CHAPTER V

Discovery, isolation and structural characterization of cyclotides from *Viola sumatrana* Miq.

5.1 INTRODUCTION

Cyclotides are known as the largest family of circular disulfide-rich peptides family. They comprise of 28-37 amino acid residues with a head to tail cyclized backbone and six conserved cysteine residues which form three interconnecting disulfide bonds (CysI-CysIV, CysII-CysV, and CysIII-CysVI). A small embedded ring is formed by two intra-cysteine backbone segments connected by two disulfide bonds (CysI-CysIV, CysII-CysV), and penetrated by the third disulfide bond (CysIII-CysVI), which is known as the cyclic cystine knot (CCK) motif. This motif makes cyclotides extremely stable to thermal, chemical or enzymatic degradation.[107, 108] In general cyclotides are classified into two major subgroups, referred to as the MÖbius and bracelet subfamilies. They are different in size, type and number of amino acid residues between cysteine loops. MÖbius subfamily cyclotide possess a *cis*-proline residue in loop 5, which causes a 'twist' in the peptide bond[109]. On the other hand, the bracelet subfamily usually comprises of a hydrophobic α-helix in loop 3 which is not found in the MÖbius subfamily.[110] Both subfamilies have high sequence homology in loop 1 (glutamic acid, E) and loop 6 (aspartic acid, D/asparagine, N).[111] In recent years, a third cyclotide subfamily (trypsin inhibitor subfamily) was discovered. This subgroup comprises two trypsin inhibitor members extracted from the seeds of *Momordica cochinchinensis*, namely the MCoTI-I and MCoTI-II. They are cyclic and also contain six conserved cysteines which formed three disulfide bonds, however

both peptides have no sequence homology when compared to two previously mentioned subgroups.[112]

In the 1960-1970s, the first cyclotide named kalata B1 was discovered by a Norwegian doctor, Lorents Gran (Figure 1A). Kalata B1 was found in the African traditional medicine (Kalata-Kalata) which was derived from boiling leaf extract of the *Oldenlandia affinis* (Rubiaceae plant family) for accelerating childbirth. Kalata B1 is a 29 amino acid residues peptide. The discovery of this peptide leads to the further characterization of its complete sequence and structural elucidation by Seather et al.[111-113] In recent years, there are several examples of kalata B1[114] and other disulfide-rich cyclic scaffolds[115, 116] being used for drug design purposes as they have potential applications in a broad range of pharmaceutical and agricultural applications.[117]

The natural role of cyclotides in plants is likely to be host defense agents, based on their insecticidal activities.[118, 119] Moreover, cyclotides have been reported to exhibit a wide range of biological activities, including uterotonic activity,[120] anti-HIV activity,[121, 122] haemolytic activity,[117, 123] antimicrobial activity,[124] antifouling activity,[125] neurotensin antagonism[126] and cytotoxic activity.[127-129] Even though the mode of action from cyclotides on these biological properties is not fully understood, there are recent studies on the membrane binding properties of cyclotide[130] which show the interaction of the peptide towards phospholipid headgroups.

To date, more than 250 cyclotides have been discovered in several plant families, including the Violaceae (173 cyclotides), Rubiaceae (50 cyclotides), Fabaceae (18 cyclotides), Cucurbitaceae (5 cyclotides) and Solanaceae (3 cyclotides).[131, 132] The

majority of known cyclotides are found in the Violaceae family as this family itself contains more than 100 cyclotides.[131, 132] Simonsen *et al.*[133] also suggested that one plant species could contained over 100 cyclotides. If so, the total number of cyclotides exist could possibly be predicted to be around 9000 members.[122, 134]

In this study, we focus particularly on the Violaceae family (violets) as it is one of the flowering plant families (Angiosperm) which consist of 25 genera and approximately 900 species. To date, this family comprises of six genera, including *Gloeospermum sp.*, *Hypanthus sp.*, *Leonia sp.*, *Melicytus sp.*, *Rinorea sp.*, and *Viola sp.* Cyclotides discovery have been reported in these genera but they are only found to be more abundant in three main genera that are *Viola sp.*, *Hypanthus sp.* and *Rinorea sp.* *Viola sp.* is the largest genus of Violaceae family which contains half of the total plant species in this family with approximately 500 species.[135] Many studies have been done on the discovery and biological properties of cyclotides in Violaceae plant family such as *Viola abyssinica*,[129] *Viola arvensis*,[127, 136, 137] *Viola biflora*,[19] *Viola hederacea*,[2] *Viola odorata*,[117, 138] *Viola tricolor*,[12] *Viola yedoensis*[11] and *Viola philippica*.[18] These *Viola sp.* were collected in different locations around the world, including Asia (China), Europe (Sweden), South America (Argentina), Africa (Ethiopia) and Australia.

