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Biological activities of a new antimicrobial peptide from *Coriandrum sativum*

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Abstract

Antimicrobial compounds from plant sources have varied therapeutic potential, as investigation on which has today been becoming an active field. Purification of peptides was done with gel filtration and reverse-phase high-performance liquid chromatography (RP-HPLC). Antimicrobial activity of new antimicrobial peptide derived from coriander leaf extract has been investigated against different organisms by Radial Diffusion Assay (RDA) and Minimum Inhibitory Concentration (MIC). In the present study, a novel antimicrobial peptide with a wide antimicrobial activity was isolated from coriander leaf extract and the greatest antimicrobial effect of it was shown on *Staphylococcus aureus* strain with MIC 1.3 mg/ml which is an antibiotic resistant prevalent in hospitals. The new peptide showed effective germicidal effects on *Klebsiella pneumonia* and halted bacterial growth in concentration 2.65 mg/ml. Among the strains investigated, *Pseudomonas aeruginosa* was the most resistant strain with MIC of 3.2 mg/ml. This peptide was also influential on the fungi. There was no growth in concentration of 2.5 and 2.3 mg/ml for *Penicillium lilacinum* and *Asperjilus niger,* respectively. This peptide showed no hemolytic activity against human red blood cells. By multiple sequence alignment, this new peptide was named Plantaricin CS. Different agents have known in the extract of *Coriandrum sativum* that have potent biological activity such as antimicrobial activity. The new peptide was acquired in this work, has wide antimicrobial and low hemolytic activity. Thus, this peptide may be a useful drug in treatment infection diseases.

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Introduction

Up until now, bacterial and fungal diseases have dramatically affected on human health (Aslan *et al.,* 2010; Lee *et al.* 2005a; Reddy *et al.* 2004; Zardini *et al.* 2012). Hence, discovery and work on antimicrobial compounds has been an active branch in medical sciences. Due to increasing antibiotic-resistant microbial strains, the discovery of new antimicrobial compounds has been more significance (Reddy *et al.* 2004). Regarding to this, various sources of poisonous secretion of animals such as snake, scorpion, spider, amphibians, insects and etc have been discovered with diverse effects (Lee *et al.* 2005a; Bulet *et al.* 1999; Chou *et al.* 2008; Toke 2005). The plants also have an equal importance with animals in this way which herbal plants have considered more in traditional medicine. Antimicrobial herbal compounds are one of the valuable medical resources that more identification of these extracts and compounds will be useful in association between spread of infectious diseases and treatment of patients (Adwan *et al.* 2010). Among different agents, antimicrobial peptides (AMPs) are alternatives for conventional antibiotic drugs (Zasloff 2002). Antimicrobial compounds with plant sources have numerous therapeutic potentials. They are effective in the treatment of infectious diseases and also simultaneously reduce a large number of side effects that are often associated with antimicrobial compounds (Ibrahim and Osman 1995; Jeevan Ram *et al.* 2004; Jin *et al.* 2009). Antimicrobial peptides have been made from various plants can be effective on a variety of pathogenic bacteria and fungi (Hancock and Diamond 2000; Marcus *et al.* 1997). Historically, this source of antimicrobial compounds can fight with infection (Webster *et al.* 2008). It is estimated that 250000 to 500000 plant species exist on Earth and some of them are widely used in medicine (Borris 1996). Different activity-related to aspects of plant compounds have been investigated against various bacteria, fungi, viruses and protozoa. Hexan extract of the stem bark of *Amona glabra* (Padmaja *et al.* 1995), alkaloid extract from dried seeds of *Semecarpus anacardium* (Chakraborty *et al.* 1995), alcoholic extract of the stem bark of *Clausena anisata* (Rana *et al.* 1997), aqueous extracts of *Azadirachta indica* leaves (Rana *et al.* 1997) and oil extract from the foliage of *Santolina chamaecyparissus* and *Aegle marmelos* (Lehrer *et al.* 1991) have shown antimicrobial activity against various bacteria and fungi. *Coriandrum sativum* is a flavoring used in foods of China, Mexico, India, South America as well as Iran. The Coriander leaf has a traditional use as flavoring, galactagogue, carminative and antiseptic (Wong and Kitts 2006; Ikeura *et al.* 2010). In Iranian traditional medicine, *Coriandrum sativum* is used for treatment of infectious wounds (Chithra and Leelamma 1997). The effect of Coriander leaf has been approved in lipid metabolism and its subsequent reduction (Chithra and Leelamma 2000; Asoodeh *et al.* 2012). Many antimicrobial peptides are reported from different plants, but there are no reports that introduce antimicrobial peptide from *Coriandrum sativum*. So, in line with above bodies of evidence, the present study has been conducted for purification and evaluation antimicrobial activity of novel antimicrobial peptides from Coriander leaf extract.

