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Synthesis, Structural Characterization, And Biological Evaluation Of Cyclic Tripeptides: Investigating Their Antimicrobial, Anti-Inflammatory, And Antioxidant Potential Through Spectroscopic And Bioactivity Assays

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ABSTRACT

The goal of this study was to create new cyclic tripeptides (CTRIP-1 and CTRIP-2) and assess their pharmacological potential for antibacterial, anti-inflammatory, and antioxidant properties. TLC, mass spectrometry, FTIR, melting point measurement, and NMR spectroscopy were used to synthesise and characterise both peptides. The agar well diffusion method was used to measure antibacterial activity against Staphylococcus aureus and Escherichia coli. Zones of inhibition for CTRIP-2 increased dose-dependently and approached those of norfloxacin at higher doses, demonstrating its better antibacterial activity over CTRIP-1. COX-1 and COX-2 inhibition assays revealed significant anti-inflammatory activity for both peptides, with CTRIP-2 showing stronger inhibition (COX-1: 82.85%; COX-2: 85.76%) relative to CTRIP-1. Antioxidant potential was evaluated using the ABTS radical decolorization assay, where CTRIP-2 (IC_{50}: 118.92 µg/mL) showed better radical scavenging ability than CTRIP-1 and was comparable to Vitamin C. The results collectively highlight CTRIP-2 as a promising candidate for further development due to its potent and broad-spectrum bioactivity.

Keywords: *Peptide, Cyclic tripeptide, Antibacterial, ABTS radical, Cyclooxygenase*

1. INTRODUCTION

Because of their innate biological compatibility, specificity, and broad-spectrum activity, peptides have become an important class of bioactive chemicals with enormous therapeutic promise. Because of their superior stability, resistance to enzyme degradation, and conformational rigidity over their linear counterparts, cyclic peptides have attracted a lot of attention among them. Because of these characteristics, cyclic peptides are useful building blocks for the creation of new pharmaceutical compounds that have uses in antibacterial, anti-inflammatory, and antioxidant treatments. Their potential for chemical alteration is further increased by their structural variety and capacity to integrate different functional groups, which enables customised interactions with biological targets (1-5).

The growing challenge of antimicrobial resistance has intensified the search for alternative therapeutic agents that can effectively combat pathogenic microorganisms. Conventional antibiotics are increasingly becoming ineffective due to the rapid evolution of resistant strains, such as multidrug-resistant *Staphylococcus aureus* and *Escherichia coli*. This calls for the creation of fresh antibacterial agents with unique modes of action. Peptides, especially cyclic tripeptides, offer a unique mode of action by disrupting bacterial membranes or inhibiting essential enzymes, thereby minimizing the likelihood of resistance development (3, 6).

Novel treatments are also desperately needed for inflammatory illnesses. Cyclooxygenase enzymes, particularly COX-1 and COX-2, catalyse the conversion of arachidonic acid to prostaglandins, which is a critical step in the inflammatory process. Widely used non-steroidal anti-inflammatory medicines (NSAIDs) that block COX enzymes are frequently linked to side effects, especially gastrointestinal issues because of their non-selective COX inhibition.

Therefore, discovering compounds that can selectively inhibit COX-2 or modulate both COX isoforms with minimal side effects remains a key goal in anti-inflammatory drug development. Cyclic peptides, with their ability to interact specifically with enzyme active sites, hold promise in this regard (7-12).

Furthermore, oxidative stress, which results from an imbalance between the body's antioxidants and free radicals, is linked to the aetiology of a number of chronic illnesses, including as cancer, neurological conditions, and cardiovascular diseases (13). Natural antioxidants such as flavonoids like quercetin and Vitamin C are commonly used to neutralize free radicals. However, there is a continuous need for novel antioxidants with greater efficacy and stability. The antioxidant potential of cyclic peptides, particularly through their ability to scavenge ABTS and other free radicals, presents an attractive area for research (8-12, 14). Given the foregoing, the synthesis and pharmacological assessment of the novel cyclic tripeptides CTRIP-1 and CTRIP-2 are the main objectives of the current investigation. Through the ABTS radical scavenging experiment, these compounds' antibacterial activity against E. coli and S. aureus, inhibitory effect on COX-1 and COX-2 enzymes, and antioxidant capacity were all systematically evaluated. The project intends to investigate these peptides' multifunctional bioactivity and find possible lead compounds for the creation of novel medicinal medicines. The findings of this study could serve as a basis for additional preclinical and clinical research and are anticipated to add to the expanding corpus of information on peptide-based medication discovery.

