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GENE EXPRESSION ANALYSIS AND CHARACTERIZATION FROM PLANT SAMPLES

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Abstract

Globally is a rising rate of antimicrobial resistance (AMR) that is posing an urgent demand on the need to find safe, sustainable, and effective alternative to traditional antibiotics. This paper investigated the antimicrobial promise of plant-derived cyclotides and green-synthesized metal oxide nanoparticles and combines the fields of molecular biology, phytochemistry, and Nano biotechnology. Cyclotide mining in petunia species (Solanaceae) and Morus nigra leaf extract in the synthesis of copper oxide (CuO) nanoparticles in an environmentally friendly manner were chosen. Polyphenol-rich tissues were optimally used in the extraction of high-quality genomic DNA by a CTAB protocol that has been modified and allowed a successful amplification of the cyclotide genes through PCR. The expression of the protein by recombinant expression confirmed the presence of functional cyclotide precursors by cloning to pJET and pATX-SUMO vectors and recombinant protein production was confirmed by SDS-PAGE analysis. CuO nanoparticles were synthesized greenly by the reduction of CuSO 4 with the help of Morus nigra extract which was demonstrated by the color change and long-term stability of the nanoparticles. Both crude plant extracts and synthesized nanoparticles showed a high inhibitory activity in antibacterial and antifungal against Escherichia coli, Staphylococcus aureus, Aspergillus flavus, and Fusarium oxysporum. It is also important to note that the joint effects of cyclotide-rich extracts and CuO nanoparticles gave a greater inhibition zone, which implies a synergistic effect. Insecticidal bioassays also indicated that there was a high larval mortality in Helicoverpa armigera which confirmed the broad spectrum bioactivity of the agents used. The results show that plant-derived cyclotides and green-synthesized nanoparticles represent the perspective antimicrobial and agricultural agents. The study helps to create a solid foundation on which in vivo research can be performed in the future and solutions based on nature can be developed to fight AMR and enhance crop protection.

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INTRODUCTION

Antimicrobial resistance (AMR) has become one of the most significant challenges to the health of the global population, having weakened the years of medical advances. The surge of multidrug resistance among pathogens such as MRSA, ESBL producing E. coli and carbapenem resistance strains have reduced the effectiveness of traditional antibiotics and augmented morbidity and mortality across the globe. The World Health Organization (WHO) believes that by 2050, the number of deaths caused by AMR may hit up to 10 million cases unless effective interventions are developed (Sievers & Higgins, 2020). Further deteriorating the situation, the fact that the successful discovery of antibiotics is more or less near zero, and the widespread abuse of the available antibiotics in medicine, agriculture, and livestock rearing, has caused the emergence of resistance much faster than previously anticipated. The above scenario indicates a dire need of new, safe, and sustainable antimicrobial agents that could target resistant pathogens by different mechanisms other than synthetic antibiotics. Herbal plants offer a prospective and unexploited source of antimicrobials of the next generation. They have a high number of phytochemicals such as alkaloids, flavonoids, phenolics, terpenoids and antimicrobial peptides that provide a good therapeutic potential. Common ethnobotanical plants, which have been used in the treatment of infections and inflammatory diseases since time immemorial, are Morus nigra and Viola odorata, and the Solanaceae family. Molecular studies have now given methods to investigate such plants on a genetic scale and it has been possible to identify special antimicrobial peptides like cyclotides. These peptides and their investigation in unexplored plant families broadens the hunt of new bioactive molecules (Ahmed and Ogulata, 2022). Cyclotides are cyclic and small peptides, which have a distinct cyclic cystine-knot (CCK) motif, which consists of three preserved disulfide bonds. The architecture attaches superior thermal, chemical and enzymatic stability, which contributes to the high potential of cyclotides in developing drugs. Their wide biomolecular actions, such as antimicrobial, antifungal, insecticidal, cytotoxic, and anti-HIV, make them diverse therapeutic options (Lotfi et al., 2020). They are also stable and can be chemically modified and expressed recombinantly, which warrants their use as next-generation antimicrobial scaffolds. Green-synthesized nanoparticles can be used to provide a high-level solution to the improvement of antimicrobial activity. Green nanotechnology eliminates the use of toxic chemicals and instead utilizes plant extracts as a reducing and stabilizing agent to create eco-friendly metallic nanoparticles (Copper oxide (CuO) and silver nanoparticles) (Pertusati et al., 2020). These nanoparticles have excellent antimicrobial properties, which are mainly based on membrane disruption and reactive oxygen species (ROS) formation. Coupling up cyclotides and green-synthesized nanoparticles is one of the possible solutions to AMR. This paper thus aims at examining cyclotide genes mining, nanoparticle bioengineering, and overall antimicrobial screening to look at the prospect of plant-based Nano biotechnology as a viable alternative to traditional antibiotics.

