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DISCOVERY AND CHARACTERIZATION OF CYCLOTIDES, PLANT-BASED PEPTIDES FROM *Viola dalatensis* Gadnep

Pham Hoa Son[1*], Tran Thi Thanh Tam[1*], Pham Nguyen Dong Yen[1], Le Xuan Binh[2], Nguyen Hung Thinh[3], Truong Quang Cuong[4], Le Tien Dung[1,2], Dang Thuy Tien[1,2]✉

[1]*Institute of Applied Materials Science, Vietnam Academy of Science and Technology, 1B TL29, District 12, Ho Chi Minh City, Vietnam.*

[2]*Graduate University of Science and Technology, Vietnam Academy of Science and Technology, Hanoi, Vietnam.*

[3]*Pham Ngoc Thach University of Medicine, Ho Chi Minh, Vietnam.*

[4]*Bidoup Nui Ba national park, Sub-area 97, Da Nhim, Lac Duong, Lam Dong, Vietnam.*

✉To whom correspondence should be addressed. E-mail: dttien@iams.vast.vn

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ABSTRACT

Cyclotides demonstrate remarkable stability due to their unique characteristic - the cyclic cystine knot motif. Cyclotides exhibit a wide range of biological activities. This study aims to explore the presence of cyclotides in *Viola dalatensis* Gadnep, a plant indigenous to Vietnam, through the utilization of LC-MS and LC-MS/MS techniques. We conducted a comprehensive analysis of three extraction methods: 50% acetonitrile with 1% formic acid, 70% ethanol, and 50% methanol. The initial method is extremely efficient for cyclotide extraction when utilizing LC-MS analysis. An ammonium sulfate salt concentration of 30% is used to enhance the cyclotide content and optimize the RP-HPLC purification procedure. The precipitates demonstrate a notable advantage in terms of antibacterial properties compared to the extracts, particularly when the antibacterial concentration is decreased by a factor of four in comparison to the extracts. The combination of cyclotides demonstrated potent antimicrobial activity against *Bacillus subtilis* and *Pseudomonas aeruginosa*. The impact was most noticeable when the concentration of the cyclotide mixture was ten times lower than the precipitates. The inhibition zones for these bacteria measured 17.17 ± 2.24 mm and 20.23 ± 0.84 mm, respectively. The identification of the primary structure of nine cyclotides through LC-MS/MS analysis was successfully achieved.

Keywords: cyclotides, cyclic cystine knot, MS/MS, plant-based peptide, *Viola dalatensis* Gadnep.

INTRODUCTION

Cyclotides are a class of peptides that come from various plant families, including Rubiaceae, Violaceae, Fabaceae, Poaceae, Solanaceae, and Cucurbitaceae (Craik *et al.*, 1999; Daly and Wilson, 2021; Dang *et al.*, 2021; Poth *et al.*, 2011b; Weidmann and

Craik, 2016). The peptide adopts a circular shape, with a cyclic backbone composed of around 30 amino acids organized in a head-to-tail arrangement. This conformation is stabilized by three intramolecular disulfide bonds, which are referred to as the cyclic cystine knot (CCK) (Clark *et al.*, 2006; Ho *et al.*, 2023a; Poth *et al.*, 2011a). The cyclotides exhibit remarkable structural stability, as indicated by their exceptional resistance to proteolytic, thermal, and chemical degradation due to their CCK structure (Casallanovo *et al.*, 2006; Clark *et al.*, 2006).

Cyclotides are classified into three distinct subfamilies: bracelet, Möbius, and trypsin inhibitor (Figure 1A and B) (Ho *et al.*, 2023b; Tran *et al.*, 2024; Tran *et al.*, 2023). The bracelet conformation is observed in the majority, specifically two-thirds, of cyclotides found in nature. The Möbius conformation is observed in approximately one-third of cyclotides, whereas the trypsin inhibitor conformation is found in a negligible proportion (Troeira Henriques and Craik, 2017). The dipeptide pair Trp-Pro (or Tyr-Pro), which is situated within loop 5, plays a crucial role in facilitating a cis conformation of the amide bond. The observed conformational change leads to a 180° rotation of the cyclic backbone, resembling a metaphorical Möbius strip. The Möbius loop 3 peptide is composed of four residues, resulting in a helix that is relatively shorter in length when compared to bracelet cyclotides. In contrast, bracelet cyclotides consist of six residues and have no cis conformation (Jagadish and Camarero, 2010). In comparison to the two first cyclotide subfamilies, trypsin inhibitors demonstrate notable variations in their sequence components and only possess a common characteristic in the form of CCK (Chiche *et al.*, 2004; Felizmenio-Quimio *et al.*, 2001; Heitz *et al.*, 2001).

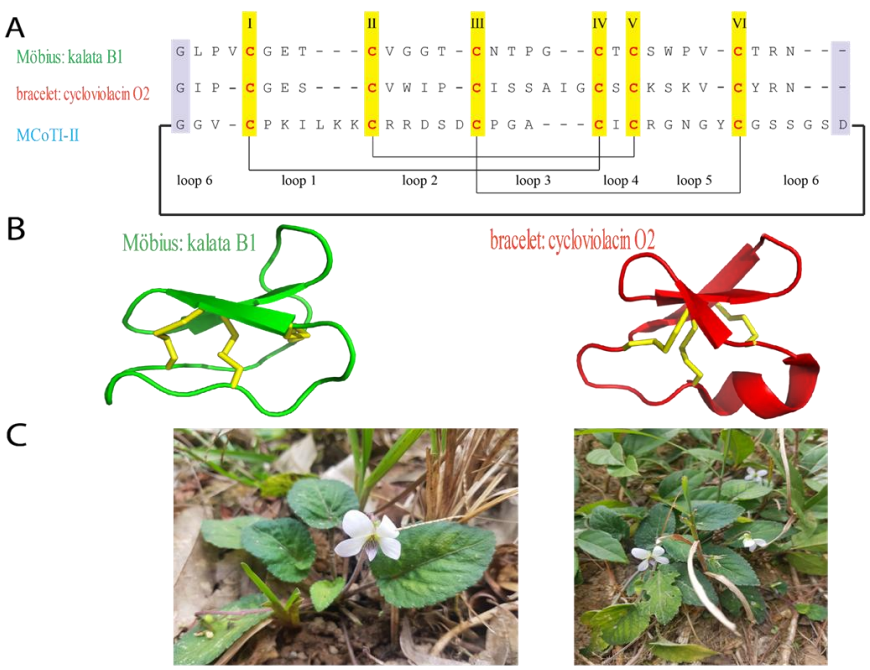


Figure 1. (A) The sequences of three cyclotide families are Möbius (kalata B1), Bracelet (cycloviolacin O2), and trypsin inhibitor (MCoTI-II). The sequence is characterized by six cysteine residues, denoted

as I-VI, and six backbone loops, and referred to as loops 1-6. (B) The 3D structure of kalata B1 (PDB 1BN1) and cycloviolacin O2 (PDB 1NBJ). (C) *Viola dalatensis* Gagnep is primarily found in Bidoup National Park, located in Lam Dong province. Its distribution is particularly concentrated in the vicinity of Da Lat.