Materials and methods

Materials

Methanol, formaldehyde, acetic acid, sodium chloride, sodium hydroxide, ethanol, trichloroacetic acid, acetone, glycerol and chloridric acid were obtained from Merck Company. Trypticase soy broth (TSB) was purchased from High Media Company. All the other chemicals used, including agarose, methyl green, agar, Triton X-100, Coomassie Brilliant Blue R-250 and bromophenol blue were prepared from analytical grades.

The extraction

Sample extraction was carried out through maceration. For this purpose, Coriander leaf was first soaked in water and then crushed in a porcelain mortar and set aside for 10 hours in the room temperature. The solution was then heated for 3 hours in medium heat and was filtered by filter paper. The extract was concentrated to one-tenth of the original volume using the vacuum distillation and was

used for peptides isolation.

Ammonium sulfate precipitation and ultra-filtration

In order to obtain the protein extract, Coriander leaf extract was brought from zero to 85% concentration according to the standard ammonium sulfate precipitation and was saturated with salt via gentle agitation at 4 °C. Under this condition, proteins of the extract were suspended in solution as colloidal particles. The deposits containing small and large proteins in the solution were then collected by centrifugation (12000 rpm for 20 min.) and were dissolved in 5 mM PBS buffer at pH 6.8. To isolate low molecular weight compounds, the protein extract was passed through an ultra-membrane with a 10 kDa cut-off. The filtrated solution was concentrated using a 1 kDa ultra-membrane and lyophilized.

Peptide isolation and purification

The peptide purification was done by C_{18} semi-preparative reverse phase-high performance liquid chromatography (RP-HPLC) column. The purification was done at a period of 70 minutes, at a flow rate of 1 ml/min. Isolation process was monitored by spectrophotometer at absorbance 220 nm. The purity of each peak was evaluated using RP-HPLC by injection of a small amount of each peak on the analytical C_{18} column. Antimicrobial activity of purified peptide was analyzed on different microorganism.

Antimicrobial assay

To investigate the antimicrobial activity were used two methods including radial diffusion and macro broth dilution. The antimicrobial activity was assessed based on radial growth inhibitions and the minimum inhibitory concentration (MIC) in radial diffusion and macro broth dilution approaches respectively.

For assessment of antimicrobial activity was used radial diffusion assay (RDA) that described in our previous articles (Lehrer *et al.* 1991; Shukla *et al.* 2012; Amiri *et al.* 2012). Briefly, two layer medium (with poor nutrient and rich nutrient) was used for growth of microorganism as well as enhancement of sensitivity. Tryptic Soy Broth (TSB) and Potato Dextrose Agar (PDA) were used for bacteria and fungi respectively. *Staphylococcus aureus* PTCC1431, *Klebsiella pneumonia, Pseudomonas aeruginosa, Penicillium lilacinum and Asperjilus niger* were used for primary assessment.

