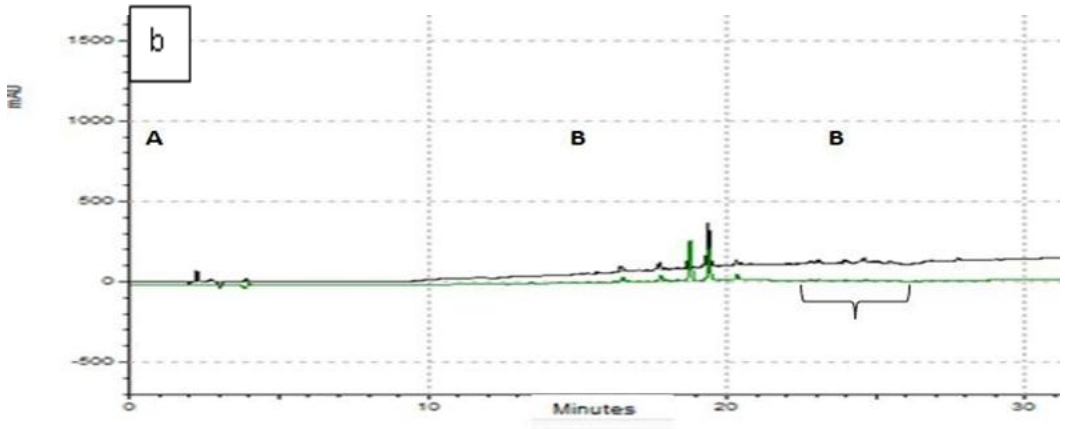

Method 3

Figure 6. RP-HPLC chromatogram. Method 1 and Method 2, SPE column washing with ethanol (a) 50 % and (b) 80 %. Method3, (a) without dialysis and (b) with dialysis. Peak intensity of polar compounds (A), which was eluted initially. The peaks are decreasing, and the peak of non-polar compounds (B) was appeared. Non-polar compound retention time is more. Non-polar compounds (B) were appeared at the time of 23-28 minutes on the charts were considered as a solution containing cyclotides.

Tricine-PAGE was applied for the analysis of proteins in the range of 1 to 100 KDa [14]. Antimicrobial activities of the same amount of cyclotide like peptides were extracted by three methods. The antimicrobial activities of the samples on gram-positive and gram-negative bacteria, including *S. aureus* and *E. coli* and the related MIC were determined (Table 2).

Table. 2. The amount of MIC (µg/ml)

Bacteria	sample resulting from SPE column washing by ethanol 50 % (Method 1)	sample resulting from SPE column washing by ethanol 80 % (Method 1)	sample resulting from SPE column washing by ethanol 50 % (Method2)	sample resulting from SPE column washing by ethanol 80 % (Method2)	sample resulting from Amonium sulfate precipitation (method 3)
***E. coli* ATTC25922**	6.71	6.28	10.83	16.9	15.6
***S. aureus* PTCC1431**	0.41	6.28	0.338	16.9	7.8

Bacteriostatic capability of samples was measured by MIC determination. It was determined that the Solid Phase Extraction (SPE) column samples washed with 80 % ethanol

showed the same MIC for *E.coli* and *S.aureus* in both the first and second method. The SPE column samples washed with 50 % and 80 % ethanol had the lower MIC in the first method in comparison with the other methods. It means that a lower concentration of samples had inhibiting effects on bacteria, and they have more bacteriostatic capability. The MIC results showed that *S. aureus* was more sensitive to these peptides than *E. coli*. Because the SPE column samples washed with 20 % ethanol had the lowest protein concentration according to the Bradford results and showing the colorless band in SDS-PAGE, these samples have not been studied by RP-HPLC. According to the SDS-PAGE and RP-HPLC results, cyclotides like peptides in terms of hydrophobicity and molecular weight properties, were more extracted in the second method, but according to the MIC results, antimicrobial effects were extracted more in the first method.

DISCUSSION

Viola tricolor is extensively employed as an herbal plant in traditional medicine [6]. As a result, a great number of researchers have been attracted to the analysis of cyclic peptides, called cyclotides [10,16].

Cyclotides are resistant to extreme environmental conditions [6,17]. Several cyclotides were extracted from the plants, and their cytotoxic effects were studied on cancer cells [16]. The antimicrobial activity of cycloviolacin O2 cyclotide of *Viola odorata* was investigated 0n gram-negative bacteria [18]. Plant antimicrobial peptides have been separated from different parts of a plant, and they were active against plants and humans pathogenesis. Cyclotides have exceptional structural properties and biological activities [18,19]. They have been extracted by various methods from numerous plants like the violet, legume, coffee, and cucurbit families [17]. Cyclotide extraction from *violaceae* for instance, *V. odorata, V. ignobilis, V. arvensis, V. philippica, V. hederaceae,* and *V.tricolor* had been performed [16-25]. Extraction in all of these methods was performed by dichloromethane or ethanol, and then the tannin was removed. In 2004, butanol extraction was passed through a SPE column by Goransson *et al*., and then cyclotide expression profiles were obtained by liquid chromatography –mass spectrometry (LC-MS) [22]. Extraction method which was used in 2008, extraction was done by dichloromethane/methanol/water (1: 1: 1) or acetonitrile/water (1: 1) and then was passed through solid phase columns and SPE-C18, in the next step, physical and chemical properties of the cyclotides such as hydrophobicity properties were evaluated [26]. In 2013, Hashempur *et al*. conducted cyclotides extraction from *Viola ignobilis*. They used a

dichloromethane/methanol (1: 1) extraction method. Finally the extraction was passed through SPE-C18. It contained fewer steps for extraction [17]. In this study, we applied the first and third methods in order to extract the cyclotides like peptide on *Viola tricolor*Because the method used by Zarrabi was time-consuming, the second method was applied, which also optimizes the cyclotides like peptide extraction processes. The second method was done through a combination of Hashempour and Zarrabi methods.

CONCLUSION

In all three methods, lower MIC amounts were obtained for the inhibition of *S. aureus than E.coli*. Method 1 was more appropriate because it was performed through fewer steps. Consequently, a fraction of the bacteriostatic ability that was eluted with 50 % ethanol was stronger than *S. aureus.*

The conflict of interest

Authors declare no conflict of interests.

ACKNOWLEDGMENT

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