Every *Viola sp.* plant that has been studied so far contains cyclotides. However, limited studies have been done on *Viola sp.* from South-East Asia. Here, we examined cyclotide expression in a common *Viola sp.* in Thailand named *Viola sumatrana* Miq. A picture of *V. sumatrana* and its phylogeny classification are represented in figure 1B. *Viola sumatrana* Miq or *Viola hossei* W. Becker or Hong-Ron (Thai local name) is generally found in South-East Asia countries, for example,

Indonesia, Malaysia, Myanmar, Vietnam, Thailand and China. At present, there are several others Viola species that have been discovered in Thailand such as *V. angkae*, *V. betonicifolia*, *V. curvistylis*, *V. diffusa*, *V. hamiltoniana*, *V. hossei*, *V. inconspicua*, *V. pilosa*, *and V. rheophila*.[139] Although some *Viola sp.* have been used as traditional medicine but no reports were found on *V. sumatrana* so far except for its morphological characterization and biological diversity of this species.[140]

Here we report the present of four known cyclotides (kalata S, cycloviolacin O9, cycloviolacin O12, and cycloviolacin O22) in *V. sumatrana* and the structure of kalata S was evaluated by NMR and showed structure homology to kalata B1 which was expected from their sequence. Furthermore, some known cyclotides isolated from *V. sumatrana* have been tested in cytotoxic assay and shown toxicity against cancer cells in low micromolar concentrations. More interestingly, it is the first discovery of kalata S from the Violaceae family of Thailand.

5.2 MATERIALS AND METHODS

5.2.1 Plant material

Fresh leaves of *V. sumatrana* were collected from Khao Soi Daw Wildlife Sanctuary, Chantraburi province Thailand in May 2013. A voucher specimen (SN065812) is deposited at the Queen Sirikit Botanical Garden Herbarium: QBG, Mae Rim, Chaing Mai, Thailand.

5.2.2 Isolation and extraction of cyclotides from *V. sumatrana*

Fresh leaves of *V. sumatrana* were added into liquid nitrogen and grounded with a motar and pestle, giving 2 g of total crude powder. The crude powder was added with 100 ml of 50% (v/v) acetonitrile in 1% formic acid and stirred at room temperature (25 °C) for 4 hrs prior to peptide extraction before the crude solution was centrifuged for 45 min at 8,000 rpm for harvesting supernatant. Finally, the supernatant was then lyophilized with freeze drier and the dried peptide material was followed by further purification and characterization of cyclotides. The crude extract also was injected to nanospray-ESI-MSMS (Applied Biosystem) for obtained a peptide separation profile.

5.2.3 Solid-phase extraction (SPE) and RP-HPLC purification

Crude peptide material was initially purified with solid-phase extraction (SPE) and followed by RP-HPLC. The crude peptide was first dissolved in 1% (v/v) formic acid before SPE extraction. C18 SPE catridges of 500 mg from Waters was activated and cleaned up with 10 mL of methanol, 70% (v/v) acetonitrile in 1% (v/v) formic acid and 1% (v/v) formic acid, respectively. The crude peptide solution was then loaded to the catridges and eluted with 20-80% (v/v) acetonitrile in 1% (v/v) formic acid for cyclotides separation. All fractions were freeze-dried after fractionation.

All dried SPE fractions were re-dissolved in 0.05% (v/v) trifluoroacetic acid in water (buffer A) then loaded to a semi-preparative column on a RP-HPLC (LC10, shimazu). A phenomenex C18 RP-HPLC column (250nm x 10nm, 10μm, 300A°) was used for achieving pure cyclotides with a gradient of 0.5% min^{-1} of 20-80% solvent B (0.45% (v/v) trifluoroacetic acid in 90% (v/v) acetonitrile) at 3 ml/min flow rate.

Absorbance of eluting cyclotides was measured with a UV detector at the 215 nm and 280 nm. The HPLC purification step was repeated several times in order to obtain pure cyclotides for further characterization and biological testing.

5.2.4 Reduction and alkylation of cyclotides

Dried cyclotide was dissolved in 100 µL of water and then added with 1 µL of 1M NH_4HCO_3 (pH 8). The solution was reduced by adding 10 µL of 100 mM dithiothreitol (DTT) and gased with N_2 gasing before 30 min incubation at 60 °C. This reduced sample was then added with 10 µL of 250 mM iodoacetamide to prevent sample from reoxidation. Next, the alkylated sample was introduced with N_2 gas and left at room temperature for 30 min incubation. Finally, the reduced and alkylated sample was desalted with C18 Ziptip (Millipore) and eluted in 10 µL of 80% (v/v) acetonitrile with 1% formic acid. The desalted cyclotide was mixed with 7 mg/mL of MALDI matrix (α-cyano-4-hydroxycinnamic acid, CHCA) in 50% (v/v) acetonitrile/ 1% (v/v) formic acid in 1:1 ratio and analyzed using MALDI-TOF MS for molecular weight determination.

5.2.5 Enzymatic digestion coupled with nanospray and MALDI-TOF MSMS sequencing

Reduced and alkylated sample above was also used for enzymatic digestion by endoprotease Glu-C, trypsin or mixture of both enzymes. For single enzyme digestion, 2 µL of 1 µL endoprotease Glu-C or trypsin enzyme was added into a 20 µL sample. Meanwhile 20 µL of sample was mixed with 2 µL of both enzymes for double enzyme digestion. All digested peptides were then incubated at 37 °C for 6 hrs and desalted by using C18 ziptip before subjected to MSMS peptide sequencing.

Finally all digested peptides were analyzed using MALDI-TOF mass spectrometry (Bruker and Applied Biosystem) and nanospray mass spectrometry (Applied Biosystem) to determine peptide sequence based on the presence of b- and y- ions fragments from N- and C- terminus.

