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# ISOLATION AND PURIFICATION OF CYCLOTIDES FROM *CALOTROPIS GIGENTEA* AS NATURAL ANTIMICROBIAL AGENT

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**ABSTRACT**

Traditional herbal medicines play a significant role in management and treatment of diseases and are gaining attention across the glob to control the diseases. *Calotropis gigantea* is a medicinal plant and have been using since ancient times for the treatment of bacterial infections, abdominal and skin diseases, wounds infections, insect bites and even for inhibiting certain cancer cells growth. Cyclotides are cyclic cyctine-rich miniproteins having three inter-locking disulphide bridges between six conserved cysteine amino acids. These cyclopeptides have exceptional properties like resistance to thermal, chemical and enzymatic degradation which is mainly due to their three-dimensional folding of polypeptide chain. The plant based bioactive cyclopeptides are novel for drug development. The current study describes the isolation and characterization of cyclotides from leaves of *C. gigentea* involving its extraction, isolation and purification by applying different techniques. Purified cyclopeptides were used as antibacterial agents against two gram positive bacteria i.e *Staphylococcus aureus* and *Bacillus subtilis* and two gram negative bacteria i.e *Escherichia coli, Pseudomonas aeruginosa.* Results showed the inhibited growth of these microbes due to antibacterial activities of cyclotides.

## Introduction

Cyclotides is a class of macrocyclic plant peptides which is characterized by a cyclic backbone having three inter-locking disulfide bonds (Slazak et al., 2021). These cyclic peptides have important properties showing exceptional resistance to chemical, thermal and enzymatic degradation which is due to their three-dimensional folding and stability due to cyclic structure and cyclic cystine knot (CCK) which is composed of head-to-tail cyclic back bone and a network of three disulfide linkages (Rajendran *et al.,* 2021). In addition to providing stability to cyclotides, the CCK arrangement, pushes a stretch of hydrophobic amino acid residues onto the surface of cyclopeptides, so these are hydrophobic in nature (Witherup *et al.,* 1994). There are also few hydrophilic amino acid residues on cyclotides surface, providing them an amphipathic character. These hydrophilic residues are important for their active membrane linking properties (Park *et al.,* 2014)

Cyclotides are miniproteins having molecular weight of 2.8 to 3.5 KDa and 28-37 amino acids (Henriques *et al.,* 2015). Their N and C termini are joined by a peptide bond to form a continuous circular backbone, having three intertwined disulfide-crosslinking bridges between CysI-CysIV and CysII-CysV to form a ladder arrangement with the disulfide bridge CysIII-CysVI running through them. This results in their signature cyclic cystine knot (CCK) motif (Puttamadappa *et al.,* 2010). Based on their sequences and structures, cyclotides can be classified into three major subfamilies: Möbius, bracelet and trypsin inhibitor subfamilies. Möbius has *cis*-pro in loop 5 while bracelet subfamily has a higher number of positively charged basic amino acids (Colgrave *et al.,* 2008; Attah *et al.,* 2016). These are characterized by their unique cyclic cystine knot motif comprising a head-to-tail cyclic backbone and a cystine knot formed by six conserved cysteine (Cys) residues (Muratspahić *et al.,* 2020).

A wide variety of cyclotides is produced in the plant kingdom, *V. anagae* showing the highest diversity while *V. cheiranthifolia* has lowest number of cyclotides (Veer *et al.,* 2017). Plant based cyclic-peptides are present in members of different angiosperm families (Rubiaceae, Apocynaceae, Fabaceae, Solanaceae, Violaceae and Poaceae) (Craik *et al.,* 1999).

A single violet species can express more than 150 cyclotides and an estimated number of over 150,000 cyclotides in violets plants (Slazak *et al.,* 2021). Cyclotides show a diverse range of bio-activities including antimicrobial, cytotoxic, anti-HIV, insecticidal, uterotonic, neurotensin inhibitory, anti-fouling and nematocidal activities (Mamusa *et al.,* 2017). These different biological properties are due variations of amino acids in cyclotides (Henriques *et al.,* 2015). Cyclotides in medicine have a novelty for designing peptide-based therapeutics. Bioactive epitopes integrated into stable cyclotide scaffolds can lead to improved pharmacokinetics and selectivity. Cyclotides grafted modulating receptor-mediated processes are made (Rajendran *et al.,* 2021). Cyclotides are used as immuno-suppressants and in the treatment of immune-related disorders (Slazak *et al.,* 2021).