2. MATERIALS AND METHODS

Chemical, Drugs, and Reagents:

The pharmaceutical compounds used in this study—Indomethacin, Amphotericin B, and Norfloxacin—were generously provided as gift samples by reputed pharmaceutical companies, ensuring the availability of high-purity drug substances for experimental purposes. All additional chemicals and reagents employed during the synthesis and evaluation processes were procured exclusively from certified and authorized suppliers. These materials were of synthetic and analytical grade, guaranteeing their suitability for laboratory use and the reliability of experimental outcomes. Strict adherence to the use of high-quality reagents and chemicals contributed to the consistency and accuracy of the synthesis and subsequent analytical procedures.

Physicochemical characterizations:

Throughout the study, all chemical reactions were tracked and examined using Thin Layer Chromatography (TLC) on plates coated with silica gel G. The mobile phase employed for TLC consisted of a mixture of methanol and chloroform in an 8:2 ratio, which provided optimal separation of the synthesized compounds. Visualization of the spots was achieved by exposing the developed plates to iodine vapors in a sealed chamber, resulting in the appearance of distinct brown patches that indicated the presence of the compounds. With the aid of a calibrated melting point device, the open capillary method was used to determine the melting points of the synthesised compounds. This approach ensured accurate measurement of thermal properties, contributing to the confirmation of compound purity and identity. Fourier-Transform Infrared (FTIR) spectroscopy was utilized for functional group analysis. A Perkin Elmer FTIR-RXI spectrophotometer was used to record the spectra of the samples, which were produced as potassium bromide (KBr) pellets. The characteristic absorption bands observed provided valuable information regarding the chemical bonds and structural features of the synthesized molecules. Additionally, proton nuclear magnetic resonance (^1H-NMR) spectroscopy helped the structural elucidation of the synthesised molecules. A Bruker NMR spectrophotometer was used to collect the ^1H-NMR spectra of samples that had been dissolved in deuterated chloroform ($CDCl_3$). Chemical shifts were referenced to the internal standard, tetramethylsilane (TMS), and were represented as delta (δ) values in parts per million (ppm). Together, these spectroscopic methods validated the synthesised compounds' purity and structural integrity, guaranteeing their appropriateness for additional pharmacological analysis.

Synthetic Procedure for Peptide:

Preparation of Boc-Protected Amino Acids:

N-tert-butoxycarbonyl (Boc)-protected amino acids were made in the first step of the synthesis to guarantee selective

reactivity in the coupling reactions that followed. A mixture of isopropanol (20 ml) and 1N sodium hydroxide (20 ml) was used to dissolve the amino acid (20 mmol). Tert-butyl dicarbonate (Boc anhydride) (26 mmol, 6 ml) was added to this basic solution after being previously dissolved in 10 ml of isopropanol. After adding 20 millilitres of 1N sodium hydroxide to keep the mixture alkaline, it was agitated for two hours at room temperature to ensure the amino group was completely protected. To get rid of any unreacted Boc anhydride and other organic contaminants, the liquid was cleaned with mild petroleum ether (40–60°C, 20 ml) once the reaction was finished. Chloroform (3 × 20 ml) was used for extraction after 2N sulphuric acid was used to acidify to pH 3.0. The crude Boc-amino acid was obtained by drying the mixed organic extracts over anhydrous sodium sulphate and then evaporating them under reduced pressure. By recrystallising it with a solvent system consisting of petroleum ether and chloroform, this was further refined. By comparing melting temperatures and spectral data with published values, the identity and purity of the Boc-protected amino acids were verified, guaranteeing their eligibility for peptide synthesis (15).

Preparation of Amino Acid Methyl Ester Hydrochlorides:

To obtain their corresponding methyl ester hydrochlorides, the amino acids' carboxyl groups were protected through esterification. To prevent excessive heat generation, 1.4 ml of 20 mmol thionyl chloride was first added dropwise to 100 ml of cool methanol at 0°C. To guarantee full conversion to the methyl ester hydrochloride, the reaction mixture was refluxed for 8–10 hours after the solution stabilised and the amino acid (20 mmol) was added. The reaction solvent was evaporated at lower pressure after cooling. To get rid of extra dimethylsulfite, a chemical byproduct, the residue—which mostly consisted of the ester hydrochloride—was triturated with cold diethyl ether at 0°C. Recrystallisation of the solid product from a methanol-diethyl ether combination at 0°C was used to purify it. Melting point analysis and spectral data were used to confirm the identity of each synthesised amino acid methyl ester hydrochloride in accordance with references from the literature (15).

