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ISOLATION OF PUTATIVE *CYCLOTIDE* GENES FROM SOME *Violacea* SPECIES USING SIMPLE PCR TECHNIQUE AND ITS PHYLOGENETIC RELETIONSHIP WITH THEIR ANCESTORS

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Abstract

Cyclotide proteins are the largest and most well-known family of cyclic proteins. These proteins have disulfide-rich bonds with 30 amino acid residues comprising C and N terminals to induce cyclization. They are found in various plant species, such as the *Violaceae* family. Bioactive peptides possess CCK (cyclic cysteine knots), which contain 28 to 37 amino acids and are useful in biotechnology and for producing peptide-based drugs. The *Violaceae* family is a distinct plant family that expresses the conserved cyclotide genes. In this study, we investigated the 14 species of the *Violaceae* family to show their antimicrobial activity against the two bacterial species, *Escherichia coli* and *Staphylococcus aureus*. Cyclotide genes were isolated from 5 species of *V. tricolor* through PCR screening. The phylogenetic analysis revealed that *viola tricolor* (white blotch), *viola tricolor* (Rose Blotch), *viola tricolor* (purple blotch), *viola tricolor* (rosalyne) and *viola tricolor* (tigerary) showed the homology with the other species of the *Violaceae* family. The discovery of the cyclotide precursor gene from the *Violaceae* plant species defined their structural sequence and evolutionary link to their diversity from other species. It opened a new research line on the evolution of cyclotide proteins. This research will foster the production of new drugs that interact with only pathogens, not humans, and to develop, produce, transport or access cheaper medications. The present research will also contribute to future exploration in agriculture.

Keywords: Antimicrobial activity, Phylogenetic analysis *Violacea* family, *Viola tricolor* Cyclotides

INTRODUCTION

Cyclotides are not universally present in all plant species. However, they have been identified in flowering plants, particularly those of medicinal significance in ethnopharmacology. These plants belong to the families *Violaceae, Rubiaceae, Cucurbitaceae, Fabaceae*, and *Solanaceae*, as documented by (Burman et al. 2015; Gruber et al., 2008; Nguyen et al., 2013; Poth et al., 2011, 2012; de Veer et al., 2019). Cyclotides, as a fascinating bioactive protein family of plants, exhibit a structural arrangement of head-to-tail cyclic backbone with inner 3 disulfide bonds knotted arrangement composed of an exceptionally stable formation (Colgrave and Craik, 2004). The *violaceae* family members are loaded with species distinguished for exhibiting

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extraordinary cyclic peptides documented with the name of cyclotides (Craik *et al*., 1999; Burman *et al.,* 2014). These are renowned for building biological activities like anti-HIV, cytotoxic uterotonic, hemolytic actions, and insecticidal and antimicrobial effects, revealing that they also combat as plant front-line defense molecules (Grover *et al.,* 2021).

In general, cyclotides are uncommon in isolation, as a single plant can generate several cyclotides (Hellinger et al., 2015; de Veer et al., 2019). Most plants possess a distinct set of cyclotides, with each cyclotide often exclusive to a certain plant species. Consequently, it is rare for a single cyclotide to be found in numerous plant species (Gruber et al., 2008). Previous studies have documented changes in cyclotide content across different tissues, seasons, and geographic regions (de Veer et al., 2019). As an illustration, the Viola tricolor (Figure 1C), belonging to the Violaceae family, was previously subjected to the isolation and characterization of 164 distinct cyclotides (Hellinger et al., 2015). The aforementioned botanical specimen is widely cultivated in horticulture. It has a long history of using traditional medicinal practices for its properties related to heat-clearing, detoxification, and alleviation of cough symptoms (Tang et al., 2010). The utilization of the Viola tricolor in official Russian pharmaceuticals is extensive, as evidenced by its inclusion in the Russian Pharmacopoeia, which features a dedicated monograph on this plant (Shikov et al., 2014, 2017, 2021). According to Hellinger et al. (2015), the abundance of violaceous plants worldwide suggests they might yield up to 150,000 unique cyclotides. Given this significant number, it is plausible to consider these commercially accessible medicinal herbs as a promising foundation for conducting bioactivity-guided screening investigations. Although research has been conducted on cyclotides, the information on indigenous plants' bioactive potential still needs to be improved, instigating new cyclotide gene variants.

Currently, > 700 cyclotides, including cyclotides (linear cyclotides), have been reported from several plant species (https://www.cybase.org.au;). About 60 species out of six families (Violaceae, Solanaceae, Rubiaceae, Fabaceae, Cucurbitaceae, and Poaceae) have been testified for the existence of cyclotides and cyclotides (Tammineni *et al.,* 2020). Related to the overall number of angiosperm species, there is a low number of species from which cyclotides and cyclotides have been isolated. Cyclotides and cyclotides have been reported most abundantly from *Violaceae* (317), trailed by *Rubiaceae* (85), then *Fabaceae* (76), almost equal in *Solanaceae* (12) and *Cucurbitaceae* (11) and only 9 reported in *Poaceae* (https://www.cybase.org.au; Gerlach *et al*.,2013; Kaas and Craik, 2010; Du *et al.,* 2019; Niyomploy *et al.,* 2018).

Despite extensive cyclotide studies, indigenous plants with bioactive cyclotides and novel gene variations must be investigated. This study sought 14 commonly available indigenous species of *Violaceae* of Pakistan to clone and express linear cyclotide precursor genes. This study used bioinformatics and molecular methods to mine cyclotide sequences from commonly found Pakistani violaceae plants. The Pakistan V. tricolor flower's cyclotide gene was extracted, cloned, and expressed in heterologous hosts. Sequences of these genes are not extensively present in a broad range of previously

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reported *violaceae* plants. Still, evolution of cyclotide gene was scrutinized by examining the analogous pattern and Phylogenetic investigation of the isolated *viola tricolor* (white blotch), *viola tricolor* (Rose Blotch), *viola tricolor* (purple blotch), *viola tricolor* (rosalyne) and *viola tricolor* (tigerary) precursor gene which augmented the evolution of cyclotide gene.