Minimal Inhibitory Concentration was determined as quantifying of antimicrobial activity. Stock serial dilutions from 0.01 to 1 mg/ml_of the extract were prepared and 20µl of the peptide stocks was added to 180 µl of solution containing 10^6 CFU/ml of bacteria which was then poured into a plate. The micro plate was incubated at 37 °C for 18 hours. Following that, the absorbance for each well was read at 630 nm using an enzyme-linked immunosorbent assay (ELISA) reader and the results were compared with control samples (Shukla *et al.* 2012; Memarpoor-Yazdi *et al.* 2013; McKeegan *et al.* 2002). The MIC was defined the minimum peptide concentration with no growth. *Klebsiella pneumonia*, *Pseudomonas aeruginosa,* and *Staphylococcus aureus* were used for MIC determination. Experiments were carried out in triplicate. As determination of MIC in association with fungi, 180 micro liters of Sabouraud Dextrose Agar culture medium along with 10 microliters of fungal suspension (10^6 CFU/ml), 10 micro liters of serial concentration of the plant extract were poured in micro plates which were then incubated at 25 °C for 7 days. The MIC was similarly defined minimum peptide concentration with no growth. *Penicillium lilacinum* and *Asperjilus niger* were used for MIC determination. Experiments were carried out in triplicate (Anderssen *et al.* 1998).

Peptide sequencing

Peptide sequencing was carried out using mass spectrometry, in positive ionization mode on a MALDI-TOF/TOF instrument. This analysis was carried out by university off Leeds.

Phylogenetic analysis

Eight amino acid sequences of different peptides were obtained from the antimicrobial peptide database

(http://aps.unmc.edu/AP/main.php). These sequences along with those for new peptide were aligned using the Blast program. The alignment was then adjusted manually. A phylogenetic tree was obtained from the CLC main workbench Ver.5.5 software using the neighbor-joining method. Bootstrap analysis, with 100 replications, was performed on the phylogenetic tree to estimate the reproducibility of the tree topology. This analysis was done according to Asoodeh *et al.* article (Asoodeh *et al.* 2012).

Hemolysis assay

The hemolytic activity of peptide was determined using human erythrocytes (Asoodeh *et al.* 2012). Human whole blood (5 ml) was added to a tube with heparin and then centrifuged at 7, 000×g for 5 min. Isolated red blood cells (RBCs) were washed five times using 4ml sterile PBS (phosphate buffered saline) and centrifuged at 7, 000×g till the solutions were clear. RBCs were diluted into PBS buffer (20 ml). Diluted peptide samples were added to microfuge tubes containing 190 µL of diluted RBC (10% cells) serially. After incubation for 30 min at 37°C, the tubes were centrifuge at 8, 000×g for 5 min. An aliquot of the supernatant solution (100 µL) was removed and then diluted with PBS buffer for obtain a final volume of 1 ml. Finally, absorbance was recorded at 567 nm. Triton X-100 was used as a positive control to compare the hemolysis effect of the identified peptides.

Results

Purification of peptides

Totally, 11 fractions were obtained from plant extract by RP-HPLC as indicated in Figure 1. Antimicrobial activities were found to more be concentrated on fractions C6. Thus, this peak was subjected to further purification by using the same RP-HPLC conditions, except for the usage a gradient of 0.5% eluent B per minute (Figure 1b). As illustrated in Figure 1ba, three peaks were eluted by C18 RP-HPLC. The eluted fraction is indicated by an asterisk (C6-2) in Figure 1b, was found to contain antimicrobial activity (Figure 2 and 3). Therefore, this peak was further analyzed by mass spectrometry and subjected to amino acid sequencing.

Table 1. The MIC values of Plantaricin CS.

Microbe	MIC(µg/ml)
S.aureus	35.2±0.25
K.pneumoniae	71.55±0.23
P.aeruginosa	86.4±0.24
P. lilacinum	67.8±0.24
A. niger	62.1±0.26