5.2.6 NMR sample analysis

Kalata S was dissolved in 90% H_2O/ 10% D_2O at pH 3.29 and 1% of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) was added as a chemical shift reference for spectral calibration. ^1H spectra and two-dimensional spectra (TOCSY and NOESY) were acquired. TOCSY and NOESY spectra were acquired with a mixing time of 80ms and 200ms, respectively and these were recorded on a Bruker Avance 600 MHz spectrometer at 298K. All NMR spectra were processed using TOPSIN (Bruker) program and analyzed using CCPNMR spectra assignment program version win32 2.3.1. The sequential assignments were done according to WÜthrich *et al.*[31] assignment procedure.

5.2.7 Evaluation of cell cytotoxic activity using MTT assay

The cytotoxic effects of cyclotides on human umbilical vein endothelial (HUVEC), two human brain cell line (U87 and U251), human colon adenocarcinoma cell line (HT29) and breast cancer cell line (MCF7) were evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. HUVEC and two human brain cell line (U87 and U251) were plated in 96 well plates at $3x10^3$ cells/well (100 µL) in 10% FBS/EBM-2 media (Lonza) and 10% FBS/DMEM (Gibco), respectively while HT29 and MCF7 were plated at $2x10^4$ cells/well (100 µL) in 10%FBS/DMEM (Dulbecco's Modified Eagle Medium). All cells were incubated at 37 °C in 5% CO_2 for

24 hrs. Before adding test compounds, media were removed and replaced with fresh 100 µL of serum-free EBM-2 and DMEM media. Cyclotides (kalata B1, kalata S, cycloviolacin O2 and cycloviolacin O12) were added in triplicates in initial concentrations ranging from 0.05 to 100 mM of to each well and incubate for 2 hrs. Controls such as vehicle control (negative control) and 1% Triton (positive control) were included in the assay. After 2 hrs of incubation, 10 µL of MTT (5mg/ml in PBS (Phosphate buffered saline)) was added to each well and further incubated for 3 hrs before removing supernatant. The MTT formazan crystals were dissolved in 100 µL dimethylsulfoxide (DMSO) and absorbance was measured using-Biotex spectrophotometer at 600 nm. Data were analyzed using GraphPad Prism program to and IC_{50} values were obtained from the sigmoidal dose response curve.

5.3 RESULTS AND DISCUSSIONS

5.3.1 Isolation, purification and mass spec sequencing of cyclotides from *V. sumatrana*

Cyclotides are most common in the Violaceae plant family. [108, 117, 122, 123, 127, 129, 130, 136-138] In this study, we isolated, purified and characterized cyclotides from *V. sumatrana*, a native plant from South-East Asia and China. This is the first report of cyclotides from South-East Asia, specifically from Thailand. Fresh leaves of *V. sumatrana* were extracted using 50% (v/v) acetonitrile in 1% formic acid and dried using freeze-dryer, giving 2 g of crude extract. Dried crude extract was then dissolved in 1% formic acid in milliQ water and subjected to nano-LC-MS. The separation profile obtained from nano-LC-MS of *V. sumatrana* leaves extracted is shown in figure 5.1. Four cyclotides (kalata S, cycloviolacin O9, cycloviolacin O12 and

cycloviolacin O22) were abundant. Three cyclotides (kalata S, cycloviolacin O12 and cycloviolacin O22) co-eluted in a single peak from nano-LC-MS whereas cycloviolacin O9 was eluted in a different peak. The crude extract was then purified using solid phase extraction (SPE) and cyclotides were eluted with approximately 30-50% acetonitrile and followed by further purification using RP-HPLC prior further characterization which include cyclotide sequencing and cytotoxicity assay.

Mass spectrometry was employed to characterize the cyclotides from *V. sumatrana*. All cyclotides were first reduced by DTT followed by alkylation with IAA. DTT was added to reduce disulfide bonds and IAA was added to prevent the refolding of peptide. An increase in 348 Da indicates the presence of six cysteines and three disulfide bonds which form the CCK motif. After the reduction and alkylation process, each cyclotide was subjected to enzymatic digestion with a range of proteolytic enzymes (endo-GluC, trypsin and mixed enzymes) before tandem MS sequencing. All isolated cyclotide sequences from *V. sumatrana* and their expected and theoretical masses are shown in Table 5.1. Sequence homology from previous studies reported was also used to identify the position of leucine and isoleucine in cyclotide molecules from *V. sumatrana*. All isolated cyclotides from *V. sumatrana* consist of two conserved amino acid residues (Glu 7 in loop 1 and Asn 29 in loop 6) especially Asn 29, which is critical for cleavage and cyclization.[141] In addition kalata S, cycloviolacin O12 and cycloviolacin O22 are categorized as Möbius cyclotides, while cycloviolacin 09 belongs to the bracelet sub-family of cyclotides based on its absence of *cis*-proline in loop 5. Figure 5.2 shows the MS/MS spectra of kalata S after reduction, alkylation and enzymatic digestion. The MS/MS fragment precursor (3242.1 Da) of kalata S (Figure 5.2A) after enzyme digestion is shown together with its *b*- and

y- ions series (Figure 5.2B and C). All isolated cyclotides sequencing results were compared to the known sequences in Cybase - a database which contains all published cyclotide information.[132]

Table 5. 1 Amino acid sequences of cyclotides from *V. sumatrana*.