The primary role of cyclotides in plants is widely recognized as host defense (Simonsen *et al.*, 2005). This specific function has sparked interest in the utilization of cyclotides for agricultural purposes, due to their wide range of biological activities, such as antimicrobial (Nguyen *et al.*, 2016; Porto *et al.*, 2012), antifouling (Göransson *et al.*, 2004), cytotoxic (Dang *et al.*, 2020), insecticidal (Pinto *et al.*, 2012), and molluscicidal activities (Plan *et al.*, 2008), provide support for this application. They also possess protease inhibition and immunosuppressive properties (Huynh *et al.*, 2024).

The Violaceae family consists of 25 genera and an estimated 1,000 to 1,100 species of herbaceous plants, shrubs, climbing vines, and trees (Dang *et al.*, 2020). Ninety-eight percent of these species are classified under the genera *Viola*, *Rinorea*, and *Hybanthus*. The *Viola* species comprise a significant majority, accounting for over half of the total number of species within this family, with estimates of 525-600 (Dang *et al.*, 2020). Throughout the course of history, numerous species of *Viola* have been utilized in the field of traditional medicine. Vietnam harbors approximately 30 species from the Violaceae family, with the majority of these species falling under the genus *Viola*. These species are commonly observed as short grasses that inhabit mountainous regions. The current study focused on the collection of *Viola dalatensis* Gagnep from Langbiang, Da Lat, Vietnam (Figure 1C). Herbaceous, shortly shooted perennial plant with ovoid, long round, or deltate leaf blades measuring 3-6 x 1.5-3.5 cm and pubescent on both surfaces. Stipulate 10-12 mm long with margins ciliate. Flowers 1-2, petiole 5-9 cm long; flower slight violet, apex dark violet, flower blooming not much open. Sepal 5-6 mm in length; lateral petal 10 mm high with dark violet stripes; spur 1,5 mm; anther 3, has pale yellow veins, 7 mm long. Capsules split along three seams. Grows on forest floors, moist forest edges, grasslands, wet places in stream valleys, 1,200-2,287 m above sea level, and endemic species to the Langbiang plateau, Lam Dong province, Vietnam, and surrounding areas (Marcussen *et al.*, 2022).

This study entailed the comparison of cyclotide-like molecules' presence in extracts obtained from various extraction methods. The plant parts were subjected to extraction using three distinct methods, namely: 50% acetonitrile and 1% formic acid (Dang *et al.*, 2024), 70% ethanol, and 50% methanol. The samples underwent salt precipitation through the addition of a 30% (w/v) solution of ammonium sulfate. The precipitates underwent LC-MS analysis to determine their molecular weight within the range of 2500-4000 Da, which corresponds to the molecular weight of cyclotides. The utilization of this precipitation method allows for a more effective purification of cyclotides using RP-HPLC as opposed to employing the solid-phase extraction (SPE) technique. The extracts, precipitates, and mixtures of cyclotides were employed to evaluate their antibacterial activity. The determination of cyclotide primary structures was achieved by employing LC-MS/MS data in conjunction with a cyclotide database available at

https://www.cybase.org.au/. A thorough examination was conducted to explore the natural compounds found in the *V. dalatensis*, specifically emphasizing polyphenols.

METHODS AND MATERIALS

Plant extraction

V. dalatensis specimen found at Bidoup National Park in Lam Dong, with coordinates: latitude N 12˚ 06' 11.93", longitude E 108˚ 21' 38.83", and altitude 1675.8 m. Plant sample deposited under voucher specimens (no. 202211_04) at the Institute of Applied Materials Science - VAST, Vietnam. Initially, the plant sample underwent a process of air drying at ambient temperature, followed by grinding into a fine powder. The entire plant powder underwent a soaking and stirring procedure utilizing various solvents, including a mixture of 50% acetonitrile and 1% formic acid, as well as 70% ethanol and 50% methanol. The extraction ratio is 0.5 g of sample per 10 mL of solvent. The extraction process was conducted over a period of six hours. Subsequently, a second extraction was conducted utilizing an equivalent volume of solvent. The extracted sample was subjected to filtration using a mesh filter in order to remove any residue, followed by evaporation to remove the solvent. Following that, the sample was subjected to centrifugation and freeze-drying.

Peptide enrichment utilizing the salting-out method

Initially, the entire plant sample of *V. dalatensis* was subjected to extraction using the aforementioned three methods. The combined extract obtained after performing double extraction was subsequently subjected to evaporation and centrifugation in order to collect the supernatant. The 30% (w/v) ammonium sulfate was added to the mixture, ensuring thorough stirring. Consistently maintained a temperature of 4 ^{0}C (Dang *et al.*, 2024). Following a 3-hour duration, the mixture underwent centrifugation, resulting in the formation of the precipitate. The precipitate was fully dissolved in a 50% acetonitrile (ACN) and 0.05% trifluoroacetic acid (TFA) buffer. Buffer A (miliQ-H_2O in 0.05% TFA) was then used to dilute the solution until the ACN concentration reaches approximately 1 - 5%. Performed centrifugation on the solution and the supernatant was proceeded to remove the salt using RP-HPLC.

Cyclotide purification via using RP-HPLC

The semi-purification of the precipitate was achieved through many rounds of the utilization of the RP - HPLC method. The samples underwent purification using RP-HPLC on a C18 Phenomenex Gemini 5 µm column (250 x 10 mm). The collected fractions were eluted using two buffers, namely buffer A (100% miliQ-H_2O/0.05% TFA, v/v) and buffer B (100% acetonitrile/0.05% TFA, v/v). A gradient of solvent B ranging from 20% to 90% was employed in a linear manner, with a flow rate of 2 mL/min. The analytes were detected using UV absorbance at wavelengths of 213 and 280 nm.