MATERIAL AND METHODS

After distilled water washing, plant leaves were dried at room temperature. The leaves were pulverized using liquid nitrogen and stored at -800 C for later research. Select plant leaves were protein extracted using extraction buffer (1.5% PVP, 100 KCl, 2mM Thiourea, 1mM PMSF) (Saravanan and Rose, 2004). Extraction buffer with 1mM PMSF and 1:3 weights by volume resuspended powdered leaves. The mixture was homogenized using a polytron on ice at 20C and centrifuged at 10,000 rpm for 30 minutes. Proteins were precipitated overnight in cold acetone with 1% PVPP, 10% TCA, and 2% β-mercaptoethanol at -400C. Next, centrifuge the sample at 10,000 rpm and 40C for 30 minutes. DMSO dissolved pellets.

The protein extract's antibacterial activity against *E. coli* and *S. aureus* was tested using disc diffusion. Cultures were inoculated in nutrient agar. Synthesized filter paper discs contained 30 microliters of retrieved protein. DMSO was the negative control for antimicrobial testing, whereas fluconazole and chloramphenicol were positive controls. Petri plates were incubated at 370C for 24 hours. Zone reader measured protein extract antibacterial activity (Rizwan et al., 2021).

CTAB was used to extract genomic DNA from the leaves of Viviaceae plants (Doyle, 1990). A developed primer was used to amplify the violaceae plant species' cyclotide gene. Thermo SCIENTIFIC components optimized the PCR reaction conditions for the primer's characteristics. These PCR primers worked:

VTF: 5' CACACTCTCTCTCTCCCTCTAT

VTR: 5' ACTCCGTTCATTACGTCATTACGTCTAACC

Amplification was performed at Initial denaturation of 3 mins at 95oC, 35 cycles of 30 seconds at 95oC 40 seconds at 50oC and 50 seconds at 72oC with final extension at 72oC of 5 minutes. Amplicons of the gene were spotted and visualization using 1 percent agarose gel following ethidium bromide staining. A gel purification mini kit was used to purify amplicons (FavorPrepTM, Cat No. FAGPK001. The Sanger method used the pjET 1.2/blunt vector to ligate the purified DNA fragments and sequences from commercial sequencing service (CAMB, Lahore) (Rizwan *et al.,* 2021). The Cyclotide gene homology was validated using Clustal in Bio Editv 7.2.5 and MEGA 6.0 software to construct the Phylogenetic tree (Hall, 2013; Tamura *et al.,* 2013).

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RESULTS

Although studies on cyclotides have been done, there is still a dearth of knowledge on the bioactive potential of native plants, which encourages the discovery of new cyclotide gene variants. This study aimed to clone and express linear cyclotide precursor genes from locally accessible Pakistani violaceae plant species. Bioinformatics and molecular methods were used to examine the plant species for mining novel cyclotide sequences. A cyclotide gene was identified, cloned, and expressed in a heterologous host from the Pakistan Viola tricolour flower. The morphology of 14 selected plant species is shown in Fig. 1

Fig 1: Varieties of *viola tricolor* species of the *Violaceae* family investigated in this study.

Table 1: Common names of different *viola tricolor* plant species of *the Violaceae* family used in the study.

Sr No.	Plant species
1.	*Viola tricolor* Rose blotch
2.	*Viola tricolor* Yellow heartsease
3.	*Viola tricolor* Clear yellow
4.	*Viola tricolor* White blotch
5.	*Viola tricolor* Blue and white blotch
6.	*Viola tricolor* Tigrary
7.	*viola tricolor* Delfth blue
8.	*Viola tricolor* Deep blue blotch
9.	*Viola tricolor* Rosalyne
10	*Viola tricolor* Yellow heartease
11	*Viola tricolor* Jumbo white blotch
12	*Viola tricolor* Mid night glow
13	*Viola tricolor* Fire
14	*Viola tricolor* Purple blotch

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Antimicrobial activity

The antimicrobial activity of 14 selected *Violaceae* Plants is evaluated with chloramphenicol (6mg/mL) applied as positive control and DMSO application as negative control through disc diffusion technique. The results represent the broad-spectrum activity of selected plants by forming a clear inhibition zone against two bacterial strains, i.e., *E. coli* and *S. aureus*. Protein extracts of *viola tricolor* purple blotch, *viola tricolor* fire, and viola tricolor yellow blotch were most active against gram-positive bacteria *S. aureus.* Overall, it was observed that *viola tricolor* purple blotch, *viola tricolor* fire, *viola tricolor* blue and white blotch, *viola tricolor* rosaline, *viola tricolor* yellow blotch showed broad spectrum activity against gram-positive bacteria *S. aureus* through developing pure inhibition zone. On the other hand, the remaining plant extracts demonstrated a significant inhibitory effect against *S. aureus*. All the varieties of *viola tricolor* were less active against a gram-negative strain of E*. coli* in contrast to the gram-positive strain. *Viola tricolor* purple blotch and *viola tricolor* yellow blotch were most active against *E. Coli*; however, the rest of the plant exhibited a clear zone of inhibition against *E. Coli*.