Cyclotides	Retention time (min)	Native mass (Da)			Amino acid sequence
		experimental[a] m/z	experimental[a]	theory	
kalata S	41.03	959.72	2876.16	2876.18	GLPVCGETCVGGTCNTPG--CSCSWPVCTRN
cycloviolacin O9	39.09	1047.13	3138.39	3138.42	GIP-CGESCVWIPCLTSAVGCSCKSKVCYRN
cycloviolacin O12	41.08	964.39	2890.17	2890.20	GLPICGETCVGGTCNTPG--CSCSWPVCTRN
cycloviolacin O22	41.47	969.06	2904.18	2904.21	GLPICGETCVGGTCNTPG--CTCSWPVCTRN

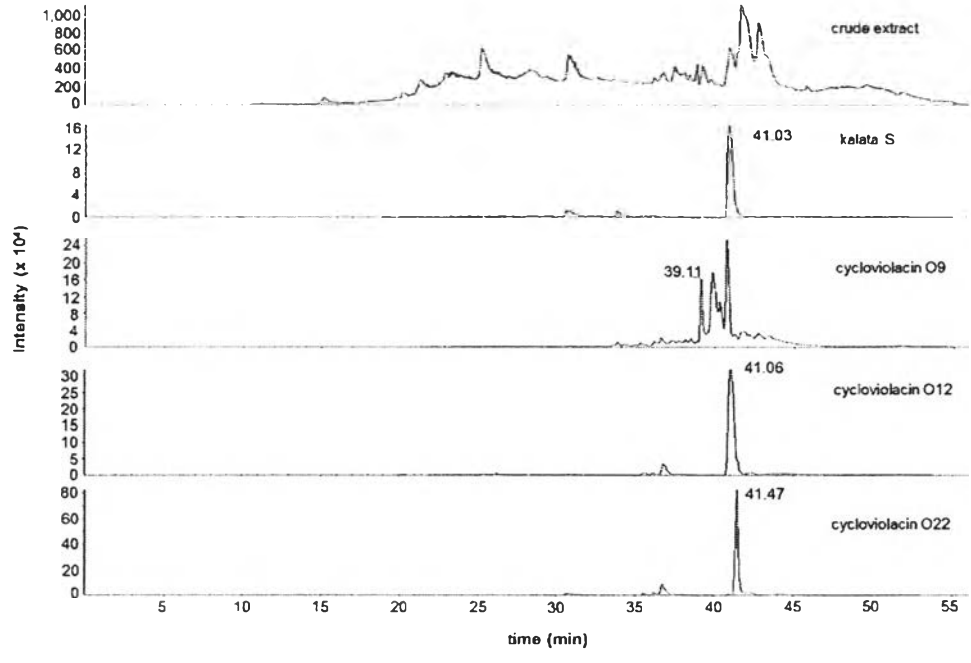

Figure 5. 1 Chromatograms of crude extract and cyclotides (kalata S, cycloviolacin O9, cycloviolacin O12 and cycloviolacin O22) isolated from *V. sumatrana*. Retention times for each known cyclotide were labeled in each chromatogram.