LC-MS analysis

The LC-MS analysis was conducted using the TripleTOF 6600+, Sciex. The analysis was carried out at a flow rate of 0.3 mL/min with the gradient starting at 10% B

(acetonitrile in 0.1% FA) and increasing linearly to 90% B over a period of 75 minutes. The buffers utilized for LC-MS analysis consist of buffer A (miliQ-H_2O in 0.1% FA) and buffer B (acetonitrile in 0.1% FA). The LC column employed is XBridge C18 (2.1 x 150 mm, 3.5 µm). The substances within the molecular weight range of 2500-4000 Da were documented based on the findings derived from MS profiles.

Determination of cyclotide sequences

Reduction and alkylation

Prepared a solution by adding 200 µL of NH_4HCO_3 buffer with a pH of 7.5 to 0.1 mg of the sample in an eppendorf tube. Added 70 µL of β-mercaptoethanol (Fisher Chemical) to an Eppendorf tube. Subsequently, placed the tube in a water bath set at a temperature of 40°C for a duration of 3 hours. Then 70 µL of 2-vinylpyridine (Thermo Scientific Chemicals) was added and the eppendorft was covered with foil to protect from light for 1 hour. Next, proceeded with the purification process by performing a thorough washing using acetone from Fisher Chemical. Performed the repetition process twice in order to eliminate superfluous substances and achieve a peptide mixture (Dang *et al.*, 2024).

Enzyme digestion

The peptides that underwent alkylation were rinsed with chilled acetone and subsequently dissolved in a 200 µL solution of 100 mM NH_4HCO_3 buffer at pH 8.0. In the single enzyme digestion process, 10 µL of either endoproteinase GluC (obtained from *Staphylococcus aureus* V8, Roche Diagnostics GmbH) or trypsin (from Merck) at a concentration of 100 µg/µL was introduced to each peptide sample. Enzymatic digestion by introducing 5 µL of endoproteinase GluC and 5 µL of trypsin into the peptide sample was also used. The specimen was subsequently placed in an incubator set at a temperature of 37 °C for a period of 16 hours (Nguyen *et al.*, 2011; Pinto *et al.*, 2021).

Sequencing by using MS/MS data combining cyclotide database

The LC-MS/MS analysis involved the examination of peptides following the processes of reduction, alkylation, and enzymatic digestion. The analysis was conducted at a flow rate of 0.3 mL/min. The elution gradient was programmed to increase the concentration of solvent B from 10% to 90% over a period of 37 minutes. The mobile phase was composed of two solvents, namely A and B. Solvent A was prepared by combining 0.1% FA (Fisher Scientific, USA) with miliQ-H_2O, v/v. Solvent B was prepared by adding acetonitrile (Fisher Scientific, USA) with 0.1% FA, v/v. The study utilized a 3.5µm 2.1 x 150mm XBridge C18 Column on a TripleTOF 6600+ mass spectrometer (AB SCIEX), equipped with TF 1.8 Analysis. The MS/MS spectra were analyzed using the ProteinPilot software combining the cyclotide database.

Determination of total protein content

The Bradford reagent is commonly employed for the quantification of total protein content (Kielkopf *et al.*, 2020; Prakash *et al.*, 2008). A standard curve was generated by utilizing bovine serum albumin (BSA) at different concentrations ranging from 2 to 10 µg/mL. Following that, 1 mL of Bradford reagent was added to each tube. A volume of 200 µL of samples was incubated

for a duration of 10 minutes with the reagent present in the tube. A measurement of absorbance was recorded at a wavelength of 595 nm, and this measurement was replicated three times.

Determination of the total phenolic compound

The determination of phenolic content in the samples was conducted using the Folin-Ciocalteu reagent method (Dang *et al.*, 2024). The substance utilized for the experiment was gallic acid, which was prepared as a solution with a concentration of 1 mg/mL. The gallic acid solution was subsequently diluted in methanol to produce a series of standard solutions with accurate concentrations: 20, 40, 60, 80, 100, and 120 µg/mL. A sample solution of 0.5 mL was prepared in a test tube. Next, 1.25 mL of a 10% Folin-Ciocalteu reagent was added to the test tube and mixed well. After the addition of the Folin-Ciocalteu reagent, the reaction was allowed to proceed for a period of 5 minutes. Next, 1 mL of a 2% sodium carbonate (Na_2CO_3) solution was added. The mixture was incubated for a period of 45 minutes at room temperature. The absorbance of the sample was measured at a wavelength of 765 nm. The experiments were performed in triplicate.

Determination of antimicrobial activity

An assessment of antibacterial activity through the utilization of the agar disk diffusion method

This study involved the testing of four bacteria known to pose a risk to human health: *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6051), *Pseudomonas aeruginosa* (B96.5), and *Escherichia coli* (ATCC 25922). An evaluation of the antibacterial efficacy of the samples was performed (Tran *et al.*, 2023). The bacterial strains were cultivated on LB agar slants for a duration of one day. Subsequently, a loop culture rod was employed to transfer a portion of the biomass into a 0.85% physiological saline solution. The suspension was then agitated until its turbidity matched that of McFarland 5 x 10^8 CFU/mL. To achieve a suspension of 5 x 10^6 CFU/mL, it was further diluted the existing suspension by a factor of 100. The suspension was evenly distributed on the MHA agar plate. The discs were perforated with a series of holes that corresponded to the test and control samples. The control sample utilized in this study was a PBS-buffered saline solution with a pH of 7.4, serving as the negative control. In contrast, the positive control was the antibiotic Levofloxacin, administered at a concentration of 10 µg/mL. The test samples were mixed with a phosphate-buffered saline (PBS) solution at specific concentrations: 50 mg/mL for precipitate samples, 200 mg/mL for extracts, and 5 mg/mL for a mixture of cyclotides. Subsequently, 50 µL of the mixture was introduced into the designated well and incubated at a temperature of 37 °C for a duration of 24 hours. The obtained results were then analyzed.