*Calotropis gigantea* (crown flower or giant milkweed, madar) is a medicinal plant of Apocynaceae family in the plant kingdom and subfamily of Asclepiadaceae. *C. gigantea*has antibacterial activity to cure a number of diseases. It has phytochemicals against the human pathogenic organisms (Gruber *et al.,* 2007). Leaves of *C. gigantea* have been using for the treatment of different diseases such as tumors, skin diseases, abdominal, wounds

and also for the treatment of insect bites (Rehman *et al.,* 2021). *C. gigantea* leaves are proven to act as therapeutic agents by inhibiting the cancer cell growth of Panc-1 cells (Uthirasamy *et al.,* 2021). The purpose of current study includes isolation and purification of cyclotides from *C. gigentea* leaves, and use of purified cyclicpeptides as antibacterial agents against two gram positive bacteria such as *S. aureus and B. subtilis* and two gram negative bacteria such as *E. coli and P. aeruginosa.*

## Material and method

### Collection of plant material

Fresh whole plant *Calotropis gigantea* was collected from a garden in Quetta, Pakistan and separated into different parts: leaves, petioles, flowers, pedicels, roots and bulbs. In our study, we only used plant leaves for cyclotides extraction while other remaining parts of plant were dried and preserved for further analysis. This work has been done in Biotechnology lab, Department of Bioinformatics and Biotechnology, Government College University Faisalabad, Faisalabad, Pakistan

### Extraction of cyclotides

100 sun dried leaves (~300 gm) of *Calotropis gigentea* were macerated for 24 hours with 100% ethanol two times at room temperature, followed by extraction of leaves components with ethanol/water (1:1 v/v) solution thrice (Purnama *et al.,* 2021). The mixture was centrifuged (~1000 rpm) for twenty minutes at 4$^{o}$C. Separation was done by using dialysis bag. 2 ml leaf extract was poured into bag and bag was hug in the beaker containing ammonium sulfate solution followed by overnight transportation of molecules from dialysis bag into ammonium sulfate solution.

### Isolation and purification

After the combined organic extracts were concentrated to 100 ml (without ethanol), the liquid extract was partitioned between $H_2O$ and petroleum ether, $H_2O$ and ethyl acetate, $H_2O$ and n-butanol. This n-butanol fraction was subjected to macro-porous resin (D101) column chromatography and eluted with increasing amounts of ethanol i.e. 30% ethanol, 70% ethanol, and 95% ethanol (Kosikowska *et al.,* 2016). The cyclotide-containing fraction was separated by polyamide chromatography, elution was achieved with the increasing concentration of ethanol i.e 20% ethanol, 50% ethanol and 80% ethanol, and by reverse phase HPLC C18 chromatography, again elution was done by increasing ethanol concentration i.e 40% ethanol, 70% ethanol and 95% ethanol, and further separation achieved using a gel permeation on sephadex LH-20 and different components eluted with 70% ethanol. All the separating steps were detected by 0.2% ninhydrin ethanol solution and Commassie brilliant blue G-250 solution using thin layer chromatography (Hemu *et al.,* 2018). After all these separating techniques cyclotides-fraction was obtained and was used for further analysis (Zhou and Tan 2000; WenYan *et al.,* 2008).

### Determination of protein and peptide concentration

To confirm the presence of peptides, sodium dodecyl sulfate polyacrylamide gel electrophoresis method was used (Hellinger *et al.,* 2015). For isolation of peptides ranging from one to hundred KDa, SDS PAGE is important. It is the desired electrophoretic method for the determination of peptides less than 30 kDa. Sodium dodecyl sulfate polyacrylamide gel electrophoresis is too worked specially for doubled SDS-PAGE, a proteomic instrument expended to separate extreme water repellent cyclotides for mass spectrometric recognition. Reverse phase high performance liquid chromatography was utilized to detached carbon-based particles built on their separating among stationary and mobile stages. These particles were immersed to the stationary stage via water repellent linkages and discarded through carbon-based diluter. The extraction of peptides from reverse level was completed through diminishing the polarization of rinsing solution. High performance liquid chromatography

investigation was achieved through C18 column.