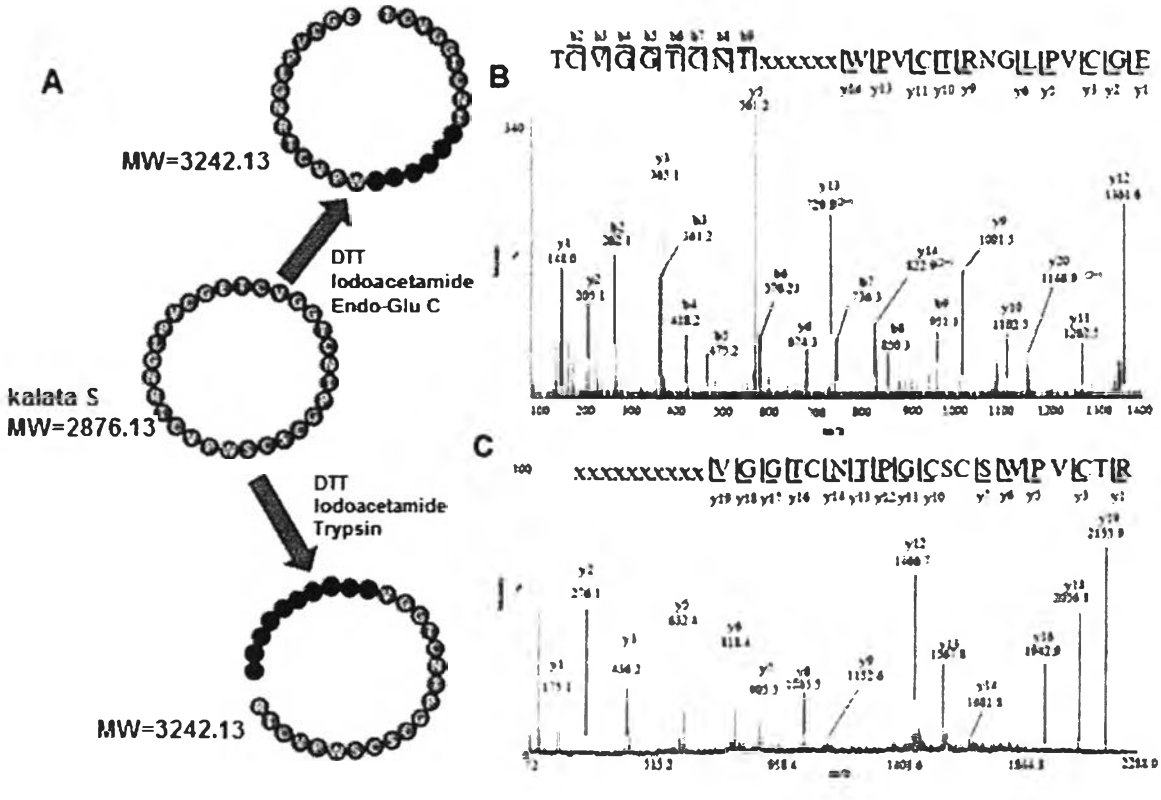

Figure 5. 2 Completed sequence of kalata S (A) derived from MS/MS spectrum of kalata S after reduction, alkylation and enzyme digestion using Endo-GluC (B) and trypsin (C) enzymes.

5.3.2 Structural analysis of kalata S by NMR

Kalata S (varv A) was the first cyclotide discovered from *V. arvensis* (Violaceae family) and is also commonly found in multiple members of the Violaceae family.[142] To date, only amino acid sequencing data and biological activities has been reported in this plant, therefore it is been interested to determine the structure of kalata S as none of NMR data have been reported so far. The sequences of kalata S and kalata B1 are similar, but different only by one amino acid residue in loop 4, i.e. serine 20 to threonine. This is supported by the amide spin system difference observed in the TOCSY and NOESY spectra of kalata S as shown in figure 5.3 and 5.4. A complete NOESY sequential assignments (αH_i-$NH_{(i+1)}$) of kalata S is shown in figure 5.4 with a complete list of chemical shifts in Table 5.2. To determine the secondary structure of kalata S, αH secondary chemical shifts of kalata B1 and kalata S were analyzed. Kalata S showed similar secondary shifts to kalata B1 which indicated both peptides possessed similar secondary structure (Figure 5.5). It is expected that both sequences only differ by one residue.

Besides the presence of *cis* peptide bond of proline in loop 5 of kalata S, it was indicate that the peptide belonged to the MÖbius subfamily. This event also found in kalata B1 but the βH resonates was slightly different since the condition of NMR analysis (pH and temperature). Consistency observed from αH chemical shifts of kalata B1 and kalata S, it was possible to postulate that kalata S had similar three-dimensional structure as kalata B1. Both NMR and mass spectrometry results correlates and provide further evidence for kalata S as a cyclic peptide which contains the unique CCK motif in its structure.

Table 5. 2 Chemical shifts of kalata S, at 298K, pH 3.29

Position	Residue	HN	Hα	Hβ	Others
1	Gly	8.63	4.25, 3.61		
2	Leu	7.77	5.07	1.94, 1.51	$_{\gamma}$H 1.71; $_{\delta}CH_3$ 0.99, 0.93
3	Pro		5.07	2.46, 1.75	CH_2 3.81
4	Val	8.16	4.67	2.6	$_{\delta}CH_3$ 0.86
5	Cys	7.99	4.47	3.36, 3.00	
6	Gly	8.52	3.85, 3.75		
7	Glu	7.17	4.81	2.01, 1.89	CH_2 2.53
8	Thr	8.44	4.58	4.46	$_{\gamma}CH_3$ 1.17
9	Cys	8.33	4.93	3.18, 2.95	
10	Val	8.51	3.9	2.05	$_{\gamma}CH_3$ 1.00
11	Gly	8.73	4.24, 3.86		
12	Gly	8.24	4.41, 4.05		
13	Thr	7.85	4.71	4.11	$_{\gamma}CH_3$ 1.16
14	Cys	8.67	4.72	3.06, 2.78	
15	Asn	10.37	4.77		
16	Thr	8.47	4.49	4.22	$_{\gamma}CH_3$ 1.34
17	Pro		4.24		CH_2 3.86, 3.61
18	Gly	8.78	4.19, 3.71		
19	Cys	7.7	5.32	3.83, 2.63	
20	Ser	9.54	4.69	3.75	
21	Cys	9.08	4.58	3.12, 2.82	
22	Ser	8.94	4.79	3.86	
23	Trp	8.02	4.09		
24	Pro		3.46	1.73, -0.13	CH_2 3.24
25	Val	8.25	4.22	1.95	
26	Cys	7.75	5.12	3.22, 2.75	
27	Thr	9.84	5.08		
28	Arg	8.75	4.76	1.67	$_{\gamma}CH_2$ 1.44; $_{\delta}CH_2$ 3.24
29	Asn	9.55	4.41	3.11, 2.82	