Determination of Minimum Inhibitory Concentration (MIC)

The determination of MIC was performed (Tran *et al.*, 2023). The bacteria were cultured overnight, following which 1-2 colonies were transferred into 5 mL of Luria Bertani Broth (LB) medium. The bacterial suspension was appropriately adjusted to the 0.5 McFarland standard in order to attain a final consistent bacterial density of 5 x 10^8

CFU/mL. The suspension was diluted by a factor of 100 using LB medium in order to attain a density of 5 x 10^6 CFU/mL. The wells of sterile 96-well plates were filled with 100 µL of Luria-Bertani (LB) medium. The 2-fold serial dilution technique was utilized to evenly distribute the concentration of the sample across multiple wells. One well was filled with 100 µL of LB medium to maintain sterility (-), while another well was designated as a control for bacterial growth (GC). The bacterial density in each well eventually reached a concentration of 5 x 10^5 CFU/mL. The plates were then sealed using parafilm and placed in an incubator set at a temperature of 37 ^oC for 20 hours. After the designated incubation period, 10 µL of a 0.015 % resazurin reagent was added to each vial and left to incubate for an additional 2 hours. Following the incubation period, the results for the MIC were determined. Subsequently, a discernible change in the color of the resazurin solution within the vials was observed, transitioning from a blue hue to a pink hue. This alteration serves as an indication of the presence of bacteria. The experiment was performed in triplicate.

RESULTS AND DISCUSSION

Plant extraction

The evaluation of cyclotides extracted from *V. dalatensis* was conducted by considering a single factor, namely the extraction solvent. When considering factors such as extraction time and temperature, it is crucial to recognize the significant role that the extraction solvent plays in achieving the desired quality and quantity of target components (Samuelsson *et al.*, 1985). The selection of a solvent is primarily based on the chemical properties of the target compounds, such as their polarity or hydrophobicity. In this study, the selection of extraction solvents was based on the amphoteric nature of cyclotide, which is characterized by the presence of hydrophobic patches and charged residues. We have selected specific concentrations for each of the three solvents, namely 50% methanol (50% MeOH), 50% acetonitrile in 1% formic acid (50% ACN), and 70% ethanol (70% EtOH) (Mahatmanto *et al.*, 2015; Poth *et al.*, 2011a).

LC-MS analysis

Initial screening of cyclotide-like molecules from V. dalatensis

The primary criterion for identifying cyclotides is a molecular weight within the mass range of 2500 to 4000 Da, based on their distinctive physicochemical properties (Koehbach *et al.*, 2013). The obtained samples, referred to as crude extracts, are initially subjected to analysis using LC-MS. The extracted samples exhibited significantly attenuated signals and did not produce mass peaks within the anticipated range of 2500 to 4000 Da (Figures 2 and S1). Hence, the utilization of ammonium sulfate salt was implemented to increase the peptide content in the extracted samples.

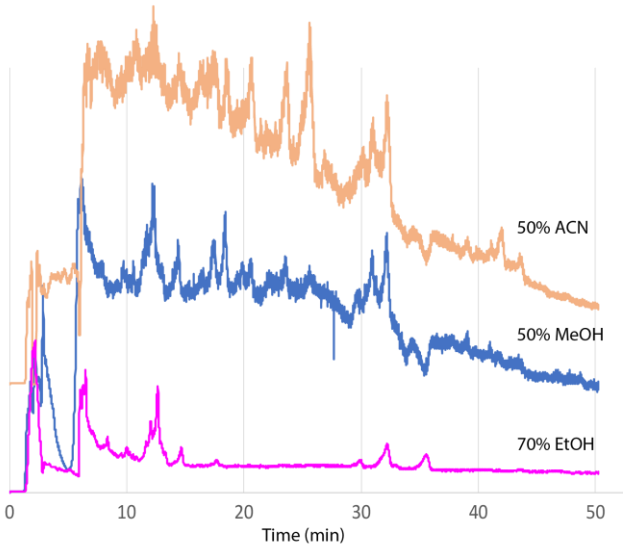
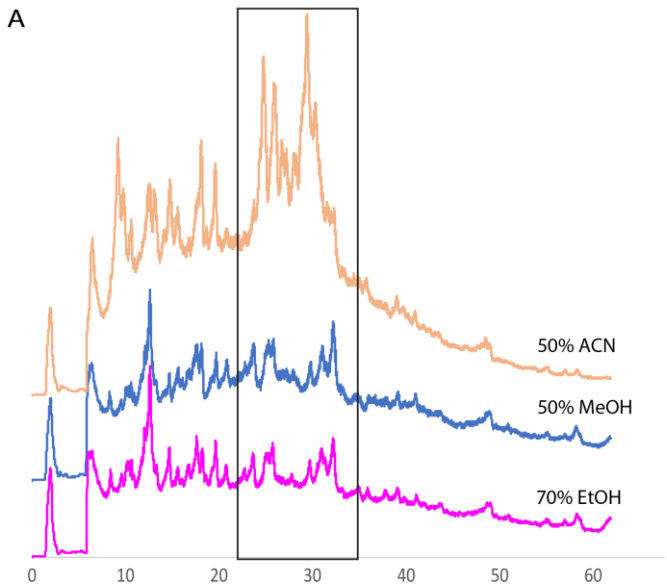


Figure 2. LC profiles of three extracts; orange (50% ACN); blue (50% MeOH); pink (70% EtOH)

Precipitates of extracts from V. dalatensis

In this study, the salt precipitation method has been chosen as the preferred technique for peptide enrichment, specifically focusing on cyclotides. Ammonium sulfate is a commonly employed salt due to its prominent position on the Hofmeister series, which leads to the production of ions that exhibit substantial interactions in aqueous solutions. As a result, the influence of these ions on the protein/peptide itself is relatively insignificant. Iodide and other ions have been found to exhibit a high level of effectiveness in the precipitation of proteins and peptides. Nevertheless, their usage is restricted as they have a propensity to cause protein denaturation or modification. A concentration of 30% ammonium sulfate salt was selected as it has been determined to be the most optimal concentration for protein/peptide precipitation, as demonstrated in previous studies (Duong-Ly and Gabelli, 2014; Santa-Coloma *et al.*, 1987). Three different types of extracts, consisting of 50% ACN, 50% MeOH and 70% EtOH were utilized for the process of ammonium sulfate salt precipitation.