Biological activity of peptide

Antimicrobial activity

The results showed that the fraction C6-2 obtained from coriander leaf has inhibitory effect on three bacteria that was studied in this work. This peptide has also antimicrobial activity against the fungi investigated in this study. The qualitative assessment results on antibacterial activity of the C6-2 are shown in Figure 2. The results indicated that antibacterial activity of this peptide effectively against the bacteria. As clear from qualitative results, *Staphylococcus aureus* and *Pseudomonas aeruginosa* have demonstrated the most and the least sensitivity to the extract respectively. As shown in Figure 2, extract-related diameter of the inhibition zone is more than 30 µg of neomycin and kanamycin antibiotics, demonstrating more effective antibacterial activity of the extract. The peptide also showed significant antifungal activity in the area around injected site at concentration of 1mg/ml (Figure 3). As mentioned before, MIC was determined for quantifying antimicrobial activity of peptide from coriander leaf extract on the microbes investigated, and the results are presented in Table 1. As shown in table 1, the results for quantitative test are well overlapping with qualitative test, as *Staphylococcus aureus* and *Pseudomonas aeruginosa* were introduced the most and the least sensitive strains in quantitative test.

Hemolytic activity

The C6-2 showed low hemolytic activity. This fraction induced 1% human RBC hemolysis in 100 µg/ml concentration. This peptide exhibited no hemolytic effect near its MIC value (≤86.4µg/ml). This result is shown in table 2. As shown, hemolysis is very

negligible so that there is 0.95% hemolytic activity in the highest MIC values of this peptide. According to result, this new peptide may have a weak interaction with membrane of RBC.

Table 2. Hemolytic activity of the antimicrobial peptide Plantaricin CS.

Concentration (µg/ml)	Hemolysis (%)
0	0.09±0.01
5	0.11±0.09
20	0.25±.05
40	0.35±.08
60	0.67±0.06
80	0.95±0.7
100	1±.08

Structural analysis

Peak C6-2 was analyzed by mass/mass spectrometry. The MS/MS spectrometry analysis of this peak was shown in Table3. The sequencing of this peptide is: GGYKNFYGSALRKGFYEGEAGRAIRR with molecular weight 2924.24 Da. There are multiple basic amino acids in the sequence of this peptide. Analysis using the CLC main workbench Ver.5.5 software showed that this new peptide had the predicted pI (isoelectric point) of 10.25. Multiple sequence alignments of C6-2 were carried out along with 8 antimicrobial peptides obtained from the antimicrobial peptide database (APD). The result of the BLAST showed that this peptide has a high homology to the Plantaricin JK (Brul and Coote 1999) from *Lactobacillus plantarum C1* (Figure 4a). The phylogenetic tree also showed that this new peptide had the highest similarity to Plantaricin JK (Anderssen *et al.* 1998) (Figure 4b). C6-2 showed no complete sequence homology to any AMP in the database, suggesting that it is a novel AMP. It's named Plantaricin CS based on a systematic nomenclature for antimicrobial peptides (Brul and Coote 1999; Conlon 2008).

Table 3. Primary structures, molecular weight, homology peptides and physicochemical features of the peptide identified from coriander leaf extract.

Peptide	Sequence	Mass(Da)	Charge	Pi	Homology peptide
Plantaricin CS	GGYKNFYGSALRKGFYEGEAGRAIRR	2924.24	+4	10.25	None found

Discussion

Plant extracts are a complex combination of different chemical elements with different values. Due to significant alteration in these compounds, biological effects of the extracts are varied. Antimicrobial properties of some herbal extracts have been identified (Lo Cantore *et al.* 2004). Regarding to these properties and other biological impacts, plant extracts was considered as an appropriate substitute for antibiotics in potential therapeutic targets (Lo Cantore *et al.* 2004) and/or health-cosmetics products and food industry (Selitrennikoff 2001). Among different compounds, antibacterial peptides have become an interesting research for the treatment of bacterial and fungal infections. Many antimicrobial peptides with different structures and functions such as cyclotides, glycine-rich proteins, snakins, albumins and purothionin derived of plants have been reported (Delaquis *et al.* 2002; Guaní-Guerra *et al.* 2010; Pelegrini and Franco 2005). Coriander is a pharmaceutical plant with proven therapeutic potential; investigation on antimicrobial effects of the plant essential oils has shown a highly effective antibacterial activity (Ceska *et al.* 1988). The results of the present study is also demonstrated antimicrobial effects of new peptide derived from coriander leaves extract which is in conformation with other researches demonstrate medicinal effects of this plant (Cevallos-Casals *et al.* 2006; Farag *et al.* 1989; Kunyanga *et al.* 2012). In addition to antibacterial effects, findings also revealed antifungal activity of this new peptide, that it eliminated the *Penicillium lilacinum* and *Asperjilus niger* effectively at low concentrations. The antifungal activity was in consistence with previous studies in this field (Anderssen *et al.* 1998; Farag *et al.* 1989; Lv *et al.* 2011; Zairi *et al.* 2009). The results exhibited that in addition to antimicrobial activity of coriander oils as