Synthesis of Boc-Dipeptide Methyl Esters:

A coupling reaction between an amino acid methyl ester hydrochloride and a Boc-protected amino acid was used to create Boc-dipeptide methyl esters. To neutralise the hydrochloride and release the free amine, triethylamine (4 ml, 28.7 mmol) was added dropwise at 0°C after the amino acid methyl ester hydrochloride (10 mmol) had been dissolved in dichloromethane (20 ml). To guarantee full deprotonation, the mixture was agitated for fifteen minutes. The Boc-amino acid (10 mmol) in dichloromethane (20 ml) solution was then added, and as a coupling agent, dicyclohexylcarbodiimide (DCC) (2.09 g, 10 mmol) was added after that. To enable the synthesis of peptide bonds, the process was agitated for 36 hours at room temperature. Dicyclohexylurea (DCU), a byproduct of the coupling process, was eliminated from the reaction mixture by filtering it after it was finished. To get rid of contaminants, the filtrate was successively rinsed with 20 millilitres of 5% hydrochloric acid, 20 millilitres of 5% sodium bicarbonate, and 20 millilitres of saturated sodium chloride. After being dried on anhydrous sodium sulphate, the organic layer was vacuum-concentrated. By dissolving the result in little chloroform and cooling it at 0°C, DCU traces were further eliminated. After filtering out the remaining DCU, the filtrate was heated to 0°C and treated with petroleum ether to recrystallise the Boc-dipeptide methyl ester, producing a very pure product that could be used further.

Synthesis of Boc-Tripeptide Methyl Esters:

Using a similar coupling strategy, Boc-tripeptide methyl esters were synthesized from the previously prepared Boc-dipeptide intermediates. The coupling reaction conditions, including solvent system, reaction time, and purification procedures, mirrored those employed in the synthesis of dipeptides, ensuring consistency and yield optimization (15).

Deprotection of Carboxyl Groups in Peptides:

To make cyclisation easier, the ester group at the carboxyl terminus was selectively deprotected. To do this, a 1:1 solution of tetrahydrofuran (THF) and water (36 ml) was used to dissolve the protected peptide (10 mmol), which was then allowed to cool to 0°C. After adding lithium hydroxide (LiOH) (0.041 g, 1.5 mmol), the mixture was agitated for an hour at room temperature. After finishing, 1N sulphuric acid was added to the solution to bring its pH down to 3.5. After using 3 × 15 ml of diethyl ether to remove the aqueous phase, the combined organic extracts were dried over anhydrous sodium sulphate and then concentrated under low pressure. This procedure effectively removed the ester group, yielding the free carboxylic acid for subsequent cyclization.

Deprotection of Amino Groups in Peptides:

Trifluoroacetic acid (TFA) was used to eliminate the Boc-protecting group from the peptide in order to reveal its amino terminus. After dissolving the Boc-protected peptide (10 mmol) in 15 ml of chloroform, TFA (0.228 g, 2 mmol) was added. After an hour of stirring at room temperature, the solution was neutralised with five millilitres of saturated sodium bicarbonate solution. Following separation, the organic layer was dried over anhydrous sodium sulphate and vacuum-concentrated. A pure deprotected peptide appropriate for cyclisation was obtained by recrystallising the result from a chloroform-petroleum ether combination.

Cyclization to Form Cyclic Tripeptides:

The linear peptide fragment was cyclized utilising a p-nitrophenyl ester method as the last step. To promote intramolecular cyclisation, the carboxylic acid of the linear peptide was first activated as its p-nitrophenyl ester. After dissolving 1.5 mmol of the Boc-protected peptide acid in 15 ml of chloroform at 0°C, p-nitrophenol (0.27 g, 2 mmol) was added. At room temperature, the reaction mixture was agitated for 12 hours. To isolate the Boc-peptide-p-nitrophenyl ester, excess p-nitrophenol was eliminated by rinsing the filtrate with 10% sodium bicarbonate solution and then again with 5% hydrochloric acid (5 ml).