**Results**

Cyclopeptides were extracted from sample leaves of plant (*Calotropis gigantea).* Cyclopeptides have pharmacological and agricultural significance. *S*un dried leaves of this medicinal plant were grind into fine powder after addition of liquid nitrogen which consequently controlled enzymatic activity as it carries around -196 ºC temperature. Resultant material was dipped in dichloromethane or methanol in equal ratio for overnight at 21 ºC in a vibratory incubator (BJPX-103B) that resulted in removal of non-polar molecules. Cyclopeptides were filtered through Whatmann filter paper. These cyclotides were isolated from sample dissolved in 90% ammonium sulphate. Separation was done by using dialysis bag. 2 ml leaf extract was poured into bag and bag was hanged in the beaker containing ammonium sulfate mixture followed by overnight transfer of molecules from dialysis bag into ammonium sulfate solution

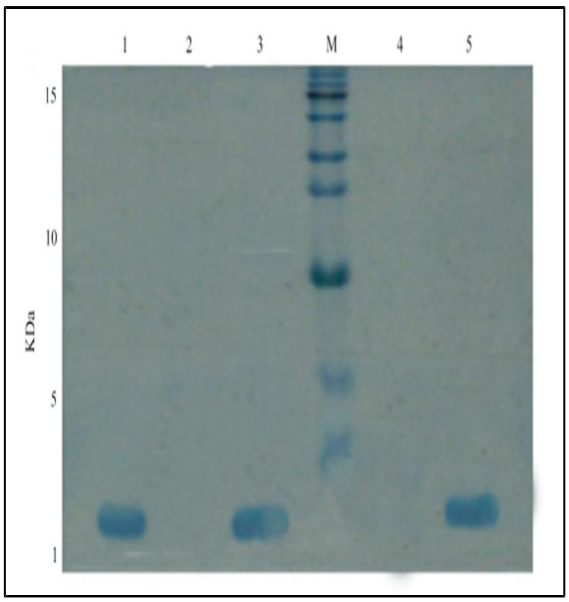



**Fig. 1** Isolation of crude peptides from leaves extract using dialysis method.

**SDS-PAGE**

Sodium dodecyl sulphate polyamide gel electrophoresis was used for the determination of molecular weight of cyclotides obtained from previous crude fractions. Sample bands were compared with the maker bands and showed that molecular weight of cyclotides ranged from 2.6-2.9 KDa.

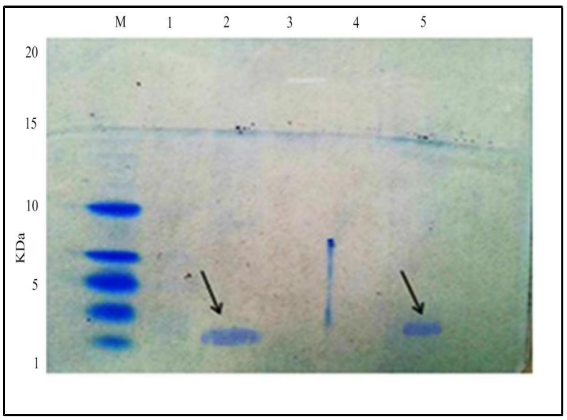
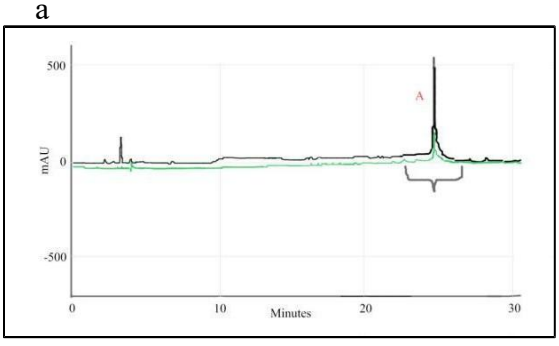