well as therapeutic potentials of other parts of the plant such as seeds, coriander leaves can be effective, particularly in terms of antibacterial and antifungal activity (Wong and Kitts 2006; Ikeura *et al.* 2010). This new peptide was named Plantaricin CS. Plantaricin CS is hydrophobic (Figure 1). The amino acid sequence of purified peptide and isoelectric point are summarized in Table 2. Most antimicrobial peptides are 10–50 amino acids in length and the new peptide Plantaricin CS contain 26 amino acids (Boman 2003; Lee *et al.* 2005b). The net charge this new peptide is +4. Net charge and number of amino acids in Plantaricin CS is similar to other reported antimicrobial peptides from toads (Bagamboula *et al.* 2004; Wang *et al.* 2005; Lee *et al.* 2005a). Computer alignments with published sequences of other antibacterial peptides revealed striking similarities between parts of this peptide and Plantaricin JK, as shown in Figure 4. Plantaricin CS also exerted antimicrobial activities against Gram negative and Gram positive bacteria and fungi (Table1). Findings associated to antimicrobial activity of Plantaricin CS indicate promising therapeutic properties of this peptide against disease pathogenesis. Antibacterial activity of coriander essential oils has been well established on the *E. coli* strain (Anderssen *et al.* 1998). The present study demonstrated more effective antibacterial property of Plantaricin CS against the *Gram negative and Gram positive bacteria* strain using macro broth dilution and disk diffusion were applied. Antibacterial activity of Plantaricin CS against resistant *Staphylococcus aureus* strain represents it can be completely effective with MIC 35.2 µg/ml against these resistant bacteria. Findings also showed effective antimicrobial activity of the Plantaricin CS in comparison with neomycin and kanamycin antibiotic on three bacteria *Klebsiella, pneumonia* and halted bacterial. In this study, it has been observed that Plantaricin CS has less antimicrobial effect on gram negative bacteria which can be probably due to the presence of cell wall polysaccharides, preventing active compounds from reaching to cytoplasmic membrane of these bacteria (Cox *et al.* 2000; Omidbeygi *et al.* 2007). Fungi are more sensitive to plant essences compared to gram negative bacteria. Generally, herbal products contribute to cytoplasm granulation (Caccioni *et al.* 1998), cytoplasmic membranes rupture (Park *et al.* 1994; Tassou *et al.* 2000) and deactivation or inhibition of intracellular and intercellular enzyme activity, cell walls disintegration (Park *et al.* 1994) and also the destruction of electron transport system (Park *et al.* 1994); these cellular events can independently or simultaneously reach to a maximum level while preventing the mycelial growth (Tassou *et al.* 2000). Mechanism of antifungal activity of this peptide may be owing to any of the reasons mentioned. Moreover, Plantaricin CS has no hemolytic activity. The most antimicrobial peptides have high or low hemolytic activity (Park *et al.* 1994; Park *et al.* 2001; Suzuki *et al.* 1995) but Plantaricin CS has no hemolytic activity as it does not interact with the blood cell membrane.