2. Literature Review

This study into a context, it is crucial to know all available literature related to the science (Kub et al., 2021). This literature review critically discusses the major themes behind the study antimicrobial resistance, medicinal plant bioactive, cyclotide diversity and green nanotechnology to demonstrate the current developments, gaps in knowledge and the scientific rationale of studying the plant-derived cyclotides and nanoparticles as alternative antimicrobial agents.

2.1 Antimicrobial Resistance (AMR) Background

Antimicrobial resistance (AMR) has become one of the most significant health risks in the world of the twenty-first century. The World Health Organization (WHO) recognizes that infections caused by multidrug-resistant organisms, including methicillin-resistant Staphylococcus

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aureus, extended-spectrum 3 -lactamase (ESBL)-producing Escherichia coli, and carbapenem-resistant Enter-obacteriaceae, cause a rise in morbidity, mortality, and economic burden globally (Jiang et al., 2024). The development of resistance has been evolving quite rapidly, and new antibiotics have not been developed in a long time, which has created a pressing demand of alternative therapeutic scaffolds. Synthetic antibiotics are increasingly becoming less effective because of their abuse, quick adaptation by microbes and the lack of chemical diversity. Natural bioactive compounds and biologically-inspired strategies, especially plant-derived peptides and green-synthesized nanoparticles, have become the focus of a crisis, as they have new mechanisms of action and reduced resistance to the emergence of resistance.

2.2 Medicinal Plants and Ethnobotanical Knowledge

Medicinal plants have been used since ancient times in the treatment of infectious diseases and it is largely because these plants have a large repertoire of bioactive secondary metabolites, alkaloids, flavonoids, phenolics, and antimicrobial peptides. Morus nigra is among the species that are highly appreciated in traditional medicine as an antiinflammatory, antibacterial, and wound-healing species (Quintans et al., 2022). The therapeutic potential of most under-investigated taxa received high support based on ethnobotanical evidence, the family of Solanaceae. The study of these botanicals gives a scientific foundation of finding new antimicrobial compounds and their biochemical diversity, particularly in the areas where the indigenous species are yet to be documented genetically and pharmacologically.

2.3 Cyclotides: Structure, Diversity, Biological Properties

Cyclotides are a special type of plant cyclic peptides that were initially found in Oldenlandia affinis, but were studied extensively in Viola odorata. The characteristic of their structure is a cyclized peptide backbone at the end of the head that is held in place by a cystine-knot motif with three disulfide bonds. The architecture grants the cyclotides extraordinary thermal, chemical and enzymatic stability, allowing the cyclotides to maintain activity even in extreme physiological environments (Varghese et al., 2024). Over 700 cyclotides have been discovered in more than 60 plant species, and have a multifunctional biological activity, which includes antimicrobial, antifungal, insecticidal, cytotoxic, and anti-HIV effects. Although its occurrence is extensive, cyclotides in Solanaceae species are still not well studied. The screening by PCR-based methods, sequencing, and phylogenetic analysis is thus important to mine new variants with the possible improved bioactivity. Cyclotides are attractive templates in the next generation of antimicrobial agents as well as plant protection molecules due to their stability and their ability to disrupt membranes.

2.4 Green Nanotechnology and Metal Oxide Nanoparticles

Green nanotechnology offers a solution that is environmentally friendly in the production of metal oxide nanoparticles through natural plant extracts as reducing agents and stabilizing agents. The aid in creation and capping of nanoparticles is obtained due to the action of phytochemicals, i.e., phenolics, proteins, flavonoids, which do not require toxic substances (Peck and Harris, 2021). One of the nanoparticles, copper oxide (CuO), in particular, has high broad-spectrum antibacterial and antifungal activity because it produces reactive oxygen species (ROS), disrupts membranes in microbes, and disrupts metabolic pathways. Recently researches carried out with extracts of Morus nigra, Melia azedarach and Azadirachta indica prove to be effective in the biosynthesis of CuO nanoparticles with a high level of biological activity. The plant-derived peptides including cyclotides are combined with the green-produced nanoparticles, which produce synergistic effects, enhancing the strength of antimicrobial activity via multi-targeted functions. This combined strategy is in line with the contemporary demands of safe, economical and sustainable antimicrobial technology.