**Fig. 2** SDS-PAGE showing molecular weight of cyclotides ranging from 2.6 to 2.9 KDa.

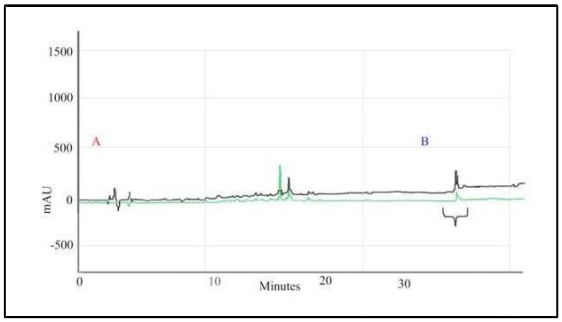
**Reverse phase-high performance liquid chromatography (RP-HPLC)**

Because of the water repellent nature of cyclotides, the reverse phase HPLC through C18 column was used for separation of cyclotides. RP-HPLC was done on mutual samples and standard solution. Non-ionic complexes that firmly affixed to the column were detached via lessening the solvent polarization. Finally the powerful non-ionic complexes were discarded. Corresponding to the standard peptides that were eluted, cyclotides were eluted after 23 to 28 minutes with reference to below figure.

a



b




c

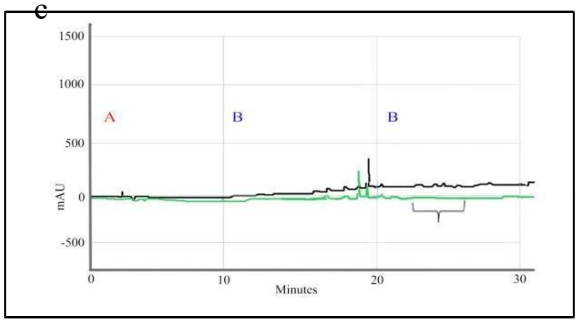



**Fig. 3** RP-HPLC chromatogram
(a) Showing the peak strength of cyclotides
(b) Peaks strength of cyclotides concentration. RP-HPLC was performed with leaves extracts for which no dialysis was done, and so additional peaks were appeared.
(c) Peaks for leaves extracts for which dialysis was done before RP-HPLC.
Point (A) crest strength of charge particles that were discarded primarily.
Point (B) crest strength of non-ionic particles

**Determination of antibacterial activity**

Results showed the antibacterial activity of cyclotides on two gram negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* and two gram positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis.*

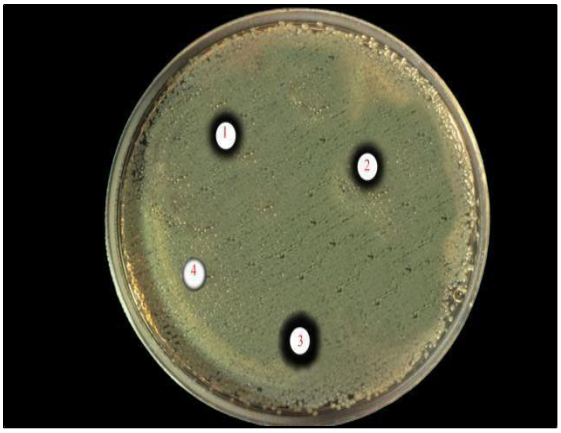
**Diameter of zones of inhibition of cyclotides and kanamycin against gram -ve and gram +ve bacteria**

**Inhibition zones of gram negative bacteria (*E. coli* & *P. aeruginosa)***

Cyclotides minimum inhibitory concentration (20 ug/ml) was applied at disks 1 and 2 and as a positive control kanamycin (20 ug/ml) was also applied at disk 3 and for negative control disk 4 was dipped in ethanol and placed on culture plate of *E. coli.* Disks 1 and 2 showed average inhibition zones of 6.5 ± 0.12 mm. While disk 3 shown the inhibition zone of diameter of 8.2 ± 0.2 mm and disk 4 didn't show any clearance zone. With the same minimum inhibitory concentration of 20 ug/ml, *P. aeruginosa* also shown the inhibition zones of diameter 11.3 ± 0.28 mm and 13.5 ± 0.34 mm for cyclotides ( disk 1 and 2) and kananmycin (disk 3) respectively.