After the completion of residual salt removal from the sample utilizing RP-HPLC, it is imperative to conduct an LC-MS analysis in order to evaluate the precipitates that contain molecules falling within the mass range of 2500-4000 Da. The LC spectra and MS information of the precipitates depicted in Figures 3 and S2 demonstrate enhanced signal quality and offer a more distinct representation of the MS information in comparison to the extracts. This study demonstrates the utilization of the salt precipitation method for the purpose of enriching peptides, with a particular focus on cyclotides. The sample of salt precipitation from the 50% ACN extract exhibits a distinct mass range of 2500-4000 Da, suggesting the presence of 26 cyclotide-like molecules. Nevertheless, the two remaining samples of salt precipitation, the 50% MeOH and 70% EtOH extracts, do not show a significant intensity in the mass range of 2500-4000 Da (Figure S2). Hence, the 50% ACN extract has been selected for further evaluation.



B

Mass (charge)	Mass/charge (charge)	Mass (charge)	Mass/charge (charge)
3113.49 (3)	1038.82 (3)	3201.44 (3)	1068.17 (3)
3127.40 (3)	1043.46 (3)	3226.44 (3)	1076.58 (3)
3138.37 (3)	1047.15 (3)	3289.41 (3)	1097.57 (3)
3143.49 (3)	1048.83 (3)	3300.45 (3)	1101.11 (3)
3149.42 (3)	1050.80 (3)	3311.43 (3)	1104.83 (3)
3165.45 (3)	1056.10 (3)	3327.43 (3)	1110.14 (3)
		3362.54 (3)	1121.88 (3)

Figure 3. (A) LC profiles of precipitates of three extracts and (B) A list of cyclotide-like molecules of 50% ACN precipitated extract; orange (50% ACN); blue (50% MeOH); pink (70% EtOH).

Alkylated precipitate of 50% ACN extract

In order to confirm the existence of three disulfide bonds within cyclotides, it is necessary to conduct a verification process. The precipitate collected from the 50% ACN extract was subjected to reduction and alkylation using 2-vinylpyridine. This process resulted in an increase in a mass shift of approximately 637 Da, indicating the presence of six cysteines (Figure S3). This observation provides strong evidence indicating the presence of cyclotides in the sample containing a 50% ACN salt precipitate.

Semi-purified mixtures of peptides (M)

The peptide mixture was obtained by performing several rounds of RP-HPLC purifications from the 50% ACN precipitate, which was designated as M. The samples were subjected to LC-MS analysis for the purpose of identifying cyclotide-like molecules. The findings are displayed in Figure 4, illustrating the existence of about nine prominent cyclotide-like compounds with molecular weights varying from 2500 to 4000 Da.

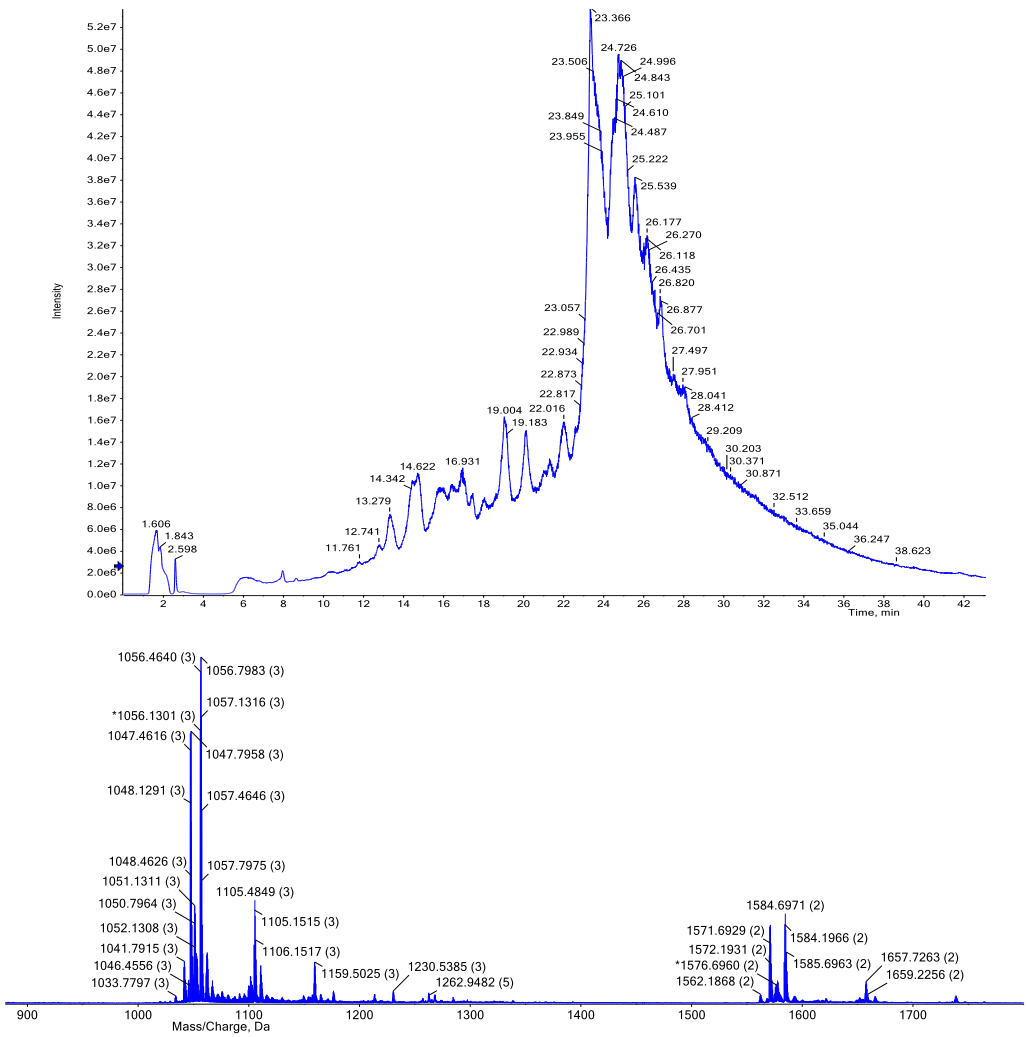


Figure 4. LC-MS profile of semi-purified mixtures of cyclotides M

Determination of cyclotide sequence by using MS tandem

A mixture of cyclotides (M) was subjected to reduction and alkylation using vinylpyridine. Subsequently, the alkylated samples were digested using trypsin, endoproteinase GluC, and a mixture of endoproteinase GluC and trypsin. The MS/MS fragments of the digested samples were sequenced using ProteinPilot Sciex software with reference to the cyclotide database (https://www.cybase.org.au/). The cyclotide sequences obtained from the M sample derived from *V. dalatensis* using the fragment assembly method have been compiled and are presented in Table 1. The analysis of samples containing chemically modified disulfide bonds involves the fragmentation pattern obtained through reduction, alkylation, and enzymatic cleavage leading to the generation of free N and C-termini (Table 2). Figure 5 depicts several digested fragments, specifically the fragments produced through the combined digestion using trypsin and end-GluC. These