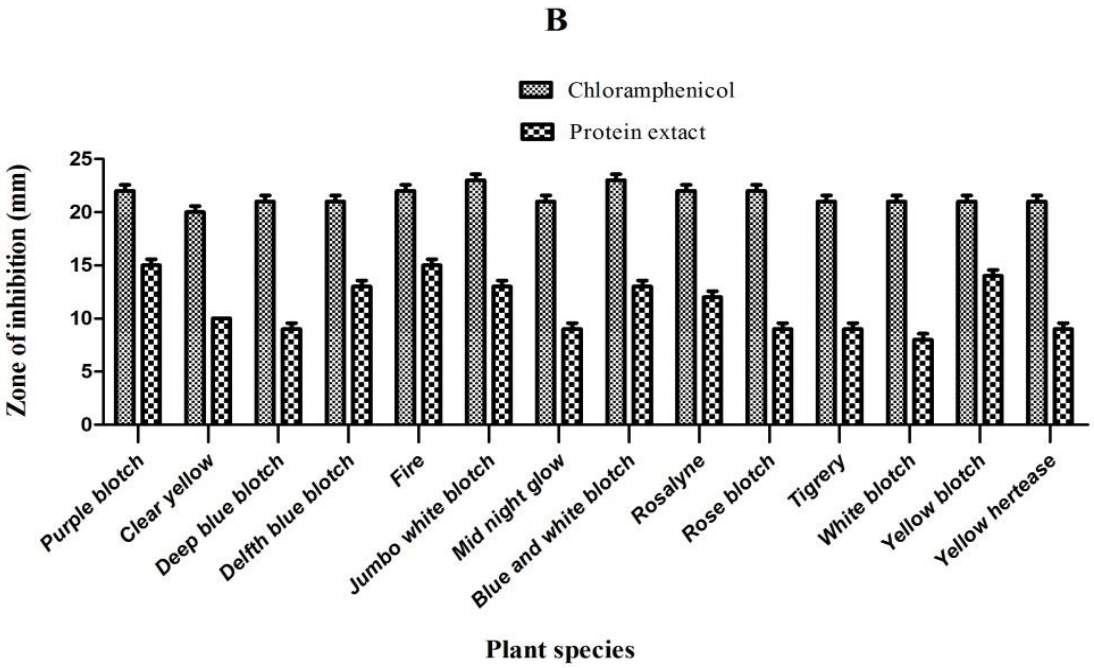

Fig 2: Effect of *S. aureus* on 14 different species of the *Violaceae*.

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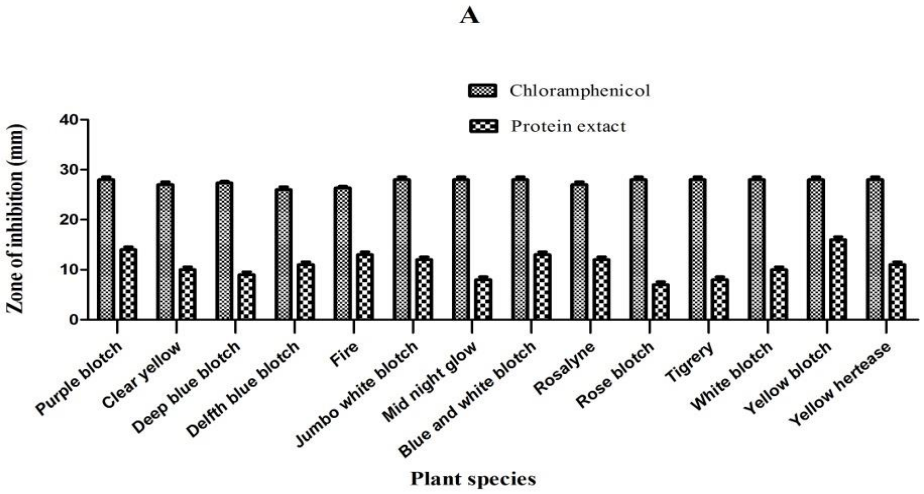

Fig 3: Effect of *Escherichia coli* on 14 different species of the *Violaceae*.

Isolation of DNA and screening of cyclotide encoding genes from *Violacea* plant species:

For cyclotide gene isolation from *Viola tricolor*, primers were designed from the conserved region of an already reported sequence of the plant *Viola baoshanesis* cyclotide precursor 2 mRNA (DQ851861). Specific forward and reverse primers were designed from the available intronic portion of cyclotide gene sequence from similar family *Violaceae* plants already submitted to GenBank (NCBI) through alignments, blast and primer 3 bioinformatics tools. Out of 14 plant *violaceae* plant species, 5 viola tricolor plant varieties showed positive results (Fig .3). Amplified bands were edited from the gel and cloned into pjET 1.2/blunt vector.

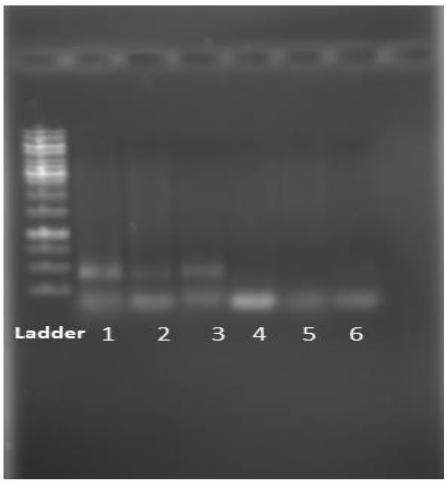

Fig 4: Cyclotide gene PCR amplification of *violaceae* plants.