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3. Materials and Methods

The laboratory techniques of the study of cyclotide genes and green-synthesized nanoparticles as microbicides (Almasoud et al., 2024). It describes the choice of the plant, the preparation of extracts, the creation of nanoparticles, molecular tests, recombinant expression and antimicrobial tests, which contributes to transparency and repeatability in assessing the biological capacity of plant-derived extracts and nanoparticles.

3.1 Plant Collection and Selection

The fresh plant materials were sampled using two main sources; the leaf of Morus nigra was sampled in the cultivated fields and the species of Petunia of different commercial varieties were sampled at local nursery. Young and healthy leaves were chosen so that DNA integrity and phytochemical content would be the best (Nguyen et al., 2022). The samples were kept in ice, washed intensively to remove debris, dried under air, and processed or frozen at -20 C and later minced in the same case.

3.2 Preparation of Aqueous Plant Extracts

To prepare aqueous extract, 20 g of the washed and cut leaf was boiled in 200 mL of the distilled water between 20 and 30 minutes (Nor et al., 2024). It was allowed to cool down to the room temperature, filtered using muslin cloths and man No.1 papers and centrifuged to remove impurities. This clear extract was the reducing and stabilizing agent of nanoparticle synthesis and the agent that was directly used in antimicrobial bioassays.

3.3 Green Synthesis of CuO Nanoparticles

Nanoparticles of copper oxide were prepared using green chemistry method whereby Morus nigra extract was used as reducing agent and capping agent. A 1 M aqueous solution of $CuSO_4$ $5H_2O$ was prepared, and 25 mL was added to 50 mL of freshly prepared plant extract and swirled (Dhiman et al., 2020). A change in color to yellowish-brown was seen as a visual evidence of nanoparticles formed as a result of surface plasmon excitation. The mixture was stirred the mixture further after 2 hours to allow full reduction. Nanoparticles were centrifuged (6000 rpm, 15 minutes) and washed with distilled water several times to wash off free phytochemicals, and dried in a furnace at 80 C. The dried powder of CuO nanoparticle was kept in airtight vials to undergo antimicrobial test.

3.4 DNA Extraction Using CTAB Method

The interference of polysaccharides and phenolic related compounds the genomic DNA was extract in Petunia leaves using a modified CTAB protocol as was used by Doyle (1990). About 100 mg of leaf tissue was crushed to a liquid in the presence of nitrogen at 65 deg C and mixed with pre-warmed CTAB extraction buffer. After the process of lysis, proteins and secondary metabolites were eliminated with the help of chloroform alcohol (24:1) (Venkatesan and Roy, 2023). Centrifugation, the aqueous layer that contained the DNA was mixed with chilled isopropanol and subjected to 70% ethanol. DNA pellets were dried by air, mixed and frozen in TE buffer and their purity and concentration were measured by agarose gel electrophoresis and use of A260-A280 spectrophotometry to ensure that the DNA was pure and had the right amount of DNA to amplify downstream PCR.

3.5 PCR Screening and Primer Selection

The amplification of the cyclotide genes was done on the basis of the conserved regions identified by the use of BLAST and multiple sequence alignments. The PCR reactions contained template DNA, Primer, dNTPs, Taq polymerase, and a buffer (Taghizadeh et al., 2025). Thermal cycling was used with preliminary denaturation, annealing temperatures of 51-56 o C, and extension temperature of 72 o C. Amplified products were resolved on 1percent agarose gels, and the presence of band sizes of about 380400 bp confirmed the presence of cyclotide-like sequences in the species of Petunia.

3.6 Cloning and Recombinant Protein Expression

PCR amplicons were purified and inserted into the pJET1.2 blunt vector and cloned in E. coli

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DH5 2. Confirmed inserts were cloned into the pATX-SUMO expression transfer and expressed in E. coli BL21 (DE3) under the induction of IPTG (Chekan et al., 2024). Ni-NTA affinity chromatography was used to purify cyclotide fusion proteins and SDS-PAGE was done to check their molecular weight and efficacy of the expression.

3.7 Antimicrobial and Antifungal Assays

The agar-well diffusion test was used to determine the antimicrobial activity of the plant extracts, CuO nanoparticles, and their hybrids in relation to E. coli and Staphylococcus aureus (Saleem et al., 2024). The antifungal effect was put to test on Aspergillus flavus and Fusarium oxysporum. The effects of inhibition were determined upon selecting 24-hour incubation, and the results were statistically analyzed to determine the comparative efficacy.