For negative control disk 4 first dipped in ethanol and then placed on culture plate, couldn't show any inhibition zone, it means *P. aeruginosa* growth was not affected at disk 4. The zones indicated that *P. aeruginosa* strain showed almost two times larger clearance zone as compared to the *E.coli* strain (Table 1).

a




b

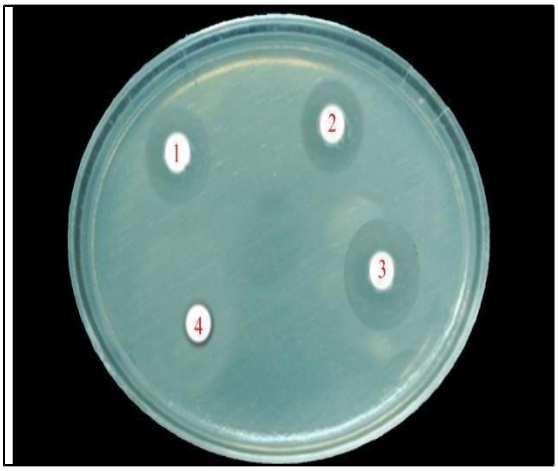
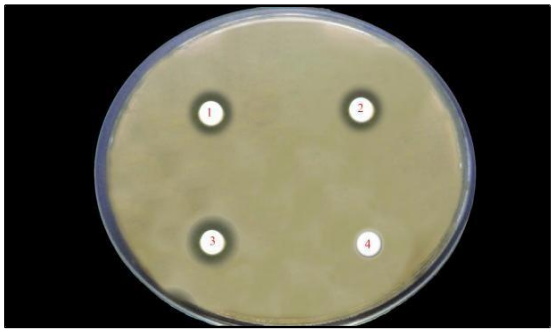



**Fig. 4** Minimum inhibitory concentration (MIC) of cyclotides inhibited the growth of *Escherichia coli* (a) and *P. aeruginosa* (b) cultures.

**Inhibition zones of gram positive bacteria (*S. aureus* & *B. subtilis)***

Cyclotides minimum inhibitory concentration (20 ug/ml) was applied at disks 1 and 2 and as a positive control kanamycin (20 ug/ml) was also applied at disk 3 and for negative control disk 4 was dipped in ethanol and placed on culture plate of *S. aureus*. Disks 1 and 2 showed average inhibition zones of 4.8 ± 0.1 mm. While disk 3 shown the diameter of 5.2 ± 0.11 mm and disk 4 didn't show any clearance zone. With the same minimum inhibitory concentration of 20 ug/ml, *B. subtilis* also shown the inhibition zones of diameter 9.7 ± 0.24 mm and 12.0 ± 0.3 mm for cyclotides (disk 1 and disk 2) and kananmycin (disk 3) respectively. For negative control disk 4 was dipped in ethanol and then placed on culture plate of *B. subtilis* couldn't show any zone of inhibition. The results clearly indicated that *B. subtilis* showed two times greater clearance zone as compared to the *S. aureus* (Table 1).

a




b

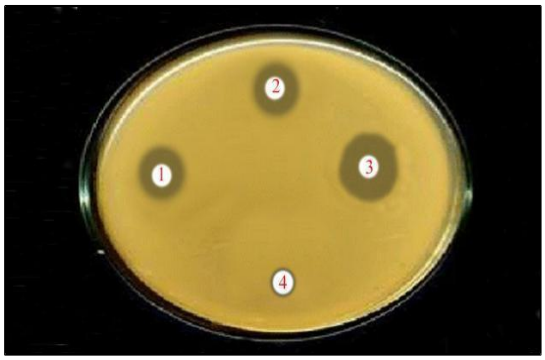



**Fig. 5** Minimum inhibitory concentration (MIC) of cyclotides inhibited the growth of *staphylococcus aureus* (a) and *Bacillus subtilis* (b) cultures.