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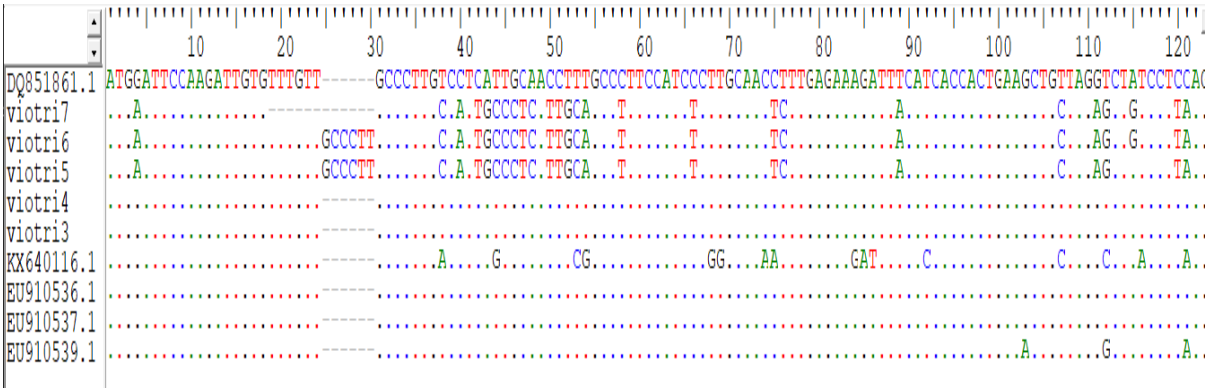
Amplicons of PCR from *viola tricolor* cultivars (*vioTRI3, vioTRI4, vioTRI5, vioTRI6 and vioTRI7*) having isoforms of approximately 380bp to 400bp amplicon size while "L" placed for 1kb DNA ladder.

Sequencing and *Insilco-based* study:

Targeted genes were screened after an extensive PCR-directed investigation. Molecular Biology Product, Inc. service was used for Commercial nucleotide sequencing of PCR products. Obtained gene sequences examination and cleaning was executed using online tools, and the Bio Edit program was used for their contigs crafting. BLASTn and alignments with different BioEdit tools were employed to deduce selected cyclotide genes**.**

Five genes were separated from the 14 *viola tricolor* plant species (*V.tricolor* Rosalyne, *V. Tricolor* tigrery, *V. Tricolor* rose blotch, *V. Tricolor* purple blotch and *V. Tricolor* white blotch) using primer designed against cyclotide precursors gene of *viola baoshenesis.* These genes were named *vioTRI 3, vioTRI 4, vioTRI5, vioTRI6,* and *VioTRI7* based on their homology (Fig. 5).

Sequence *vioTRI3 and vioTRI4* from viola tricolor rosalyne and tigrery have 390 bp amplicon and showed 100% similarity to Accession No. DQ851861.1 and 82.87% to KX640116.1. Similarly, sequence vioTRI5 isolated amplicon from *viola tricolor* purple blotch, vioTRI6 rose blotch and vioTRI7 showed amplicon 392 bp to 400bp in length. Nucleotide sequences are further analyzed by TBLASTN (http://blas.ncbi.nlm.nih.gov/Blast.cgi), which confirmed that it has the highest homology to the Cyclotide gene and showed 79% to 87% similarity to Accession No. DQ581861.1 and EU910536.1 from all three sequences of Cyclotide gene, DQ85186.1 is used as a reference sequence from which primers are designed for this research. Bio Edit software is further used to translate and compare these sequences with related reference translated sequences shown in Fig 5.



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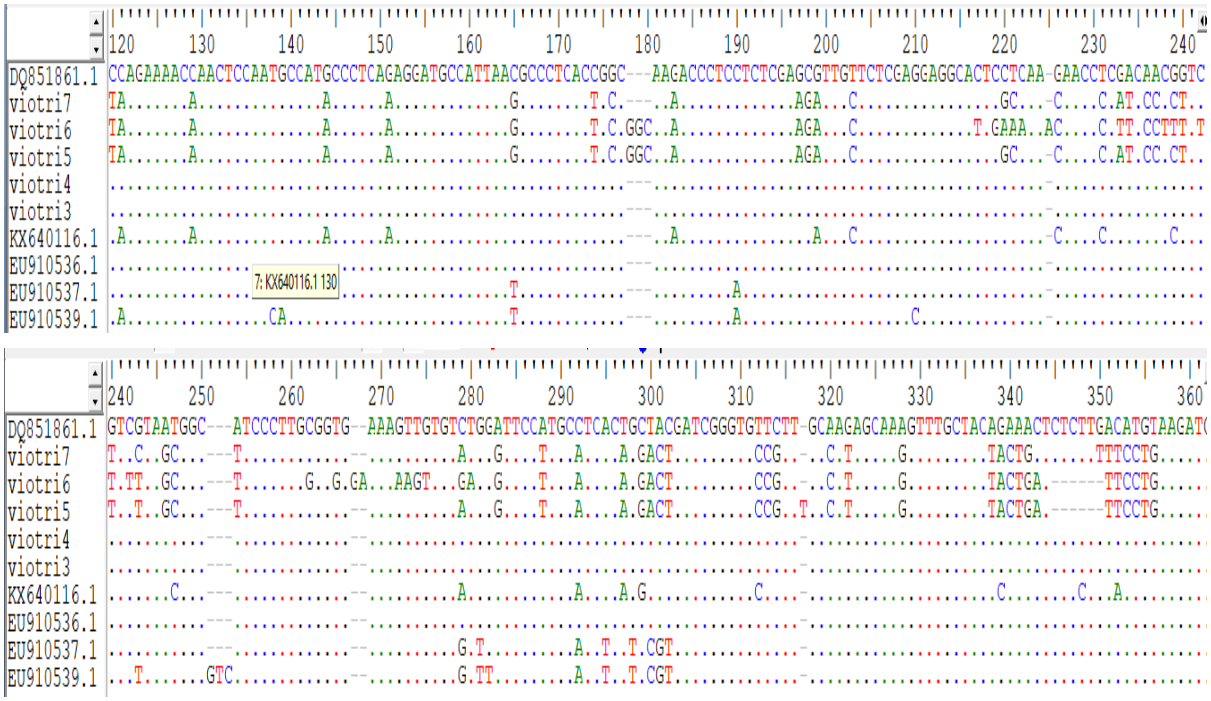

Fig 5: Alignment of sequence A with reference sequence

Phylogenetic Analysis of Cyclotide gene:

In this study, 14 *violaceae* plants were investigated for cyclotide evolution. Five precursor sequences were ascertained from *viola tricolor* species (Table 1.3). These five precursor sequences revealed in this study were grouped with nine formerly recognized most related precursor sequences recouped from the NCBI database. Phylogenetic profiling to study evolutionary links among vioTRI 3, viotTRI 4*, vioTRI 5, vioTRI 6 and vioTRI 7* precursor *cyclotide* genes with other already reported *violaceae* cyclotide genes was created through software MEGA6.0 (Fig. 6). The Phylogenetic construal based on Maximum likelihood method using Tamura and Nei-based distance model.