*chemical shifts were referenced to DSS

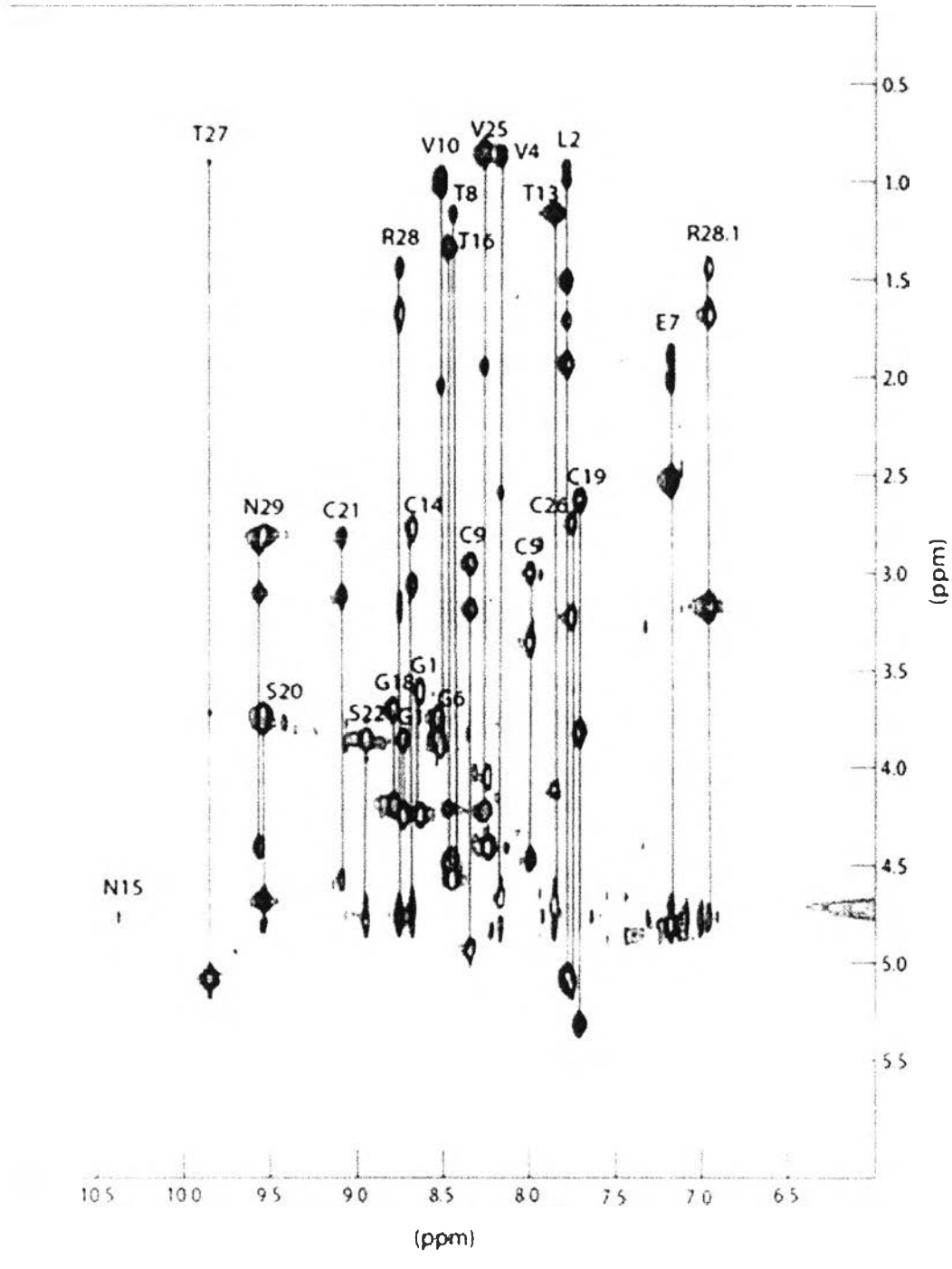

Figure 5. 3 The TOCSY spectrum of kalata S

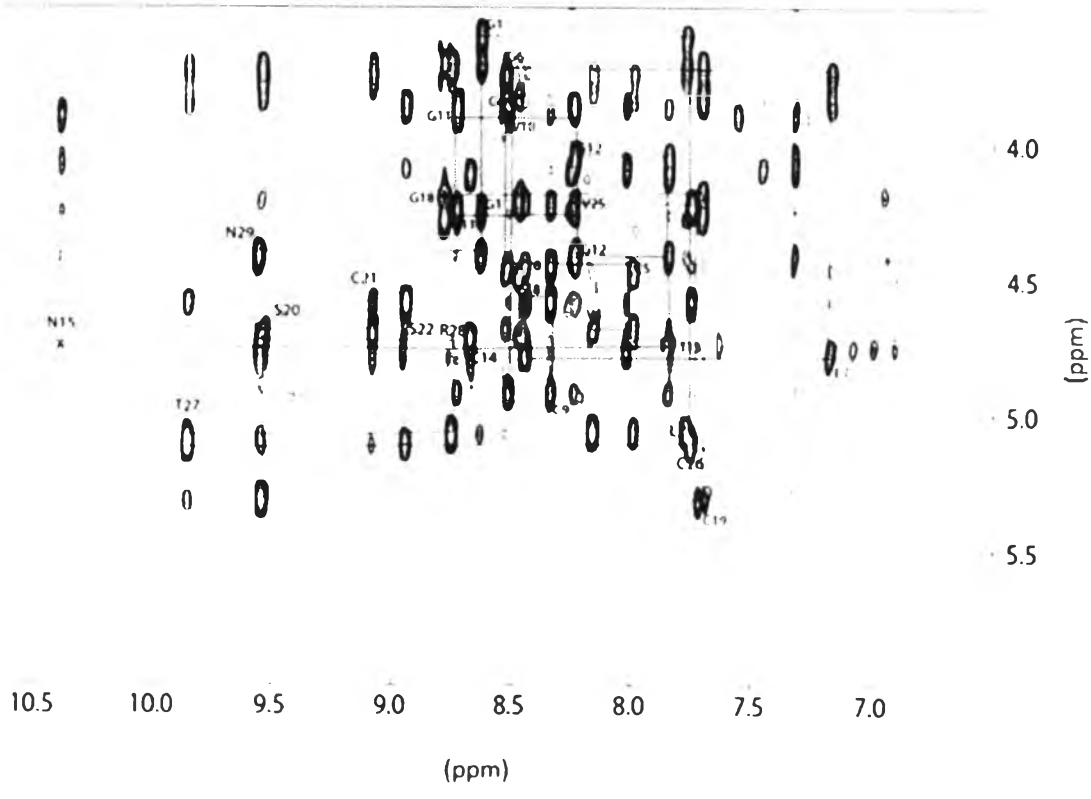

Figure 5. 4 The fingerprint of NOESY spectrum of kalata S

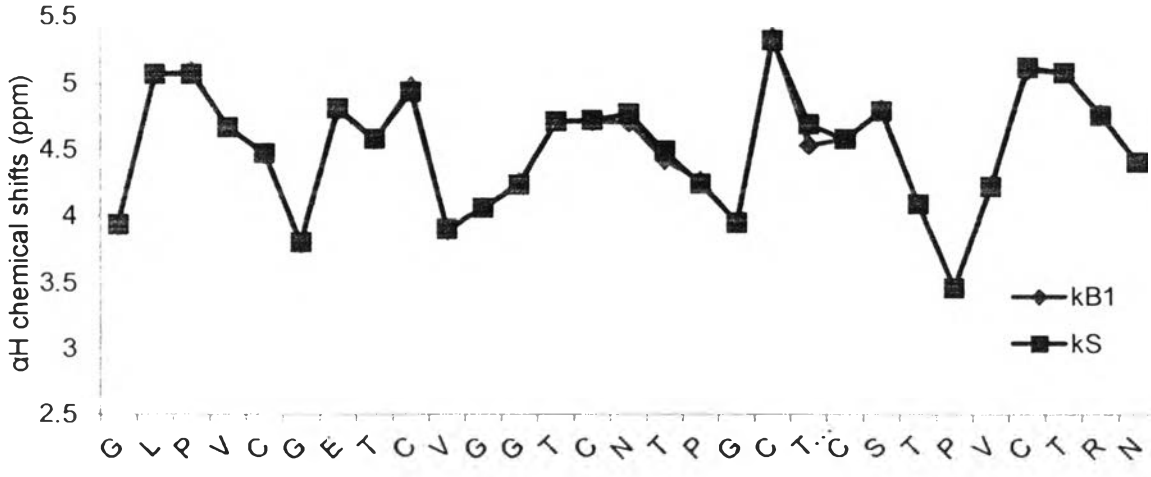

Figure 5. 5 αH secondary chemical shifts comparisonof kalata S and kalata ^{1}H NMR spectra were recorded at 298K and the αH secondary shifts were calculated by substracting the random coil ^{1}H NMR chemical shifts of Wishart et al.[143] from the experimental αH chemical shifts.

5.3.3 Cytotoxicity activity

Cytotoxicity of all isolated cyclotides were evaluated against HUVEC (non-cancer cell) and four different human cancer cell lines (U87, U251, HT29 and MCF7) using MTT assay as described in the experimental section. Cycloviolacin O2 and kalata B1 were chosen based on their effect on cell cytotoxicity. Cycloviolacin O2 was previously shown as the most cytotoxic cyclotide in the bracelet family. Meanwhile, kalata B1 was used to compare the results with kalata S since there was only one amino acid difference (Thr or Ser) in residue position 20 of the sequence. Cycloviolacin O9 and O22 were excluded from this assay due to limited amount of material available. Interestingly, previous publications have reported cytotoxicity screening of cycloviolacin O2 against U251, HT29 and MCF7 using SRB,[123] FMCA[144] and MTT