

## Original Article

# Novel cyclotides from *Hedyotis biflora* has potent bactericidal activity against Gram-negative bacteria and *E. coli* drug resistance

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**Abstract:** Background: *Hedyotis biflora* is a well-known herb in traditional Chinese medicine (TCM) which is used to treat various diseases, including cancer and inflammation. Recently, nine cytotoxic cyclotides were found in *H. biflora*, suggesting cyclotides may be bioactive ingredients in this herb. Materials and methods: In this present study, another two novel cyclotides: hedyotide B10 (HB10) and HB11 from the root of *H. biflora* were isolated, besides the known HB1 to HB9. By Edman degradation sequencing and gene cloning, we confirmed their amino acid sequences and obtained precursors of hedyotides. Radial diffusion assays (RDAs) and minimum inhibitory concentration (MIC) assays were used to screen their antimicrobial ability against four bacterial strains and its effect to bacterial drug resistance of *Escherichia coli* which generates extended-spectrum-lactamases (ESBLs). Results: Both cyclotides were bactericidal to *E. coli* and *Streptococcus salivarius* at low micromolar concentration, and both had the reverse effect to bacterial drug resistance. Conclusions: These results suggest that HB10 and HB11 might have potent activity against Gram-negative bacteria and bacterial drug resistance.

**Keywords:** Cyclotide, *H. biflora*, bacteria, drug resistance

## Introduction

Cyclotides are plant-derived cyclic peptides containing 28 to 37 residues [1]. They can be considered as mini-proteins because of their well-defined 3D structures with an end-to-end circular peptide backbone cross-braced by three disulfide bonds in a cysteine-knot arrangement of cysteine I-IV, II-V, III-VI [1, 2]. The absence of both termini and the presence of the knotted disulfide arrangement in a cyclic structure attribute to the extraordinary proteolytic, thermal or chemical stability of the cyclotides [3].

The discovery of the first cyclotide, kalata B1, from the Rubiaceae plant, *Oldenlandia affinis*, dated back in the early 1970s [4]. Kalata B1 produces an oxytocic activity and constitutes one of the active ingredients that caused the

uterotonic decoction kalata-kalata to accelerate child delivery in the tribal women in Congo. Since then, over 300 cyclotide sequences [2] had been found in the Rubiaceae, Violaceae, Cucurbitaceae and Fabaceae families [5-8]. Cyclotides likely played a role of host defense in plants and exhibited diverse biological activities such as anti-HIV [5], anti-cancer, antimicrobial, hemolysis, uterotensin, insecticidal, and nematocidal [6-10].

*H. biflora* was widely used in traditional Chinese medicine for a long history. Based on the old medical documents, it could be used to treat infection, inflammation and cancers. Recently, nine cyclotides were isolated and sequenced from *H. biflora*. Tam et al. reported HB1 and HB2 had a potent antimicrobial activity [11] and Qian et al. reported that HB3 to HB9 had strong cytotoxic activity [7].

The rapid development of antibiotic resistance in pathogenic bacteria had stimulated interest in the development of alternative pharmaceuticals that might complement or replace commonly used antibiotics. Antimicrobial peptides (AMPs) were discovered relatively recently, but they had become an intensive research field where much effort is focused on finding potential novel and future antibacterial drugs. Some studies about cyclotides suggested that this class of peptides might share the novel structure and had reverse effect of drug resistance to bacteria [8].

In the current study, we try to isolate more cyclotides from *H. biflora*, and further investigate its anti-bacteria effect. Finally, we find another two novel cyclotides from root of *H. biflora*. After confirming their sequence, we have a series of assays to investigate its anti-microbial effect and reverse effect on drug-resistance.

### Materials and methods

#### Screening cyclotides in *H. biflora*

We used previous method by Tam et al. [11], briefly, 500 mg material of each plant species was macerated and extracted with 2 mL of 50% ethanol. The extracts were diluted five-fold and subjected to C18 solid phase extraction (SPE) columns. The SPE columns were washed with 20% acetonitrile (ACN) and eluted with 80% ACN. The eluted fractions were subjected to MALDI-TOF MS to scan for mass signals between the 2-4 kDa ranges. Plant extracts showing positive signal in the desired mass range were subjected further for S-reduction and S-alkylation to verify the disulfide number.