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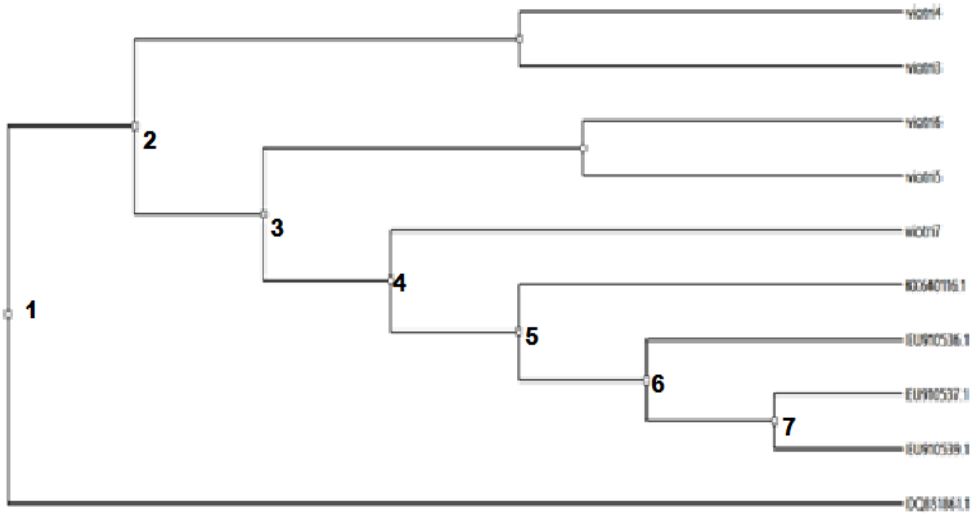

Cyclotide Gene name	Total Nucleotides (bp)	Accession Number	Source
Viola tricolor cyclotide gene complete cds	374	KX640116.1	Viola tricolor
Viola baoshanesis cyclotide precursor 2b mRNA,complete cds	527	EU910536.1	Viola baoshanensis
Viola baoshanesis cyclotide precursor 2 mRNA, complete cds	676	DQ851861.1	Viola baoshanensis
Viola baoshanesis cyclotides precursor 2c mRNA, complete cds	528	EU910537.1	Viola baoshanensis
Viola baoshanesis cyclotide precursor 2e mRNA, complete cds	523	EU910539.1	Viola baoshanensis
Viola baoshanesis cyclotide precursor 2a mRNA, complete cds	528	EU910535.1	Viola baoshanensis
Viola baoshanesis cyclotide precursor 2d mRNA, complete cds	528	EU910538.1	Viola baoshanensis

Fig 6: Molecular-based Phylogenetic assessment through Maximum Likelihood method.

The Maximum Likelihood mode based on the Tamura-Nei model revealed the evolutionary background. The tree was constructed in MEGA6.0, presenting the similarities and differences among cyclotide genes reported earlier from *Violaceae* plant species. The investigation concerned 10 sequences, including 5 previously submitted most relevant sequences and 5 novel isolated *viola tricolor* precursor sequences from this study (Table 3).

DISCUSSION

Viola tricolor synthesized several antimicrobial compounds such as flavonoids (Hassanvand *et al.,* 2021), phenylboronic acids, polysaccharides, salicylic acid derivatives, coumarins and catechins. Additionally, the *Violaceae* plants, in which *V.*

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tricolor distinctively spectacled as prolific for macrocyclic peptides, entitled as cyclotides (Hellinger *et al.,* 2015), which is a contributing factor for exhibiting significant antibacterial potential of viola tricolor. In the current study, we assayed *Violaceae* plants' leaf protein extracts for antimicrobial activity by focusing on 14 plant species of *viola tricolor.* Following the antimicrobial screening of these plants, the putative cyclotide gene was isolated through PCR, cloned in a pJET vector and sequenced for further *insulin* studies.

Antimicrobial susceptibility

Semi-purified Viola odorata cyclotide was evaluated against S*. aureus, P. aeruginosa,* and *E. coli* (Zarrabi *et al.,* 2013). The large inhibition zone against *E. coli* and *S. aureus* shows the broad-spectrum action of selected plants. Gram-positive bacteria B. Subtili were susceptible to *Vivia tricolor* purple, fire, and yellow splotch protein extracts. The zones of inhibition of *viola tricolor* purple blotch, fire, blue and white, rosalyne, and yellow blotch against gram-positive Bacillus subtilis were extensive. Most xenobiotics inhibited *B.subtilis*. Psychopharmacological plant extracts are more efficient against Gram-negative than Gram-positive bacteria (Mehta *et al.,* 2022). All *viola tricolor* verities examined in this study were less active against E. coli than gram-positive bacteria. E. Coli was highly aggressive against *viola tricolor* purple and yellow spots, although the rest of the plant exhibited a zone of inhibition.