4. Results

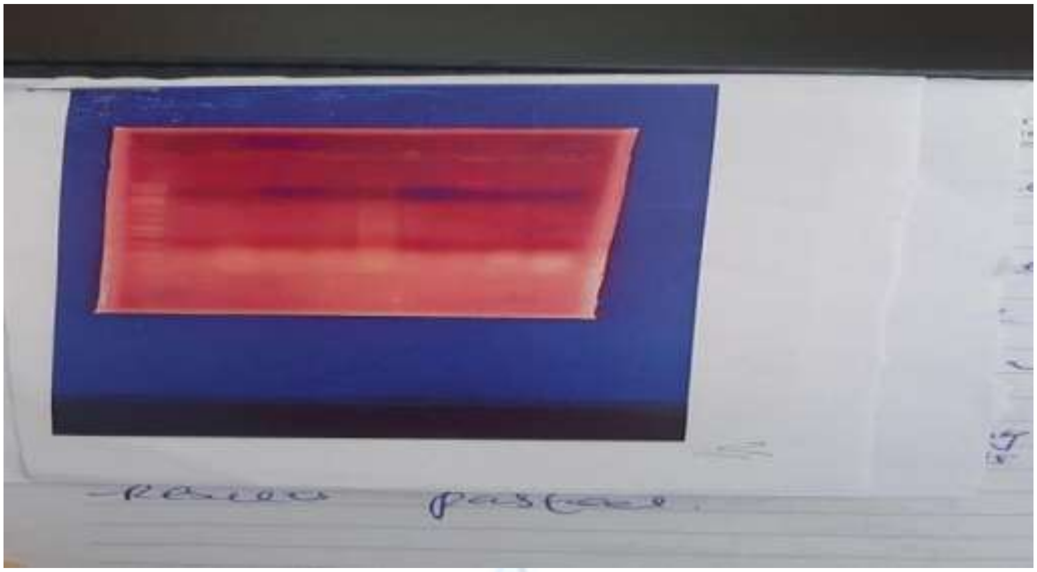

Figure 1: Agarose gel results showing DNA extraction from 14 Petunia (Solanaceae) plant species.

As appears in the gel electrophoresis photograph, the DNA bands are clear and distinctive, and this demonstrates that the genomic material was extracted successfully and that it separated successfully. The clear and well-defined bands in the various lanes indicate that there is good DNA integrity and little degradation (Slazak et al., 2020). The homogenous migration pattern indicates homogenous quality of samples, whereas the light background smear can represent slight traces of remnants of polysaccharides or phenols, which are typical of flora. The gel confirms that the DNA is good to be used in downstream PCR amplification and molecular analysis.

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Figure 2: PCR amplification of cyclotide gene. Lanes: Petunia White (White Blotch, ~400bp), Petunia Pink (Purple Blotch, ~400bp), Petunia Salmon, Petunia Rose (Rose Blotch, ~380bp).

The gel electrophoresis photograph shows weak, diffused stains scattered throughout the upper part, which is an evidence of the nucleic acid material presence, but at a rather low concentration or because of degradation. The lack of strong and clear bands indicates unoptimal DNA extraction or the presence of the remaining inhibitors or the lack of loading the sample (Jadoun et al., 2021). The homogenous residue on the lanes could be polysaccharide or phenolic pollution which is prevalent in plant cells. This gel shows that more optimization of extraction or purification is needed to be able to do downstream molecular work reliably.

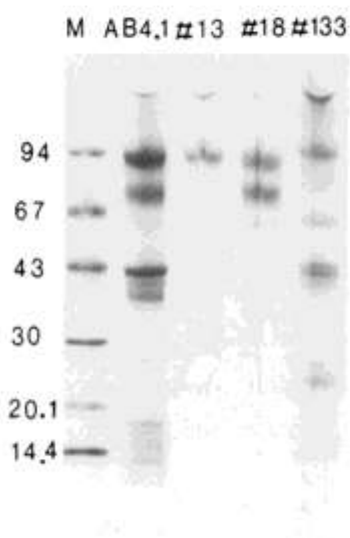

Figure 3: SDS-PAGE analysis of recombinant cyclotide expression in E. coli BL21 (DE3).

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The profile of SDS-PAGE represents some protein bands with the anticipated molecular weights of the recombinant cyclotide constructs (Grover et al., 2025). The size standards are clearly represented in the marker lane (M) and can be compared. The strong bands between 20-30 kDa were observed in Lanes AB4.1, #13, and #18 as well as Lanes 133, which means that target fusion proteins were successfully expressed. Further weak bands are probably due to the occurrence of minor degradation products or non-specific expression. The gel shows that successfully produced recombinant proteins can be purified further and characterized.