assays[145], respectively. Despite the test on HT29 of kalata B1 by FMCA assay,144 however none of the screening of all cyclotides against U87 cells have been reported. The cytotoxicity of cycloviolacin O2 has been reported on MCF7 using MTT assay by Samantha et al.,[145] but different experiments, conditions were used when compared to current study. Therefore, it is interesting that this could be the first study to gather all information on cytotoxicity screening of four well-known cyclotides (kalata B1, kalata S, cycloviolacin O2 and cycloviolacin O12) against different human cell lines. The complete cytotoxicity results are illustrated in figure 5.6 and a summary result of cell cytotoxicity IC_{50} values is shown in table 5.3. Among all tested cyclotides, cycloviolacin O2 has the lowest IC_{50} value compared to other cyclotides. The IC_{50} values of cycloviolacin O2 for HUVEC, U87, U251, HT29 and MCF7 are 0.35, 0.45, 0.79, 0.87 and 0.29 μM, respectively. Cycloviolacin O2 was found to be the most cytotoxic against MCF7 cell line, consistent with previous published data.[146]

This might due to the different dispersion of hydrophobic amino acid on the cyclotide surface[16], although both bracelet and MÖbius families have similar ratio of hydrophobic residues. This might also indicates higher percentage of hydrophobic residues arrangement in cyclotide has an effect on cells cytotoxicity.