The synergistic association between V. Tricolor's active components revealed low to substantial antibacterial activity against diverse microbes in crude herb extracts with differential polarity. Several studies have found plant-derived antibacterial cyclotides. Pränting *et al.* (2010) showed that cycloviolacin O2 and kalata B1 killed *E. coli* but not *S. aureus*. Gran *et al.* (2008) discovered that Kalata was ineffective against *S.aureus* but strong against gram-negative bacteria, including the Pränting group. Another study by Tam *et al.* (1999) indicated that four synthesized cyclotides were effective against S. aureus but not gram-negative bacteria. Zarrabi *et al.* investigated *V. odorata* semi-purified cyclotides against S*. aureus, E.coli,* and *P. aeruginosa. S. aureus* was also more sensitive (Zarrabi *et al.,* 2013). Roshan *et al.* (2014) found similar results.

Cyclotide encoding gene of *viola tricolor* plants.

Cyclotides were isolated from *Rubiaceae, Violaceae, Cucurbitaceae,* and **Fabaceae** (Kohehbech *et al.,* 2013).

Cyclotides were isolated from *Rubiaceae, Violaceae, Cucurbitaceae*, and *Fabacea* (Kohehbech et al., 2013). Viola tricolor, or pansy, is a beautiful plant. Viola has 585–625 species in tropical and southern temperate regions. Most recently found cyclotides are in the *violaceae* family, which has 30 thousand species (Zhang *et al.,* 2015). Of 25 genera in the *Violaceae* family, only 6 species (*Melicytus, Viola, Gloeospermum, Hybanso, Leonia,* and *Rinorea*) contain cyclotides (Burman *et al.,* 2015). Cycloviolacin O12 & O2, kalata S, tricyclon A & B, vitrine A-F, and varv (A, D, E, F, H, Hm & He) were among the unusual cyclotides from V.tricolor studied until now (Azmi *et al.,* 2022).

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Different plant species of *viola tricolor* (*violaceae* family) were isolated by special primers VTF/ VTR from Viola *baoshanesis* cyclotide precursor 2 mRNA DQ85186 predicted cyclotide amplicon size of 400 bp of entire CDS of cyclotide precursor without introns. For the cyclotides precursors gene of viola baoshanesis' by using primers, five genes were isolated from 14 *viola tricolor* plant species: vioTRI 3 from white blotch, vioTRI 4 from purple blotch, vioTRI5 from rose blotch, vioTRI6 from tigrery, and vioTRI7 from Rosalyne. BLAST search for recruitment homologous patterns within extracted cyclotide transcripts of these viola genomes showed similarities from 79% to 100% to Accession No. DQ851861.1, a reference sequence.

In the current study, all five varieties of the *V. tricolor* were found to be conserved cyclotide domains in sequence with amino acid variation (fig. 5). Sequences from *vioTRI6 from V. Tricolor* tigrery and *vioTRI7 from V.tricolor* Rosalyne are homologous to DQ851861 precursors sequence. *V. Tricolor* purple blotch, *vioTRI5 from V. Tricolor* rose blotch sequences have mutated six cysteine boxes. *vioTRI 3 from V. Tricolor* white blotch has conserved all six cysteine residues with small variation in their loops. When *vioTRI 3* is translated into protein, its 3-D structure shows 75 percent similarity with Cycloviolacin O2, as shown in Fig. 8.

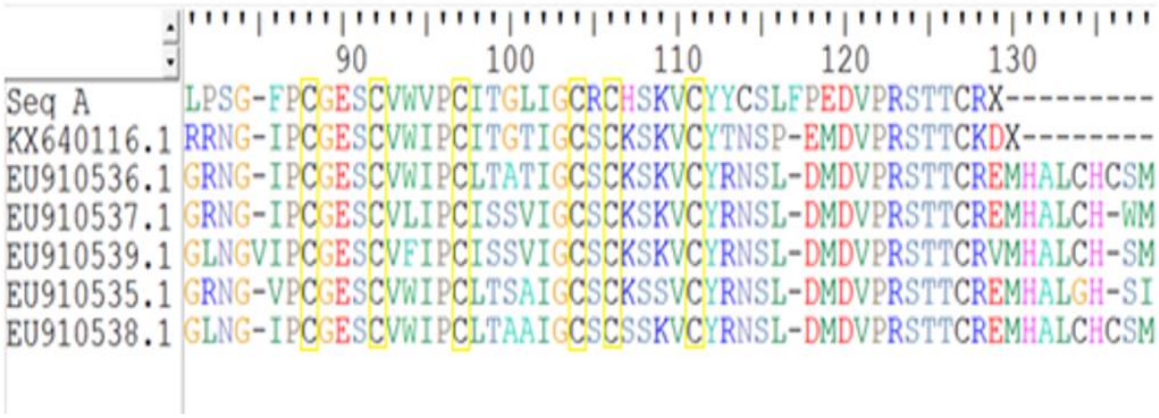

Fig 7: Conserved 6 cysteine residues of sequence A highlighted in yellow columns

Cycloviolacin O2 is a prototypic cyclotide due to its well-documented and potent anti-tumor and antibacterial activity and optimized protocols for identifying and purifying proteins.

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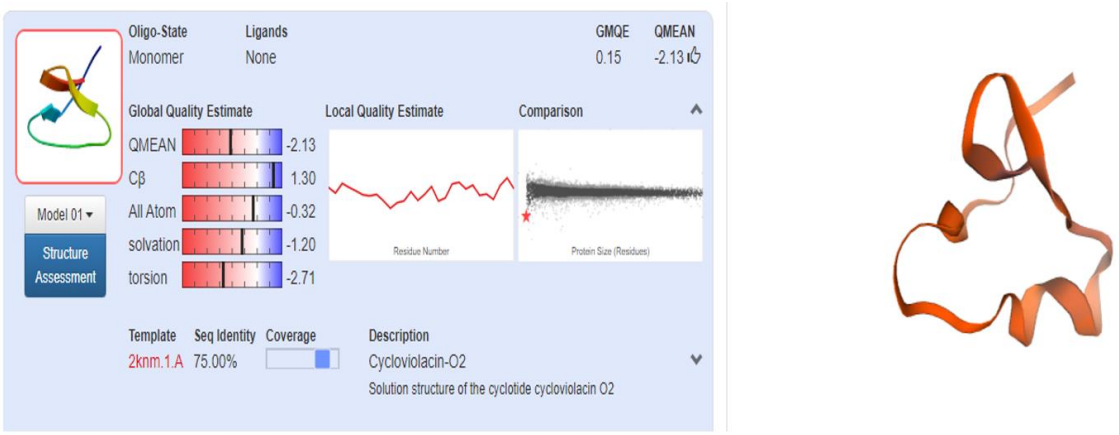