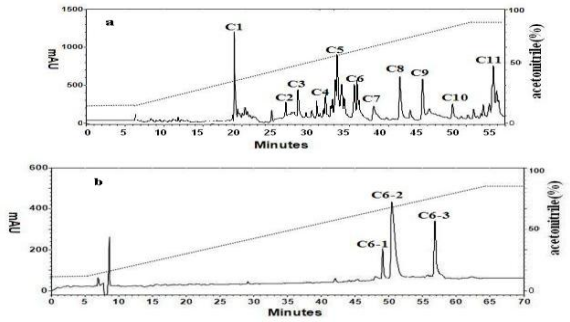

Fig. 1. Isolation of peptides from Coriandrum sativum. 400 µl aliquot of plant extract was loaded onto a semi-preparative C18 reverse-phase column. The elution was performed with a 1% acetonitrile gradient at a flow rate of 2 ml/min. The absorbance was monitored at 220 nm. Totally, 11 peaks were collected (a). The active fraction (C6) was further purified using an analytical C18 column by applying a mild slope of the eluent B, at a flow rate of 1 ml/min (b).

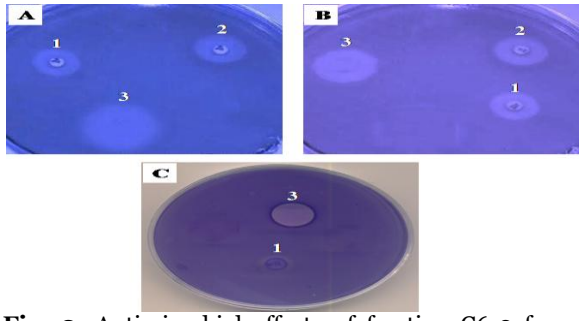

Fig. 2. Antimicrobial effects of fraction C6-2 from

coriander leaves extract against Gram-positive and Gram-negative bacteria.1: kanamycin, 2: neomycin, 3: fraction C6-2. A: *Klebsiella pneumonia*, B: *Pseudomonas aeruginosa*; C: *Staphylococcus aureus*. *Staphylococcus aureus* is resistant strain.

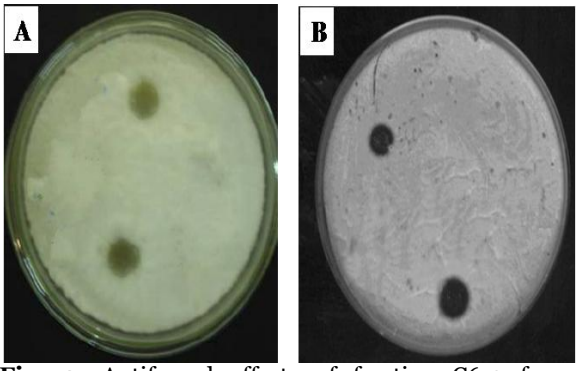

Fig. 3. Antifungal effects of fraction C6-2 from coriander leaves extract against fungi.
a:*Penicillium lilacinum*, b: *Aspergillus ni*ger.

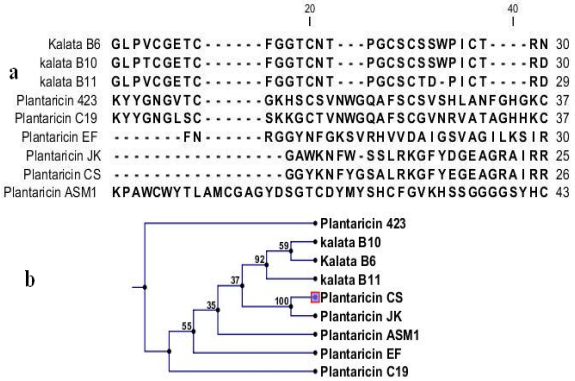

Fig. 4. The alignment and phylogenetic tree of Plantaricin CS (a) the alignment of amino acid sequences of Plantaricin CS with the sequences of other antimicrobial peptides. The alignment was carried out with CLC Main Work Bench Ver.5.5 software. (b) Phylogenetic tree of Plantaricin CS. Amino acid sequences of the 8 peptides obtained from the APD database were incorporated into the tree using the neighbor-joining method. The name of each sequence is typed at the end of the corresponding branch. Reliability of the tree was assessed by bootstrap analysis with 100 replications.

Conclusion

Plantaricin CS from Coriander extract has effective antimicrobial impact against Gram negative and Gram positive bacteria as well as fungi. It revealed more effective antimicrobial activity compared to common antibiotics like neomycin and kanamycin.

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