fragments have molecular weights of 794.33, 2176.01, and 1420.63 Da, respectively. These findings have enabled the determination of the sequence of cycloviolacin O4. In a similar manner, this particular method successfully identified a total of nine cyclotides that had been previously reported (https://www.cybase.org.au/), as shown in Table 1 and Table 2. This study has contributed to the advancement of understanding cyclotide diversity within the Violaceae family, particularly in relation to the discovery of cyclotides in *V. dalatensis*.

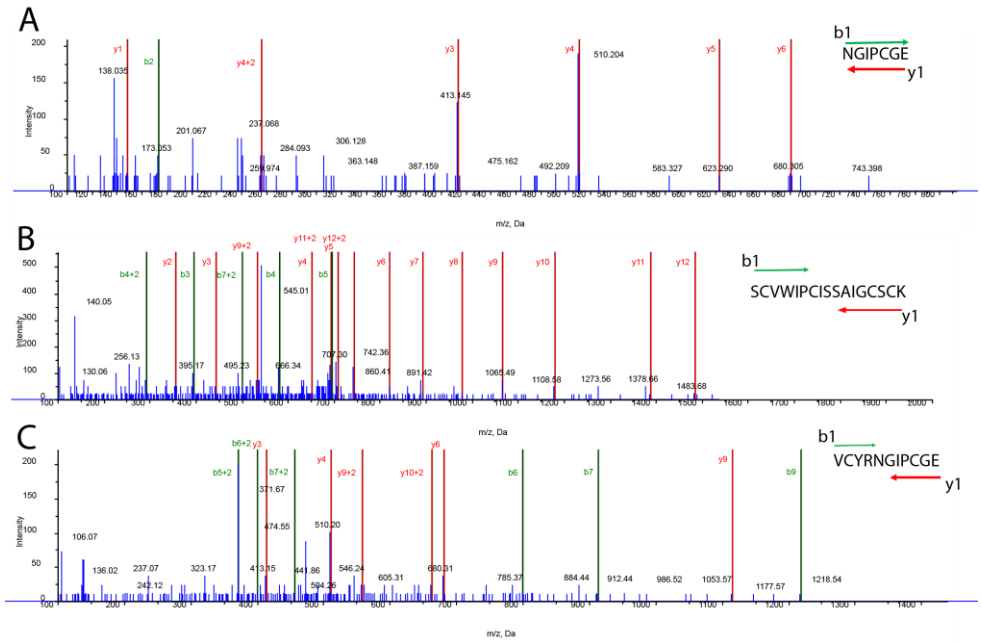


Figure 5. Three fragments from the digestion with both endoproteinase Glu-C and trypsin of the reduced and alkylated form of cycloviolacin O4, with specific masses 794.33 Da (A), 2176.01 Da (B), and 1420.63 Da (C).

The sequence was subsequently deduced and determined to be consistent with the previously reported sequence (Fernández-Bobey *et al.*, 2022; Herrmann *et al.*, 2008; Ireland *et al.*, 2006). The other sequences have also been verified to be equivalent to the studies reported, as shown in Table 1 and Table 2.

Table 1. Sequences of cyclotides in the M sample that have been identified from *V. dalatensis*. Obs. MW: observed molecular weight; Theor. MW: theoretical molecular weight.

Obs. MW (Da)	Theor. MW	Sequence	Cyclotide's name
3138.35	3138.37	GIPCGESCVWIPCLTSAVGCSCKSKVCYRN	cycloviolacin O9
3148.35	3148.39	GIPCGESCVWIPCLTSAVGCPCKSKVCYRN	vibi K
3151.37	3151.33	GIPCGESCVWIPCISGVQGCSCSNKICYRN	Phyb E
3165.36	3165.38	GIPCGESCVWIPCISSAIGCSCKNKVCYRN	cycloviolacin O4
3166.37	3166.40	GIPCGESCVWIPCLTATIGCSCKSKVCYRN	cycloviolacin B2

3181.36	3181.40	GTLPCGESCVWIPCISAAVGCSCKSKVCYKN	cycloviolacin O6
3199.37	3199.41	GSIPCGESCVFIPCISSVVGCSCKNKVCYKN	mram 4
3210.37	3210.43	GVIPCGESCVFIPCITAAVGCSCKNKVCYRD	psyle F
3226.38	3226.41	GTLPCGESCVWIPCISSVVGCSCKSKVCYKN	cycloviolacin O8

Table 2. MS/MS profile of cyclotide sequences identified in the M sample from *V. dalatensis*. Obs. MW: observed molecular weight; Theor. MW: theoretical molecular weight; Conf: confidency score; Obs. m/z: observed mass/charge.

Obs. MW	Obs. m/z	Best sequence	Enzyme	Conf	Theor. MW
3791.76	632.97	SCVWIPCITSAVGCSCKSKVCYRNGIPCGE	E	99	3792.77
3804.76	635.13	SCVWIPCLTSAVGCPCKSKVCYRNGIPCGE	E	99	3802.79
3808.76	545.12	SCVWIPCISGVQGCSCSNKICYRNGIPCGE	E	99	3806.72
3820.77	637.80	SCVWIPCISSAIGCSCKNKVCYRNGIPCGE	E	99	3820.77
3820.77	637.80	SCVWIPCLTATIGCSCKSKVCYRNGIPCGE	E	99	3820.80
3836.78	549.12	SCVWIPCISAAVGCSCKSKVCYKNGTLPCGE	E	99	3835.80
3854.79	643.47	SCVFIPCISSVVGCSCKNKVCYKNGSIPCGE	E	99	3854.80
3863.80	644.97	SCVFIPCITAAVGCSCKNKVCYRDGVIPCGE	E	99	3865.81
3878.78	647.47	SCVWIPCISSVVGCSCKSKVCYKDGTLPCGE	E	99	3880.81
2951.34	492.90	NGIPCGESCVWIPCISSALGCSCK	T	99	2951.35
2952.34	493.06	GTLPCGESCVWIPCISSVVGCSCK	T	99	2952.37
3578.63	597.44	VCYRNGIPCGESCVWIPCISSALGCSCK	T	99	3578.63
3020.38	605.08	VCYRDGVIPCGESCVFIPCITAAVGCSCK	T	99	3622.69
2952.34	739.09	NGIPCGESCVWIPCISSALGCSCK	T	99	2951.35
2952.34	739.09	NGIPCGESCVWIPCITSAVGCSCK	T	99	2951.35