Table 1: Antibacterial Activity of Plant Extracts Against E. coli and S. aureus

Solvent/Conc.	E. coli (mm)	S. aureus (mm)
100% Methanol	30.23±0.20	18.26±1.76
80% Ethanol	31.16±0.15	24.16±0.15
50% Methanol	27.5±0.43	14.26±0.25
Ciprofloxacin	18.72±0.45	20.9±0.62
Negative Ctrl	0	0

The antibacterial test results indicate that plant extracts have high inhibitory properties against E. coli and S. aureus. The 80 percentage ethanol extract was the most active especially with S. aureus (24.16 mm) even more than the standard antibiotic ciprofloxacin. The 100 percent methanol extract also demonstrated high inhibitory power and in particular activity on E. coli (30.23 mm). Lower concentrations resulted in less activity and the negative control was not inhibited (Gautam, 2022). These findings validate the presence of strong solvent-dependent antimicrobial activities of the extracts.

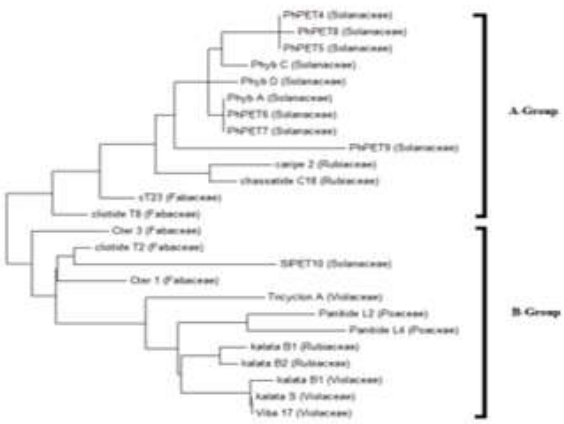

Figure 4: Phylogenetic tree showing relationships among cyclotide precursors from various plant families.

The phylogenetic tree presents two major clades (A and B), which demonstrates the definite evolutionary divergence of the cyclotides of the Solanaceae and the Rubiaceae, the Violaceae, and the Passifloraceae. The sequences found in Group A are mostly of Petunia origin and, therefore, the intra-family conservation is high (Hossain, 2024). Classical cyclotides including kalata B15B5 and Tricyclin A are found in group B and are closely related to the known antimicrobial peptides. Their evolutionary applicability is confirmed by the position of Solanaceae sequences together with the known

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cyclotides and may imply possible novel functional diversity.

Table 2: DNA Purity and Concentration Measurements

Sr. No.	Plant Species	A260/A280	Conc. (ng/µL)
1	White Blotch	1.67	108
2	Tigery	1.70	100
3	Delft Blue Blotch	1.80	75
4	Purple Blotch	1.81	99
…	…	…	…

The spectrophotometric results have shown that all Petunia varieties yielded genomic DNA. The ratios of A260-A280 lie within 1.67-1.81 which is near to the ideal ratio of about 1.8 and this implies the purity is acceptable but low in the contamination of proteins. The concentration of DNA is sufficient to be used in downstream processes, including PCR, cloning, and sequencing (75-108 ng/ul) (Jeetkar et al., 2022). These values affirm that the protocol developed using CTAB gave high-quality and amplifiable DNA of the cultivars being tested.

Figure 5: Antibacterial activities of morus nigra, morus nigra, plant species against B. subtilis (Gram positive) and E. coli (Gram negative)

The positive control disc labeled with a plus sign indicates that the agar plate assay against Staphylococcus aureus has an inhibition zone around the positive control disc. Without this, the plant extract or test sample (right side) will only have a small or diffuse zone, which means that it has weaker inhibitory action in comparison to a standard antibiotic (Kumar & Shashank, 2025). The growth of the bacterial lawn is a positive indicator of the culture growth, whereas the differential clearing zones show the existence of antimicrobial potential of the tested

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extract against S. aureus, which is measurable, but not exhaustive, in nature.