#### Isolation and purification of hedyotide B10 to B11

Fresh aerial materials (5 kg) of *H. biflora* were macerated with 5 L of 50% ethanol and partitioned with 2.5 L of dichloromethane for defatting. After removal of plant debris, the ethanol/water fraction was dried in vacuum and dissolved in 200 mL of 10% ethanol. The concentrated extract was subjected to flash column packed with 100 g of C18 media (Agilent, USA). The column was washed with 20% ACN and eluted with 80% ACN to obtain the cyclotide-

enriched fraction containing main hedyotide B10 and B11. Isolation of individual peptides was then achieved by repetitive RP-HPLC using Agilent system.

#### S-reduction and edman degradation sequencing

20 µg of each peptide was dissolved in 100 µL of  $\text{NH}_4\text{HCO}_3$  buffer (100 mM, pH 7.8) containing 10 mM dithiothreitol (DTT), and incubated for 1 h at 37°C. Two-fold excess of iodoacetamide (IAA) over the total thiol was added and incubated for 1 h at 37°C. S-alkylated peptides were purified by RP-HPLC. All the purified peptides were sent to Zhang Jiang Biotechnology Co., Ltd (Shanghai, China) for Edman degradation sequencing.

#### RNA cloning

RNA was prepared from fresh plants and converted to single-stranded cDNA. The first seven amino acid sequence of cyclotides precursor was highly conserved, which was MAHFIKY [12], based on that, we designed the degenerative primers to perform 5' RACE (rapid-amplification of cDNA ends) to get the precursor genes, the sequence was 5'ATGGCYCATTTYAT-HAARGT3'.

#### Preparation of bacterial strains and *E. coli* strains producing ESBLs

Four bacterial strains from the ATCC were used, including *Staphylococcus aureus* ATCC 12600, *Streptococcus salivarius* ATCC 13419, *Streptococcus epidermidis* ATCC 14990, and *Escherichia coli* ATCC 25922. All dilutions were performed in PBS and cells were spread on Luria agar (LA) for viable counts. Seventy *E. coli* strains producing ESBLs were selected from strain samples in our laboratory based on Performance Standard for Antimicrobial Susceptibility Testing proposed by Clinical And Laboratory Standards Institute, USA [9, 10], strongest resistant strain was selected for reversal assay.

#### Radial diffusion assay (RDA)

A modified version of the RDA described by Lehrer et al. [13] was used to evaluate the antibacterial activities of the examined peptides. In brief, bacteria were grown in trypticase soy

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**Table 1.** Biological characteristics of three novel cyclotides from *Hedyotis diffusa*

Hedyotide	Sequence	MW (Da)	Net Charge
HB10	GISCAETCVKLPCLSSVIGCTCQNKRCYKD	3223	+2
HB11	GISCGETCVLLPCLSSVIGCTCQNKRCYKN	3193	+2

Note: Molecular weight is expressed as the monoisotopic mass of the molecule. Cysteine residues are highlighted in yellow.

broth (TSB, Becton Dickinson and Company, Sparks, USA) to an optical density at 600 nm of 0.3. 1 mL of culture was centrifuged at 3400×g for 10 min at 48°C, and the bacteria were washed once and resuspended in cold sodium phosphate buffer (SPB, 10 mM, pH 7.4). Approximately 4×10<sup>6</sup> cfu were added to 10 mL of previously autoclaved (42°C) SPB containing 0.03% w/v TSB, 1% w/v SeaKem LE agarose (Cambrex Bioscience Inc., Rockland, USA) and 0.02% v/v Tween 20 (Sigma-Aldrich, St Louis, USA). The mixture was vortex and poured into a Petri dish (diameter 85 mm). Evenly distributed wells were made using a 3 mm gel punch and 5 mL of peptide sample (concentration 200, 100 and 50 μM) or control (5 mL 10% DMSO) was added to each well. The agarose plates were incubated for 3 h at 37°C to allow diffusion of the peptides, after which the surface was covered with 10 mL of overlay gel solution (42°C) consisting of 6% w/v TSB and 1% w/v agarose in 10 mM SPB. After 18-24 h of incubation, the diameter of the clear zone around the wells was measured.

### MIC assay

Minimum Inhibitory Concentration (MIC) assays were performed in 10 mM SPB supplemented with 0.1% TSB in 96-microwell plates (round bottom; Nunc A/S, Roskilde, Denmark). Bacteria were grown overnight (10 mM SPB/0.1% TSB) and diluted to 5×10<sup>5</sup> cfu/mL. Then, 90 mL of bacterial solution was mixed with 10 mL of peptide solution of different concentrations (the highest concentration of each peptide was 1 mM). The microwell plates were incubated with continuous shaking at 37°C. The assay medium allowed sufficient growth of bacterium to visually determine the MIC of the different peptides. Experiments were performed with duplicates of each sample on at least two separate occasions and the average MIC was calculated.