Fig 8: 3-D Structure of translated sequence A from *viola tricolor* (White Blotch)

Although out of five sequences, only two are similar to the precursor sequence of Accession No. DQ851861.1 and three other sequences may help extend the knowledge of plant Cyclotide distribution. Our finding of cyclotide precursors could be compared with the reported novel cyclotide precursors from the *Violaceae* family. This finding could change the evolutionary point of view on the genetic ancestors of cyclotides and related gene precursors of cyclotides.

Phylogenetic profiling for Homology comparison of novel cyclotide encoding genes from *the Violacea* family:

Nucleotide sequences from *the viola tricolor* were aligned with early identified reference sequences of *the violaceae* family. The deduced nucleotide sequences for each gene were compared with *the baoshanesis* sequence taken as a reference using the Clustal tool of BioEdit software (Hall, 2013).

Five homologous sequences encoding *cyclotide* genes with slight base pair differences had been reported. This decree revealed that more than one *cyclotide-encoding* gene prevails within the *viola tricolor* plants. Further gene sequences analysis based on *silicon* study after alignment directed to the idea that the presence of more than one type of cyclotides gene guided that even within similar plant species, the copies of gene sequences are becoming different from each other, which might be the outcome of overtime accumulation of mutations. In contrast, five plant species of *viola tricolor* resulted in only a single PCR product during isolation using the same pair of primers verified having a single gene locus (Khoshkam *et al.,* 2020). Multiple alignments with seven already reported *precursor* genes displayed that all of our *viola tricolor* genes *viotri3*, *viotri4*, *viotri5*, *viotri6* and *viotri7* were homologous of accession no DQ851861. The entire gene sequences exhibited unique novel sequences with some degree of homology. This review also verified clearly that all species of *Viola tricolor* did not possess *cyclotide* encoding genes as only five plants gave positive amplification out of 14 plant species of *Viola tricolor*; this confirmed the non-ubiquitous nature of cyclotides within the *Violacea* family (Ravipati *et al.,* 2017). Direct confirmation of the role of this

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gene can be exposed by monitoring the expression patterns of cyclotides encoding genes of *the Violaceae* family.

It is anticipated from our results that the gene sequence variation among precursor of intimated molecular species was little; however, it is quite large in the rest of the species. Incipit of these small variations, precursor sequences are homologous. This hypothesis supported that these molecular species are interrelated rather than their small sequence variation. These results elaborated from the punctuated theory of equilibrium, which discussed organism evolutionary trends (Eldredge and Gould, 1997). MEGA6.0 software was used for Phylogenetic profiling of *5 viola tricolor cyclotide* genes (Tamura *et al.,* 2013) as cyclotides conserved domain assumed as signature sequences, which is highly conserved throughout evolution. This conserved signature sequence generated a Phylogenetic pedigree of plant families (Park *et al.,* 2017). The Phylogenetic tree showed that 5 selected and 5 isolated *cyclotide* genes of *Viola tricolor* have a close relationship with *Violacea* family members, considering their similarities and dissimilarities pattern of nucleotide sequences (Fig.6). *Cyclotide* precursor genes branched into two distinct clad at base point. Nine precursor genes of *Violacea* grouped into one clad at the base branch point. *Viotri 3 and viotri 4* were separated from all other sequences at the second branched point as having 100% similarities with our reference sequence DQ851861.1, and the remaining 7 sequences clad differently as having less than 100% similarity index with a reference sequence. However, at the third branched point, viotri 5 and viotri 6 clad differently from others as having similar sequences. At the fourth branch point, viotri7 is clad differently from others. At the fifth branch point, kx640116.1 clad differently from other early reported sequences of *violaceae* plants. Phylogenetic pedigree confirms that isolated *viola tricolor* cyclotides genes have a common ancestor with reference sequence DQ851861.1 but are clad differently due to successive accumulation of variations generation over generations.

CONCLUSION

A screening of cyclotide-bearing indigenous 14 plant species found that most plant species had potent bioactive potential. Two homologous and three novel cyclotide gene sequences were isolated from five varieties of *viola tricolor.* Bioinformatics analysis revealed that the deduced amino acid sequence of the viotri7 gene from *viola tricolor* (white blotch) had conserved all 6 cysteine residues. Its bioinformatics analysis showed that this sequence had 75% structural homology with Cycloviolacin O2 when translated into protein. Cyclovialacin O2 is a cyclotide already isolated from other viola plants. Cyclotides have garnered significant interest in the medication and agricultural industries due to their diverse biological properties and remarkable tolerance for sequence variability. *V. tricolor*, a plant with medicinal properties, serves as the subject of this research, which aims to contribute to the future exploration of herbal preparations for various pharmacological activities.

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Reference

1) Azmi, S., Mustafa, M., Shoaib, S., & Hussain, M. K. (2022). Structures, Functions and Therapeutic Potential of Cyclotides. *Journal of Exploratory Research in Pharmacology*, *7*(4), 234-242.

2) Burman, R., M.Y. Yeshak, S. Larsson, D.J. Craik, K.J. Rosengren & U. Göransson. (2015). Distribution of circular proteins in plants: large-scale mapping ofcyclotides in the *Violaceae*. *Frontiers in plant science*, (6), 855.