It is interesting to compare the IC_{50} value of kalata S and kalata B1 as these cyclotides differ only in one amino acid residue (Table 5.3). Both cyclotides have similar IC_{50} values for all cell lines. It could be due to both amino acid residues (Ser/Thr) in loop 4 of kalata S and kalata B1 are not hydrophobic[21] and loop 4 does not normally interact with cell membrane. Furthermore cycloviolacin O12 was also compared to kalata S on cytotoxicity since both cyclotides have only one residue difference in loop 6 (isoleucine/valine). Both cyclotides also showed similar IC_{50} values on U87, U251 and HT29 cancer cells, except cycloviolacin O12 has more potent cytotoxicity against HUVEC and MCF7 cells when compared to kalata S. From these results, it could explain the change in amino acid hydrophobicity in loop 6, which normally interacts with cell membrane, might have an effect to cells like the case of cycloviolacin O12. In addition, the cytotoxicity of cyclotides on U87 and U251 is also interesting for comparison as both cell lines are human glioblastoma cells. It was known that the U87 is a primary glioblastoma cell line and U251 is derived from a malignant glioblastoma tumour. The IC_{50} values of cyclotides (Table 5.3) on U251 cells are 2-3 fold higher compared to U87. It might be these cyclotides has a better effect on targeting early stages of cancers since it has better potency on primary glioblastoma.

Table 5. 3 Cytotoxic activity of known cyclotides (kalata B1, kalata S, cycloviolacin O2 and O12) against non-cancerous and cancer cells.

Cyclotides	IC_{50} (μM) ± S.D				
	HUVEC	U87	U251	HT29	MCF7
kalata B1	6.43±0.04	3.21±0.07	10.88±0.03	11.43±0.08	5.76±0.05
kalata S	9.73±0.06	4.63±0.07	8.35±0.04	10.69±0.04	5.46±0.02
cycloviolacin O2	0.35±0.06	0.45±0.02	0.79±0.06	0.87±0.08	0.29±0.04
cycloviolacin O12	1.47±0.04	2.94±0.12	6.44±0.02	7.69±0.08	1.50±0.04

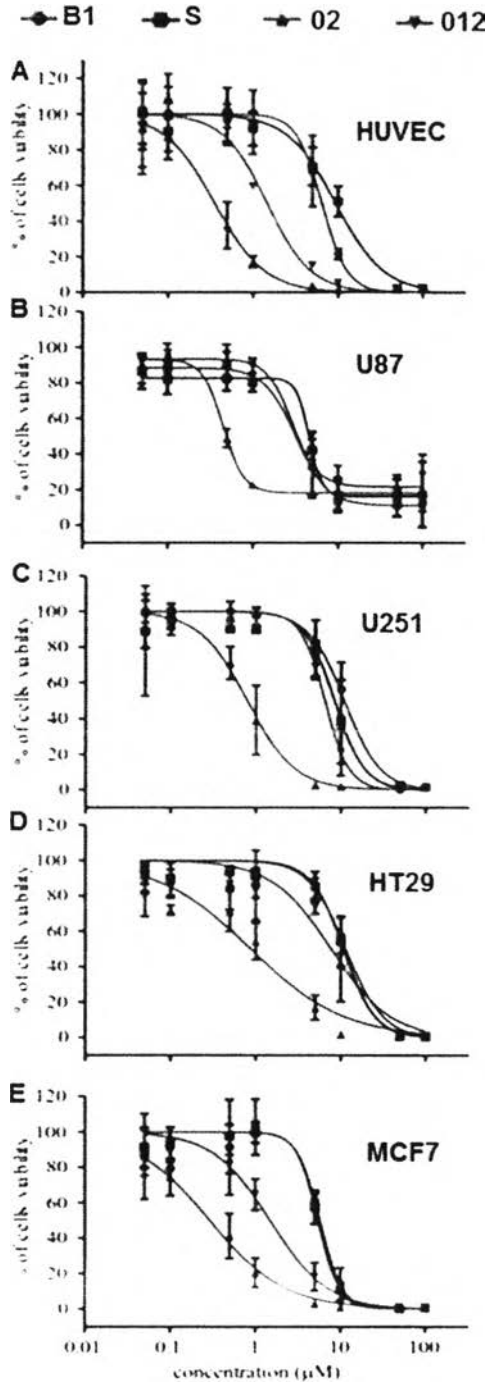

Figure 5. 6 Cytotoxic activity of known cyclotides (kalata B1, kalata S, cycloviolacin O2 and cycloviolacin O12). (A) Human umbilical vein endothelial cell (HUVEC), a non-cancerous cell line; (B-C) Human brain cancer cell line, U87 and U251; (D) Human colon adenocarcinoma cell line (HT29), and (E) Breast cancer cell line (MCF7). IC_{50} values were obtained from plotting % of cells viability versus peptide concentration using GraphPad Prism.

5.4 CONCLUSION

Cyclotides were found in *V. sumatrana*. This result supports many previous publications that every viola genus contains cyclotides. Cyclotide has a wide range of biological properties and anticancer activity is one of important properties of cyclotide. So far, cycloviolacin O2 has the most potency on cell cytotoxicity. Some publications explain that the structure of cycloviolacin O2 (bracelet) might effect to hydrophobic residue arrangement in the molecule so it's important to understand more about the mechanism of cycloviolacin O2 against cancer cell line for using cycloviolacin O2 as a protein framework in drug design for pharmaceutical or agricultural applications. In addition we have found two new cyclotides from this plant which should be characterized for further study on their structure and biological activities.