### Reversal of drug resistance of *E. coli* by cyclotide hedyotide B10 and B11

Drug resistant *E. coli* strain was incubated with cyclotide at 10 μM for 18 to 24 h before being transferred to nutrient agar plates. After another 24 h culture, single colony on plates was picked and cultured in LB liquid for 12 h. Then suspension of strains was spread on M-H plates. The results were analyzed according to the method proposed by Kirby-Bailer [14].

### Statistical analysis

All values were expressed as Mean ± SD. The significance of the data was determined by one-way analysis of variance (ANOVA) followed by Student's paired t test or Student-Newman-Keuls analysis. A *P* value was less than 0.05 was considered significant.

## Results

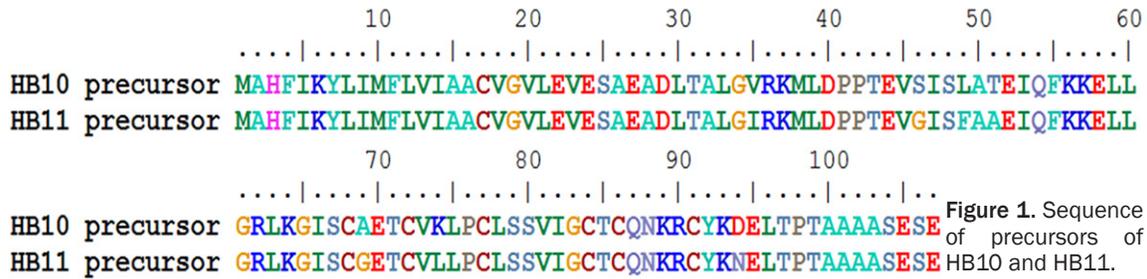
### Screening for novel cyclotide from *H. biflora*

Based on the previous report, cyclotide distribution are region-specific, even same plant, if they are from different regions, generally, they produce various cyclotides. We purchased the *H. biflora* from Zhe Jiang province of China, aimed to find more novel cyclotides. Finally two novel peaks which molecular weight was between 2800 Da to 3300 Da were found in mass spectrometry. They were confirmed as cyclotides by comparing the mass difference before and after S-alkylation with IAA. Each S-alkylated half-cysteine residue caused a mass increase of 58 Da. All of them displayed a mass shift of 348 Da indicating the presence of three cysteine bonds.

### Isolation and sequence determination of hedyotide B10 and B11

Two novel cyclotides which had m/z of 3193 and 3223 Da were isolated from the *H. biflora* by C18 reverse-phase HPLC. They were designated as hedyotide B10 to B11 respectively because HB1 to HB9 had been reported previously. All the cyclotides was sequenced by Edman degradation with help of Zhang Jiang biotech Ltd, Shanghai, China.

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**Figure 1.** Sequence of precursors of HB10 and HB11.

**Table 2.** RDAs with four strains treated by cyclotides

Strains	Diameter of clear zone in mm (well diameter subtracted from each zone)					
	HB10			HB11		
	200 $\mu$ M	100 $\mu$ M	50 $\mu$ M	200 $\mu$ M	100 $\mu$ M	50 $\mu$ M
<i>E. coli</i>	9.4 $\pm$ 0.24	6.8 $\pm$ 0.15	5.5 $\pm$ 0.42	9.2 $\pm$ 0.38	5.8 $\pm$ 0.34	5.1 $\pm$ 0.29
<i>S. salivarius</i>	7.5 $\pm$ 0.41	6.3 $\pm$ 0.38	4.6 $\pm$ 0.26	7.9 $\pm$ 0.51	6.9 $\pm$ 0.18	5.1 $\pm$ 0.17
<i>S. epidermidis</i>	0	0	0	0	0	0
<i>S. aureus</i>	0	0	0	0	0	0

results from the RDA, the MIC of each cyclotide against *E. coli* and *S. salivarius* was determined in a microdilution assay. The resulting MICs were presented in **Table 3**. In accordance with the RDA results, both hedyotide 10 and 11 could inhibit the growth of gram-negative strains.