2952.34	739.09	NGIPCGESCVWIPCISSALGCSCK	T	99	2952.33
2978.39	497.41	NGIPCGESCVWIPCLTATIGCSCK	T	99	2980.36
2952.33	591.47	NGIPCGESCVWIPCISSALGCSCK	T	99	2951.35
3108.44	519.08	RNGIPCGESCVWIPCISSALGCSCK	T	99	3108.43
894.39	448.20	NGTLPCGE	TE	99	894.39
859.44	430.73	SKVCYR	TE	99	859.44
2192.00	549.01	SCVWIPCISSALGCSCK	TE	99	2192.01
1420.63	474.55	VCYRDGIPCGE	TE	99	1420.63
2419.12	484.83	SCVWIPCISSALGCSCKNK	TE	99	2419.14
2176.02	363.68	SCVWIPCISSALGCSCK	TE	99	2176.01
2951.34	492.90	NGIPCGESCVWIPCISSALGCSCK	TE	99	2951.35
2176.01	545.01	SCVWIPCISSALGCSCK	TE	99	2176.01
3578.63	597.45	VCYRNGIPCGESCVWIPCISSALGCSCK	TE	99	3578.63
1304.62	435.88	SCVWIPCISSALGCSCK	TE	99	2176.01
3820.76	637.80	NKVCYRNGIPCGESCVWIPCISSALGCSCK	TE	99	3820.77
2176.01	436.21	SCVWIPCISSALGCSCK	TE	99	2176.01
2951.34	738.84	NGIPCGESCVWIPCISSALGCSCK	TE	99	2951.35
794.33	398.17	NGIPCGE	TE	99	794.33
881.36	441.69	NGSIPCGE	TE	99	881.36
886.45	444.23	NKVCYR	TE	99	886.45
892.41	447.21	NGVIPCGE	TE	99	892.41
894.39	448.20	NGTIPCGE	TE	99	894.39
2151.02	538.76	SCVFIPCLTSAIGCSCK	TE	99	2151.02
2176.01	545.01	SCVWIPCISSAIGCSCK	TE	99	2176.01
1420.63	474.55	VCYRDGIPCGE	TE	99	1420.63
2176.00	436.21	SCVWIPCISSAIGCSCK	TE	99	2176.01

All cysteines were reduced and alkylated by using vinyl pyridine.

T: digestion by using trypsin; E: digestion by using Endo GluC; TE: digestion by using a mixture of trypsin and Endo GluC.

The total protein concentration (TCP)

The total protein content was determined using the Bradford method to validate the effectiveness of the salt precipitation method. The 50% ACN extract and salt precipitate demonstrated the highest protein content when compared to the other samples (Figure 6). The extract obtained with 50% ACN showed a value of 32.62 ± 0.66 µg/mg dry weight (DW), while the precipitate exhibited a value of 65.25 ± 2.60 µg/mg DW. The above results have demonstrated the effectiveness of the salt precipitation method for protein enrichment. Furthermore, the ethanol and methanol samples exhibited relatively low total protein values. The obtained results align with the LC-MS findings previously discussed. The recommended approach for extracting cyclotides from *V. dalatensis* is the utilization of a 50% ACN solution.

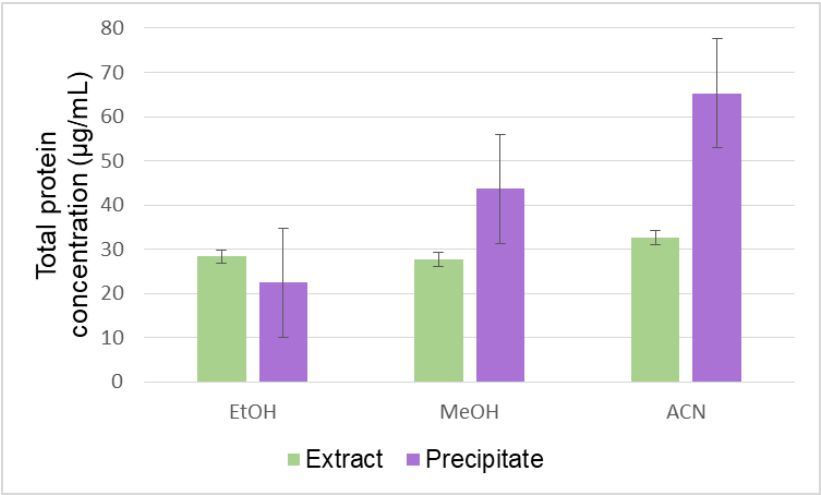


Figure 6. The total protein concentration (µg) of 1 mg samples

The total phenolic content

Phenolic compounds play a significant role as essential constituents of plants, as their properties greatly contribute to a wide range of biological activities (Jung *et al.*, 2007; Kim *et al.*, 2013a; Kim *et al.*, 2013b; Soobrattee *et al.*, 2005). The phenolic content in each sample was quantified by employing the Folin–Ciocalteu reagent. The concentration of phenolic compounds in the 50% ACN precipitate is measured to be 60.30 ± 1.99 mg GAE/g, whereas the phenolic content of the 50% ACN extract is determined to be 28.44 ± 0.93 mg GAE/g, indicating a difference of approximately two-fold. Hence, the salt precipitation method facilitates the extraction of proteins and polyphenols from plant extracts.