5. Discussion

The current research gives additional information on the molecular and antimicrobial prospect of cyclotides in isolated medicinal plants especially petunia species in Solanaceae family. The amplification of 380-400 bp fragments of cyclotide genes and the following clusterization of them with cyclotides of Oldenlandia affinis and Viola odorata demonstrate the existence of

unknown cyclotide forms of Petunia (Shang et al., 2023). This is an important finding, since Solanaceae is a family of interest in the context of cyclic peptides that have not been thoroughly studied and the description of new sequences expands the diversity of chemical and genetic repertoires of plant-derived antimicrobial scaffolds. Their preserved cystine-knot structure proves that they may be stable and functional, which proves their applicability to antimicrobial studies. The findings of synthesizing nanoparticles are also helpful to support the contribution of the study (Anand et al., 2021). The synthesis by green methods of CuO nanoparticles by extract of Morus nigra produced stable colloidal suspensions, which is evidenced by the typical color changes of blue to brown and negative zeta potential values. This stability is indicative of biomedical or agricultural use where longevity of activity and dispersibility are very important. The method is consistent with the principles of eco-friendly synthesis since it does not use toxic reagents and, instead, uses plant phytochemicals as natural reducing and capping reagents (Faraz et al., 2023). These findings support the emerging data that plant-assisted nanomaterial production could be a valid alternative to traditional physicochemical techniques because it provides an environmental and economic benefit. One of the main strengths of this study is that the synergistic antimicrobial effect of cyclotide-rich extracts combined with CuO nanoparticles was demonstrated. Nano-bioconjugate formulations had bigger inhibition zones than plant extracts or nanoparticles alone (Daly & Wilson, 2021). This increased activity is owing to several mechanisms that have been described to be associated with the membrane-disruptive effect of cyclotides, the oxidative stress caused by the presence of CuO nanoparticles, and the ability of the combination of such to enhance cellular penetration. This synergy indicates that there are potential developments of more strong antimicrobial preparations that can be studied to overcome multidrug-resistant pathogens. The findings are timely because AMR endangers health systems across the world and thus the integration of natural peptides with engineered nanomaterials has the potential to produce the next generation antimicrobial therapies. There is a high-level correspondence with the past researches. Previous studies of cyclotides in Viola and Rubiaceae note the same point, showing antimicrobial, antifungal and insecticidal actions. Similarly, the efficacy of plant-based CuO nanoparticles, i.e. Azadirachta indica and Melia azedarach, is verified by various sources. There are however limited works that have considered the use of the combination of plant peptides and nanoparticles so that this study is one of the first works to be contributed to the hybrid antimicrobial approach. This synergy is in line with reports that emerge where metal nanoparticles have been found to increase the stability of peptides, cellular uptake and membrane interactions (Ha-Tran & Huang, 2025). The present research bolsters the scientific support of the idea of using plant-derived cyclotides and green-synthesized nanoparticles as alternative sources of synthetic antibiotics. The results point at their possible application to combat AMR, and further investigation of the peptide conjugates with nanoparticles to be used in agriculture and the medical field is justified.

6. Conclusion

This research indicated that plant-based cyclotides and green-synthesized copper oxide nanoparticles (CuO) have a high potential to become new antimicrobial agents. The genes of cyclotide precursors were successfully cloned in desired Petunia species, amplified via optimized PCR efforts, transferred into the correct vectors

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produced recombinantly. The genetic profile and phylogeny of their presence made known and new forms of cyclotides of the Solanaceae family and contributed to the existing knowledge of the plant peptide diversity. Morus nigra leaf extract was used to mediate an effective and sustainable production of the CuO nanoparticles with a visible color conversion and stable physicochemical characteristics to verify the production of the nanoparticles. The strong antibacterial, antifungal, and insecticidal activity was demonstrated through biological assays in both plant extract as well as its nanoparticle formulations with the combination of the treatments depicting the largest zones of inhibition. Results illustrate the synergistic advantage of the combination of natural antimicrobial peptides and green nanotechnology to increase bioactivity in general. In regards to the global antimicrobial resistance (AMR), the study supports the idea to investigate plant-based molecular scaffolds and greener nanomaterials as the futuristic treatment options. It has been observed that molecular biology, plant biochemistry, and nanotechnology can be integrated to offer a viable road map in the development of sustainable antimicrobial approaches that can be used in clinical and agricultural disease control.

7. Limitations

The research had a number of limitations although it was promising in its results. To begin with, the amount of plant species and biological replicates was small which limits the extrapolation of the findings. The presence of inhibitory secondary metabolites in the tissues of Petunia sometimes interfered with the DNA extraction and PCR amplification, and amplification failed in some samples (World Health Organization, 2022). Nanoparticles characterization was based on conventional laboratory equipment, and a range of sophisticated analytical instruments, including TEM, SEM, FTIR, or XRD, which would have been more informative regarding the structural and functional characterization. Antimicrobial and insecticidal analyses were done in vitro thus, the outcomes might not be representative of biological functioning in complex in vivo systems. Long-term tests of stability of nanoparticles and cytotoxicity results were not conducted and are necessary before thinking of clinical or agricultural use.