**Table 3.** Cyclotide susceptibility of bacterial strains, as determined by MIC assays

Strains	MIC ( $\mu$ M)	
	HB10	HB11
<i>E. coli</i>	1.5	1.8
<i>S. salivarius</i>	2.1	2.2
<i>S. epidermidis</i>	>100	>100
<i>S. aureus</i>	>100	>100

### Reversal of drug resistance of *E. coli*

Both cyclotide hedyotide B10 and B11 reversed the drug resistance significantly, the results were shown in **Table 4** (Supplemental Figure 2), both hedyotide had more than 50% reversal rate.

### Discussion

Cyclotides are a large family of plant-derived mini-proteins that have been discovered and characterized over the last decade [1]. They range in size from 28 to 37 amino acids and possess several characteristics that are advantageous from a drug development point of view. In addition, cyclotides are stable against proteases such as trypsin, pepsin, thermolysin and endoprotease Glu-C, and their cyclic structure protects against exopeptidases [3, 15]. In the present study, we have screened cyclotides in *H. biflora* and found two novel cyclotide, HB10 and HB11. The two novel cyclotides had m/z of 3193 and 3223 Da, respectively and the amino acid sequence of HB10 and HB11 was determined by Edman degradation sequencing and gene cloning (**Table 1**). Database search revealed that these two peptides were novel and belonged to the cyclotide family.

The bactericidal activity against Gram-negative bacteria of HB10 and HB11 were tested with RDA and MIC. Both cyclotides at even concen-

The primary sequences of hedyotide B10 and B11 were summarized in **Table 1**. Database search revealed that these two peptides were novel and belonged to the cyclotide family.

### Encoding cDNAs of hedyotide B10 and B11

Degenerative primers were used to run a 5' RACE to get precursor genes of HB10 and HB11. Finally, we obtained their precursors, which were shown in **Figure 1**. The precursor sequence help us to further confirmed the primary sequence of two novel cyclotides.

### RDA and MIC assay

Activity against four bacterial strains above was evaluated and the resulting zone sizes could be found in **Table 2** (Supplemental Figure 1). Both cyclotides at concentrations of 200 and 100  $\mu$ M inhibited the growth of *E. coli* and *S. salivarius*, resulting in zone sizes of about 9 and 6 mm, respectively. To confirm and expand the

**Table 4.** Reversal of drug resistance by cyclotide HB10 and HB11

Cyclotide	Optimal concentration ( $\mu\text{M}$ )	Diameter before reversal (mm)	Diameter after reversal (mm)	Reversal rate (%)
HB10	100	16 $\pm$ 1.21	24.9 $\pm$ 1.88	55.6
HB11	100	16 $\pm$ 1.58	24.4 $\pm$ 2.27	52.5

trations of 50  $\mu\text{M}$  could inhibit the growth of *E. coli* and *S. salivarius*, resulting in zone sizes of 7 and 6 mm, respectively. In contrast, *S. aureus* and *S. epidermidis* was not inhibited by any of the cyclotides tested. A disadvantage with the RDA is the difficulty in interpreting the potency of the peptides, e.g. the peptides may diffuse differently in the media. It is also difficult to estimate how many cells have actually been killed/inhibited. To confirm and expand the results from the RDA, the MIC of each cyclotide against four strains was determined in a microdilution assay (**Table 3**). In accordance with the RDA results, *E. coli* and *S. salivarius* were more susceptible, while the Gram-positive *S. epidermidis* and *S. aureus* grew as well in cyclotide-supplemented media as without any effects. A possible explanation for the differences in susceptibility between the Gram-negative strains might be their ability to modify their membrane charges in the assay media. One example of a resistance pathway was the PhoP-PhoQ two-component system, which was regulated by extracellular  $\text{Mg}^{2+}$  concentrations [16].

Reversal of drug resistance of *E. coli* by HB10 and HB11 was tested with MIC. Significantly improvement of drug sensitivity of *E. coli* was found after incubated with HB10 and HB11. Generally, the drug resistance mechanism of Gram-negative bacteria species was complicated, including the synthesis of extended spectrum beta-lactamases ESBLs, AmpC, KPC, MBLs [17]. *E. coli* strains used in the present study could all generate ESBLs, which suggesting the potential of HB10 and HB11 in interfering the related pathways.

In the current study, two novel cyclotides are obtained from *H. biflora*, demonstrating plants from different region produce various cyclotides. By Edman degradation sequencing and gene cloning, we confirm their amino acid sequence. By a series of *in vitro* assays, we provide evidence that HB10 and HB11 may have good anti-bacteria bioactivities, especially for

gram-negative strains. Meanwhile, preliminary analysis suggests HB10 and HB11 could reverse the drug-resistant *E. coli* caused by ESBLs. Their anti-microbial effects are associated with their net charge status.