Antimicrobial activity

According to published studies, both polyphenols and cyclotides have been found to possess antibacterial properties (Ganesan *et al.*, 2021; Henriques *et al.*, 2011; Manso *et al.*, 2021; Miklasińska-Majdanik *et al.*, 2018). According to the data presented in Figure 7 and Table S1, the extract and precipitate obtained through the 50% ACN

extraction method exhibit superior antibacterial efficacy compared to the other methods. The inhibition rings observed for these samples measure 10-10.87 mm and 22-25 mm, respectively. Simultaneously, the extract was subjected to testing at a concentration of 200 mg/mL, while the precipitate underwent testing at a concentration of 50 mg/mL. Hence, the precipitate sample exhibited a notable antibacterial advantage in comparison to the extract sample, despite having an antibacterial concentration that was four times lower. Furthermore, the samples obtained through the 70% EtOH and 50% MeOH extraction methods did not exhibit any activity as extracts, despite being tested at a concentration of up to 200 mg/mL. However, activity was observed only when the enrichment method was employed, resulting in inhibition rings ranging from 13-17 mm. This demonstrates the efficacy of the salt precipitation method in enriching biologically active compounds and augmenting the test sample's capacity to exhibit antibacterial activity. Nevertheless, none of the samples exhibited resistance against two bacterial species, namely *E. coli* and *S. aureus*. Khoshkam *et al.* (2016) demonstrated that semi-purified cyclotides and crude extracts exhibited antimicrobial properties, highlighting the antibacterial effectiveness of fully isolated compounds from *V. tricolor* against gram-negative bacteria, especially *E. coli* and *P. aeruginosa*. The peptides had a significant inhibitory effect, resulting in an inhibition zone width of 13 mm when evaluated at a dose of 25 mg/mL. The efficacy of crude ethanol and butanolic extracts was found to be higher against gram-negative bacteria *E. coli* and *P. aeruginosa* compared to *S. aureus* (Khoshkam *et al.*, 2016).

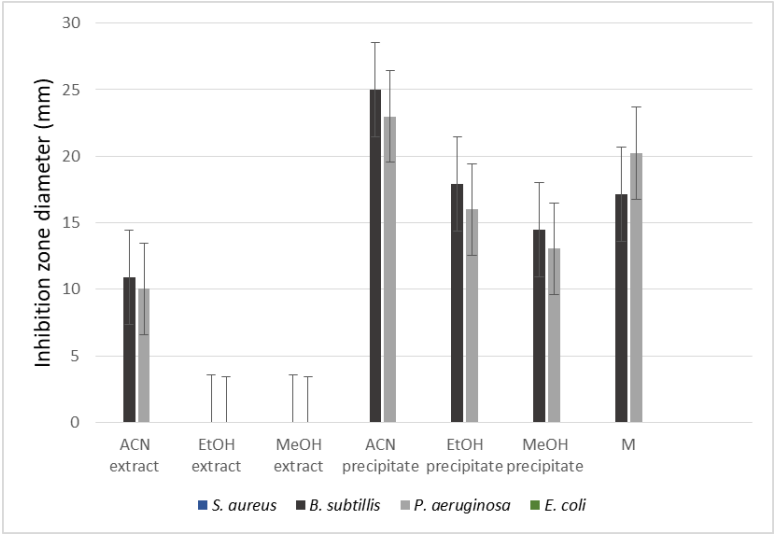


Figure 7. The antibacterial activity of samples derived from *V. dalatensis* was demonstrated by the agar well diffusion method.

Following the purification process, the peptide mixture sample underwent testing to evaluate its antibacterial activity at a concentration of 5 mg/mL. Peptide sample

M exhibits sensitivity to two bacterial species, namely *B. subtilis* and *P. aeruginosa*, as depicted in Figure 7. Upon comparing the resistance of each bacterium, sample M exhibited a higher level of antibacterial activity against the *P. aeruginosa* strain in comparison to the *B. subtilis* strain. The precipitate samples were tested at a concentration of 50 mg/mL, whereas the peptide mixture M was tested at a concentration of 5 mg/mL, which is ten times lower. In summary, the results suggest that sample M, after undergoing the purification procedure, displayed a notable presence of cyclotides, indicating an increase in antibacterial effectiveness.

Minimum inhibitory concentration (MIC)

According to the data presented in Table 3, the MIC value for the *B. subtilis* strain of 50% ACN extract is 1.6 times lower compared to that of 50% ACN precipitate. In contrast, the MIC value of the 50% ACN extract for *P. aeruginosa* was found to be twice as high as that of the 50% ACN precipitate. *P. aeruginosa* is capable of causing various acute and chronic infections in humans, and has emerged as a significant contributor to both infection rates and antibiotic resistance (Al-Wrafy *et al.*, 2017). This study has demonstrated that the precipitate obtained from a 50% ACN extract exhibits antibacterial activity against *P. aeruginosa*, with a MIC value of 25 mg/mL. In another study, a cyclotide derived from *V. odorata* was also examined for its effectiveness against *S. aureus* (Zarrabi *et al.*, 2013). The MIC was determined to be 1.6 mg/mL. The peptide mixture (M) exhibit remarkable antibacterial activity compared to other extracts due to the utilization of multiple purification procedures to increase the concentration of cyclotides. They form a strong inhibitory ring at a concentration of only 5 mg/mL.

Table 3. MIC values of 50% ACN extract and precipitate from *V.dalatensis*

Samples \ Bacteria	***B. subtilis***	***P. aeruginosa***
50% ACN extract	31.25 mg/mL	50 mg/mL
50% ACN precipitate	50 mg/mL	25 mg/mL

CONCLUSION

This study enhances our understanding of the extraction strategies utilized for cyclotide isolation in *Viola dalatensis*. In the present study, the solvent containing 50% ACN demonstrated the highest extraction of cyclotide-like molecules within the mass range of 2500-4000 Da. The application of the ammonium sulfate salt precipitation technique has been observed to be highly efficient in enrichment of the abundance of cyclotides and secondary compounds with antibacterial properties. This was demonstrated through the determination of total protein content, the total phenolic compounds, LC-MS analysis, determination for cylotide sequences, and assessment of its antibacterial activity. Furthermore, the cyclotide mixture, acquired through multiple RP-HPLC purifications of the precipitate sample, demonstrated effectiveness against both gram-positive and gram-negative

bacteria. This study shows significant potential in the development of antibiotics for both medical and agricultural purposes.

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AUTHOR CONTRIBUTIONS

S.H.P. and T.T.T.T. performed experiments, processed data, wrote the manuscript, and prepared the figures. Others performed experiments and reviewed the manuscript. T.Q.C. collected and validated *Viola dalatensis* Gadnep. T.T.D. provided scope, guidance, and critically reviewed the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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