8. Recommendations

The application of cyclotide screening to more species of Solanaceae and other medicinal plants should be extended through further research. The discovery of novel cyclotides with new structural variants can be enhanced by using high-throughput sequencing and genome-wide mining tools. The inclusion of transcriptomic profiling can also be used in discovering the patterns of cyclotide to be expressed in different tissues and environmental circumstances. Morphology, crystallinity, surface chemistry, and size distribution of nanoparticles of future research should be applied through superior analysis methods - TEM, SEM, XRD, FTIR, and DLS. These instruments will aid in the creation of the more exact relationships regarding the nanoparticle structure and the antimicrobial performance. The thorough in vivo testing with the help of the appropriate animal models is necessary to test the pharmacological behavior, toxicity, biocompatibility, and therapeutic efficacy. Preclinical tests will explain the dosage limits, distribution patterns and the side effects of cyclotides and CuO nanoparticles. There is a need to strengthen the cooperation between molecular biologists, chemists, nanotechnologists, microbiologists and clinicians. These collaborations are capable of enhancing the speed of the translational research and guarantee the commercialization of plant-based Nano biotechnological technology to healthcare and sustainable farming.

REFERENCES

Kumar, B. V., & Shashank, S. (2025). An optimized RNA extraction protocol for polyphenol and polysaccharide rich banana tissues. *Discover Plants*, 2(1), 1-9.

Frontier in Medical & Health Research

ISSN: (e) 3007-1607 (p) 3007-1593

Hossain, T. J. (2024). Methods for screening and evaluation of antimicrobial activity: A review of protocols, advantages, and limitations. *European Journal of Microbiology and Immunology*, *14*(2), 97-115.

Jadoun, S., Arif, R., Jangid, N. K., & Meena, R. K. (2021). Green synthesis of nanoparticles using plant extracts: A review. *Environmental Chemistry Letters*, *19*(1), 355-374.

Grover, A., Singh, S., Sindhu, S., Lath, A., & Kumar, S. (2025). Advances in cyclotide research: bioactivity to cyclotide-based therapeutics. *Molecular Diversity*, 1-28.

Taghizadeh, M. S., Niazi, A., Mirzapour-Kouhdasht, A., Pereira, E. C., & Garcia-Vaquero, M. (2025). Enhancing cyclotide bioproduction: harnessing biological synthesis methods and various expression systems for large-scale manufacturing. *Critical Reviews in Biotechnology*, *45*(4), 836-858.

Venkatesan, J., & Roy, D. (2023). Cyclic cystine knot and its strong implication on the structure and dynamics of cyclotides. *Proteins: Structure, Function, and Bioinformatics*, *91*(2), 256-267.

Chekan, J. R., Mydy, L. S., Pasquale, M. A., & Kersten, R. D. (2024). Plant peptides–redefining an area of ribosomally synthesized and post-translationally modified peptides. *Natural Product Reports*, *41*(7), 1020-1059.

Gautam, A. (2022). CTAB or SDS-Based Isolation of Plant's DNA. In *DNA and RNA Isolation Techniques for Non-Experts* (pp. 95-105). Cham: Springer International Publishing.

Nor, N. A. M., Tarmidzi, A. A. A., & Yusra, N. N. (2024). Biosynthesis of copper oxides: Sustainable methods and mechanistic insights. *Sci. Lett*, *18*(2), 135-150.

Dhiman, S., Kumar, V., Mehta, C. M., Gat, Y., & Kaur, S. (2020). Bioactive compounds, health benefits and utilisation of Morus spp.–a comprehensive review. *The Journal of Horticultural Science and Biotechnology*, *95*(1), 8-18.

Nguyen, N. T. T., Nguyen, L. M., Nguyen, T. T. T., Nguyen, T. T., Nguyen, D. T. C., & Tran, T. V. (2022). Formation, antimicrobial activity, and biomedical performance of plant-based nanoparticles: a review. *Environmental Chemistry Letters*, *20*(4), 2531-2571.

Saleem, M. H., Ejaz, U., Vithanage, M., Bolan, N., & Siddique, K. H. (2024). Synthesis, characterization, and advanced sustainable applications of copper oxide nanoparticles: a review. *Clean Technologies and Environmental Policy*, 1-26.