**Diameter of zones of inhibition of cyclotides and kanamycin against gram -ve and gram +ve bacteria**

| | Disk Content | Gram -ve (Diameter mm) | | Gram +ve (Diameter mm) | |
|---|---|---|---|---|---|
| | | *E. coli* | *P. aerugi nosa* | *S. aur eus* | *B. sub tilis* |
| Cyclotides | 20 ug/ml | 6.5 ± 0.12 | 11.3 ± 0.28 | 4.8 ± 0.1 | 9.7 ± 0.24 |
| Kanamycin (Positive control) | 20 ug/ml | 8.2 ± 0.2 | 13.5 ± 0.34 | 5.2 ± 0.11 | 12.0 ± 0.3 |

**Table 1**. Effects of cyclotides and kanamycin on gram +ve and gram -ve bacteria showing diameter of zones of inhibition in millimeter (mm).

**Discussion**

Plant antimicrobial cyclic-peptides (host defence peptides) have promising potentials in novel drug development due to their wide range of bioactivity and ability to induce very little resistance. These plant based cyclotides have synergistic effect when used in combination therapy with antibiotics against pathogens, and optimize the bioactivity of these antipathogenic agents (Zhao *et al.,* 2012). Cyclic-peptides have side chains of basic amino acids i.e arginine, histidine, lysine and aromatic amino acids such as tryptophan, phenylalanine and tyrosine, which were detected during chemical analysis (Rosengren *et al.,* 2003). These amino acids play an important role in the bioactivity and physico-chemical properties of antimicrobial peptides by facilitating their solubilisation in aqueous media. Arginine and lysine-rich cationic side chains form about 20% of plant antipathogenic peptides as these have high stability due to three disulphide bridges (WenYan *et al.,* 2008). These amino acids increase the binding interactions of cyclotides and hence have improved bioactivity. Cyclic-peptides have hydrophobic nature due to certain amino acids present on their surface, so RP-HPLC (C18 column) was used for their purification (Herrmann *et al.,* 2006). Cyclotides were

eluted after 23 to 28 minutes using elution buffer. A mixture of peptides obtained after RP-HPLC was used for further analysis. SDS-PAGE showed that these have molecular weight of 2.6 to 2.9 KDa.

During current study, antimicrobial activity of cyclotides was checked on two gram positive bacteria i.e *S. aureus* and *B. subtilis* and two gram negative bacteria i.e *E. coli* and *P. aeruginosa.* The minimum inhibitory concentration of 20 ug/ml was used for both cyclotides and kanamycin in all cultures. Cyclotides have more antibacterial activity against gram negative bacteria (*P. aeruginosa)* as compared to all other gram negative and gram positive bacteria. Some reported cyclotides have inhibitory activities against certain fungi such as *Candida albicans* and bacteria such as *Mycobacterium tuberculosis, Enterococcus faecalis* and few others (Craik *et al.,* 1999). These cyclotides can inhibit the growth of microbes that form biofilms. Plant based cyclopeptides (antimicrobial peptodes) are now called Host Defence Peptides (HDP). These HDP have been isolated and purified from many other plants, and used in traditional medicines in ancient times (Gerlach *et al.,* 2013). Due to their specific bio-activities synthetic cyclotides have been developed for medicinal uses. Some cyclopeptides are used peptide-based antimicrobial therapy (Hellinger *et al.,* 2015).

**Conclusion**

This study presents the first report on the presence of cystiene-rich bioactive peptides in *Calotropis gigantea*, as well as the antimicrobial activity of the peptide enriched eluates against the gram-negative and gram-positive bacteria. *S. aurreas* strain was found to have low sensitivity to the tested extracts and the standard drug (Kanamycin). Roots of *Crescentia portoricensis* also exhibited the antimicrobial effects. *C. gigantea is* widely used in conventional drugs. As a consequence, these kinds of plants are in great interest because of the availability of cyclotides in them. These peptides are used for the purpose of destroying insects, nematodes and microbes, consequently their natural task is connected to host resistant mechanism. In this beneficial procedure, cyclotides exhibited to intermingle with plant's cell membrane and perform its biological activity. The antibacterial action of *Calotropis gigantea*'s cyclotides extraction, prification and examination on gram positive and gram negative bacteria proves that these cyclotides have excellent scaffold features and biochemical actions. Different techniques for the isolation and characterization plant based cyclopeptides are being developed.

**Conflict of Interest**

All the authors contributed equally to this work. So there is no conflict of interest.

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