3) Colgrave, M.L. &D.J. Craik. (2004). Thermal, chemical, and enzymatic stability of the cyclotide kalata B1: The importance of the cyclic cystine knot. *Biochem,* (43), 5965-5975.

4) Craik, D.J., &U. Malik. (2013). Cyclotide biosynthesis.Curr.Opin. *Chem. Biol*, (17), 546-554.

5) Craik, D.J., J.S. Mylne & N.L. Daly. (2010). Cyclotides: macrocyclic peptides with applications in drug design and agriculture. *Cell Mol Life Sci*, (67), 9-16.

6) Craik, D.J., N.L. Daly, T. Bond& C.Waine.(1999). Plant cyclotides: a unique family of cyclic and knotted proteins that defines the cyclic CCK knot structural motif. *J Mol. Biol.* (294), 1327-1336.

7) Daly, N.L., K.J. Rosengren& D.J. Craik. (2009). Discovery, structure and biological activities of cyclotides. *Adv Drug Deliv Rev*, (61), 918-930.

8) Doyle, J.J. (1990). A rapid total DNA preparation produced for fresh plant tissue. Focus. 12), 13-15.

9) Gerlach, S.L. & D. Mondal. (2013). the bountiful biological activities of cyclotides. *Chronicles of Young Scientists*, (3), 169-177.

10) Gran, L., Sletten, K., & Skjeldal, L. (2008). Cyclic peptides from Oldenlandia affinis DC. Molecular and biological properties. *Chemistry & biodiversity*, *5*(10), 2014-2022.kha

11) Grover, T., Mishra, R., Gulati, P., & Mohanty, A. (2021). An insight into biological activities of native cyclotides for potential applications in agriculture and pharmaceutics. *Peptides*, *135*, 170430.

12) Hall, B.G. (2013). Building phylogenetic trees from molecular data with MEGA. *Mol. Biol. Evol.* (30), 1229-1235.

13) Hassanvand, A., Saadatmand, S., Lari Yazdi, H., & Iranbakhsh, A. (2021). Biosynthesis of NanoSilver and Its Effect on Key Genes of Flavonoids and Physicochemical Properties of *viola tricolor* L. *Iranian Journal of Science and Technology, Transactions A: Science*, *45*, 805-819.

14) Hellinger, R., Koehbach, J., Soltis, D. E., Carpenter, E. J., Wong, G. K. S., & Gruber, C. W. (2015). Peptidomics of circular cysteine-rich plant peptides: analysis of the diversity of cyclotides from Viola tricolor by transcriptome and proteome mining. *Journal of proteome research*, *14*(11), 4851-4862.

15) Jackson, M.A., E.K. Gilding, T. Shafee, K.S. Harris, Q. Kaas, S. Poon. (2018). Molecular basis for the production of cyclic peptides by plant asparaginyl endopeptidases. *Nat Commun,* (9), 24-11.

16) Khoshkam, Z., M. Zarrabi, Z. Sepehrizade& M. Keshavarzi. (2016). The Study of Antimicrobial Activities of Partially Purified Cyclotide Content and Crude Extracts from Viola tricolor. *J Med Bacteriol,* 29-35.

17) Khoshkam, Z., Zarrabi, M., Sepehrizadeh, Z., Naghdi, E., & Aftabi, Y. (2020). Reporting a transcript from Iranian Viola tricolor, which may encode a novel cyclotide-like precursor: molecular and in silico studies. *Computational Biology and Chemistry*, *84*, 107168.

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18) Kumar, A., K. Pulicherla, K. Seetha Ram &K. SambasivaRao. (2010). Evolutionary trend of thrombolytics. *International Journal of Bio-science and Bio-technology*, (2), 51- 68.

19) Mehta, L., Shambhawi, Kumar, S., & Mohanty, A. (2022). In silico Analysis of Native Cyclotides with Antibacterial Activity against Gram-negative Bacteria. *Applied Biochemistry and Microbiology*, *58*(6), 715-725.

20) Mushtaq, A. & A. Jamil. (2012). Cloning of a β-glucosidase gene from thermophilic fungus *Cheatomiumthermophilum*. *Pak. J. Life. Soc. Sci. (*10), 98-101.

21) Rizwan, Z., Aslam, N., Zafar, F., Humma, R., & Jamil, A. (2021). Isolation of novel Cyclotide encoding genes from some Solanaceae species and evolutionary link to other families. *Pakistan Journal of Agricultural Sciences*, *58*(1).

22) Roshan, A., Zarrabi, M., Asgarani, E., & Kanaani, M. R. (2014). Sequence analysis of gene encoding cyclotides in three species of Violaceae and determination of their anti-microbial activities. *J Biochem Microb Technol*, *2*, 1-7.

23) Saravanan, R. S., & Rose, J. K. (2004). A critical evaluation of sample extraction techniques for enhanced proteomic analysis of recalcitrant plant tissues. *Proteomics*, *4*(9), 2522-2532.

24) Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution*, *30*(12), 2725-2729.

25) Tang, J., Wang, C. K., Pan, X., Yan, H., Zeng, G., Xu, W., ... & Tan, N. (2010). Isolation and characterization of cytotoxic cyclotides from Viola tricolor. *Peptides*, *31*(8), 1434-1440.

26) Zarrabi, M., Dalirfardouei, R., Sepehrizade, Z., & Kermanshahi, R. K. (2013). Comparison of the antimicrobial effects of semipurified cyclotides from Iranian Viola odorata against some of plant and human pathogenic bacteria. *Journal of applied microbiology*, *115*(2), 367-375.

27) Zhang, J., Li, J., Huang, Z., Yang, B., Zhang, X., Li, D., ... & Liao, B. (2015). Transcriptomic screening for cyclotides and other cysteine-rich proteins in the metallophyte Viola baoshanensis. *Journal of plant physiology*, *178*, 17-26.