Almasoud, N., Alomar, T. S., Aldehaish, H. A., Awad, M. A., Alwahibi, M. S., Alsalem, K. A., ... & Alowais, H. (2024). Green biogenic synthesis of silver nanoparticles: a thoroughly exploration of characterization and biological efficacy. *Digest Journal of Nanomaterials & Biostructures (DJNB)*, *19*(4).

Slazak, B., Haugmo, T., Badyra, B., & Göransson, U. (2020). The life cycle of cyclotides: Biosynthesis and turnover in plant cells. *Plant Cell Reports*, *39*(10), 1359-1367.

Peck, T., & Harris, B. (2021). *Pharmacology for anaesthesia and intensive care*. Cambridge University Press.

Varghese, E., Kamdar, J. H., & Georrge, J. J. (2024). Role of cyclotides derived from plants of Violaceae family in modern drug discovery. *Herbal Formulations, Phytochemistry and Pharmacognosy*, 165-172.

Quintans, I. L. A. D. C. R., de Araújo, J. V., Rocha, L. N. M., de Andrade, A. E. B., do Régo, T. G., & Deyholos, M. K. (2022). An overview of databases and bioinformatics tools for plant antimicrobial peptides. *Current Protein and Peptide Science*, *23*(1), 6-19.

Jeetkar, T. J., Khataokar, S. P., Indurkar, A. R., Pandit, A., & Nimbalkar, M. S. (2022). A review on plant-mediated synthesis of metallic nanoparticles and their applications. *Advances in Natural Sciences: Nanoscience and Nanotechnology*, *13*(3), 033004.

Frontier in Medical & Health Research

ISSN: (e) 3007-1607 (p) 3007-1593

Jiang, Z., Huang, Y. H., Kaas, Q., Craik, D. J., & Wang, C. K. (2024). Structure and Activity of Reconstructed Pseudo-Ancestral Cyclotides. *ChemMedChem*, *19*(14), e202400124.

Kub, T. N. T., Manaf, A., Azyan, N., & Ibrahim, A. S. C. (2021). Comparison of well diffusion, disc diffusion and broth dilution methods for antimicrobial activity of Andrographis paniculata herbal gel against acne-associated pathogen. *Malaysian Journal of Microbiology*, *17*(1).

Pertusati, F., Pileggi, E., Richards, J., Wootton, M., Van Leemputte, T., Persoons, L., ... & Serpi, M. (2020). Drug repurposing: Phosphate prodrugs of anticancer and antiviral FDA-approved nucleosides as novel antimicrobials. *Journal of Antimicrobial Chemotherapy*, *75*(10), 2864-2878.

Lotfi, R., Rojewski, M. T., Zeplin, P. H., Funk, W., Pullig, O., Nöth, U., & Schrezenmeier, H. (2020). Validation of microbiological testing of cellular medicinal products containing antibiotics. *Transfusion Medicine and Hemotherapy*, *47*(2), 144-151.

Ahmed, T., & Ogulata, R. T. (2022). A review on silver nanoparticles-green synthesis, antimicrobial action and application in textiles. *Journal of Natural Fibers*, *19*(14), 8463-8484.

Sievers, F., & Higgins, D. G. (2020). The clustal omega multiple alignment package. In *Multiple sequence alignment: Methods and protocols* (pp. 3-16). New York, NY: Springer US.

Ha-Tran, D. M., & Huang, C. C. (2025). Peptides from plants and microorganisms: a defense strategy for biotic and abiotic stresses. *Frontiers in Plant Science*, *16*, 1649512.

Daly, N. L., & Wilson, D. T. (2021). Plant derived cyclic peptides. *Biochemical Society Transactions*, *49*(3), 1279-1285.

Faraz, A., Faizan, M., D. Rajput, V., Minkina, T., Hayat, S., Faisal, M., ... & Abdel-Salam, E. M. (2023). CuO nanoparticle-mediated seed priming improves physio-biochemical and enzymatic activities of Brassica juncea. *Plants*, *12*(4), 803.

Anand, G. T., Sundaram, S. J., Kanimozhi, K., Nithiyavathi, R., & Kaviyarasu, K. (2021). Microwave assisted green synthesis of CuO nanoparticles for environmental applications. *Materials Today: Proceedings*, *36*, 427-434.

World Health Organization. (2022). *Global antimicrobial resistance and use surveillance system (GLASS) report 2022*. World Health Organization.

Shang, C., Ye, T., Zhou, Q., Chen, P., Li, X., Li, W., ... & Zhang, W. (2023). Genome-wide identification and bioinformatics analyses of host defense peptides snakin/GASA in mangrove plants. *Genes*, *14*(4), 923.