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#### Disclosure of conflict of interest

None.

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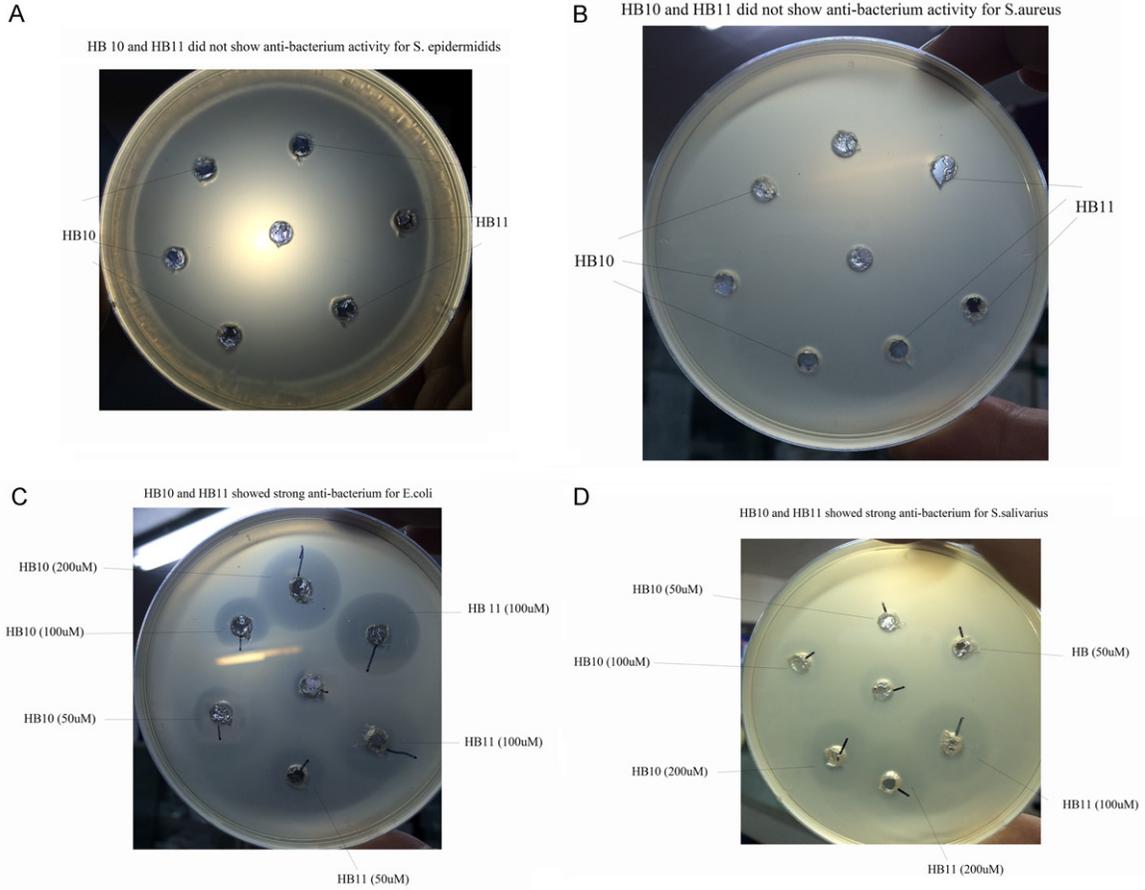
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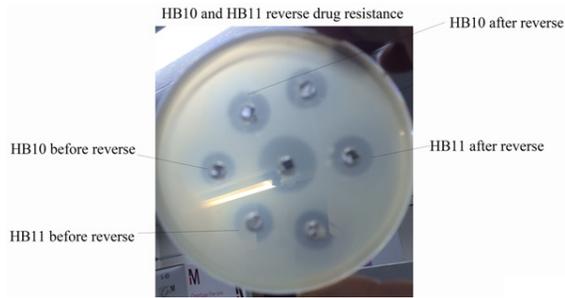
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**Supplemental Figure 1.** A, B. HB10 and HB11 did not show anti-bacterium activity for *S. epidermidis* and *S. aureus*. C, D. HB10 and HB11 showed strong anti-bacterium for *E. coli* and *S. salivarius*.



**Supplemental Figure 2.** HB10 and HB11 reverse drug resistance.