To remove the Boc group, TFA (0.274 g, 2.4 mmol) was added to this activated ester (1.2 mmol) that had been dissolved in 15 ml of chloroform. The mixture was then agitated for an hour. After removing TFA from the solution using a 10% sodium bicarbonate solution, the organic layer was dried using sodium sulphate. Following solvent removal, the deprotected peptide-p-nitrophenyl ester in chloroform (15 ml) was mixed with pyridine (1.4 ml, 2 mmol), and the combination was kept at 0°C for 10 days to permit cyclisation. After removing the unreacted p-nitrophenol from the reaction mixture, it was rinsed with a 10% sodium bicarbonate solution and then 5% hydrochloric acid (5 ml). The final refined cyclic tripeptide was obtained by recrystallising the crude cyclic peptide product from chloroform and n-hexane after drying the organic phase over sodium sulphate and eliminating solvents under vacuum (Belagali et al., 1995; Dahiya et al., 2006).

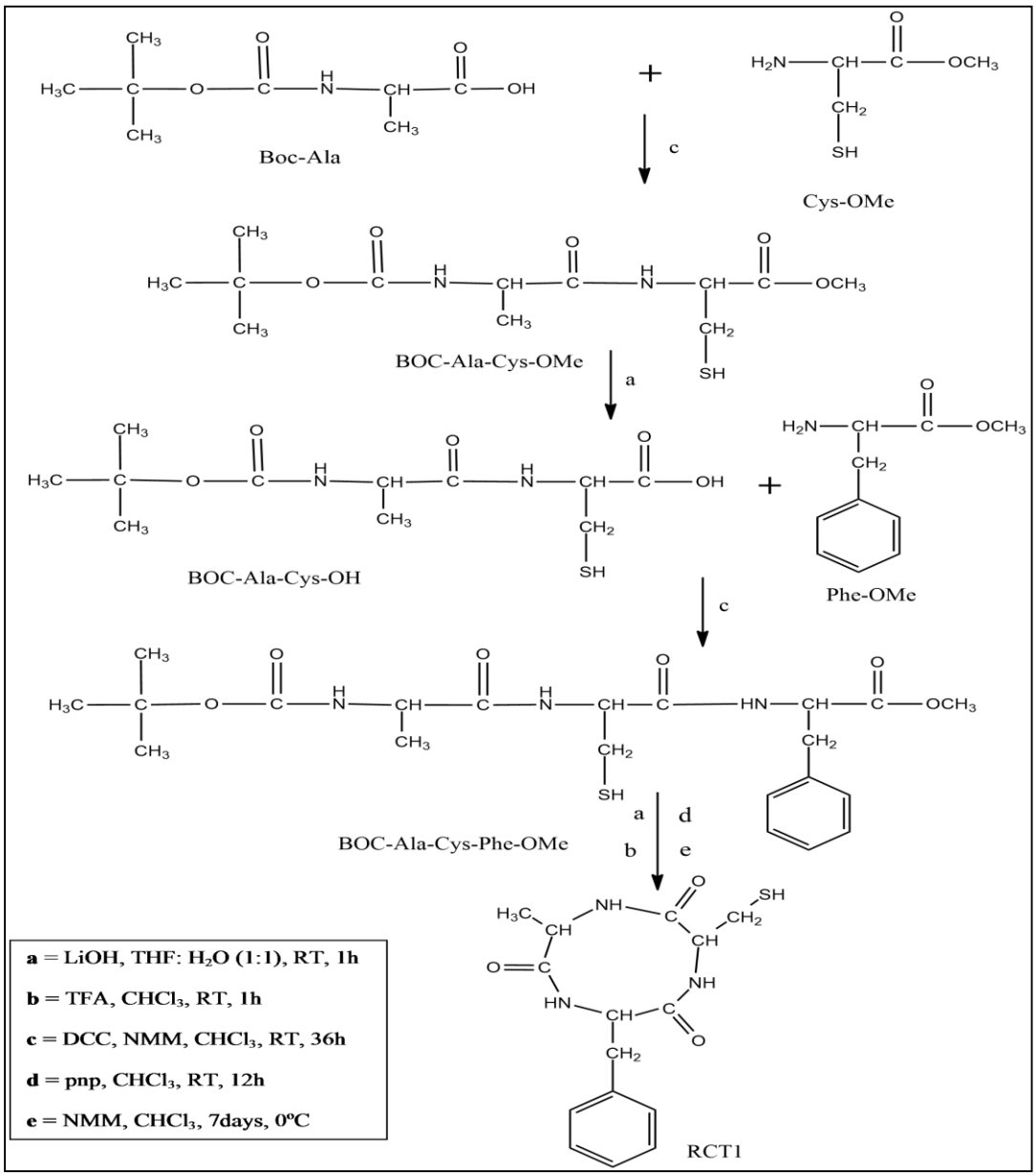


Figure 1. Pathway for synthesis of cyclic tripeptide (CTRIP-1)

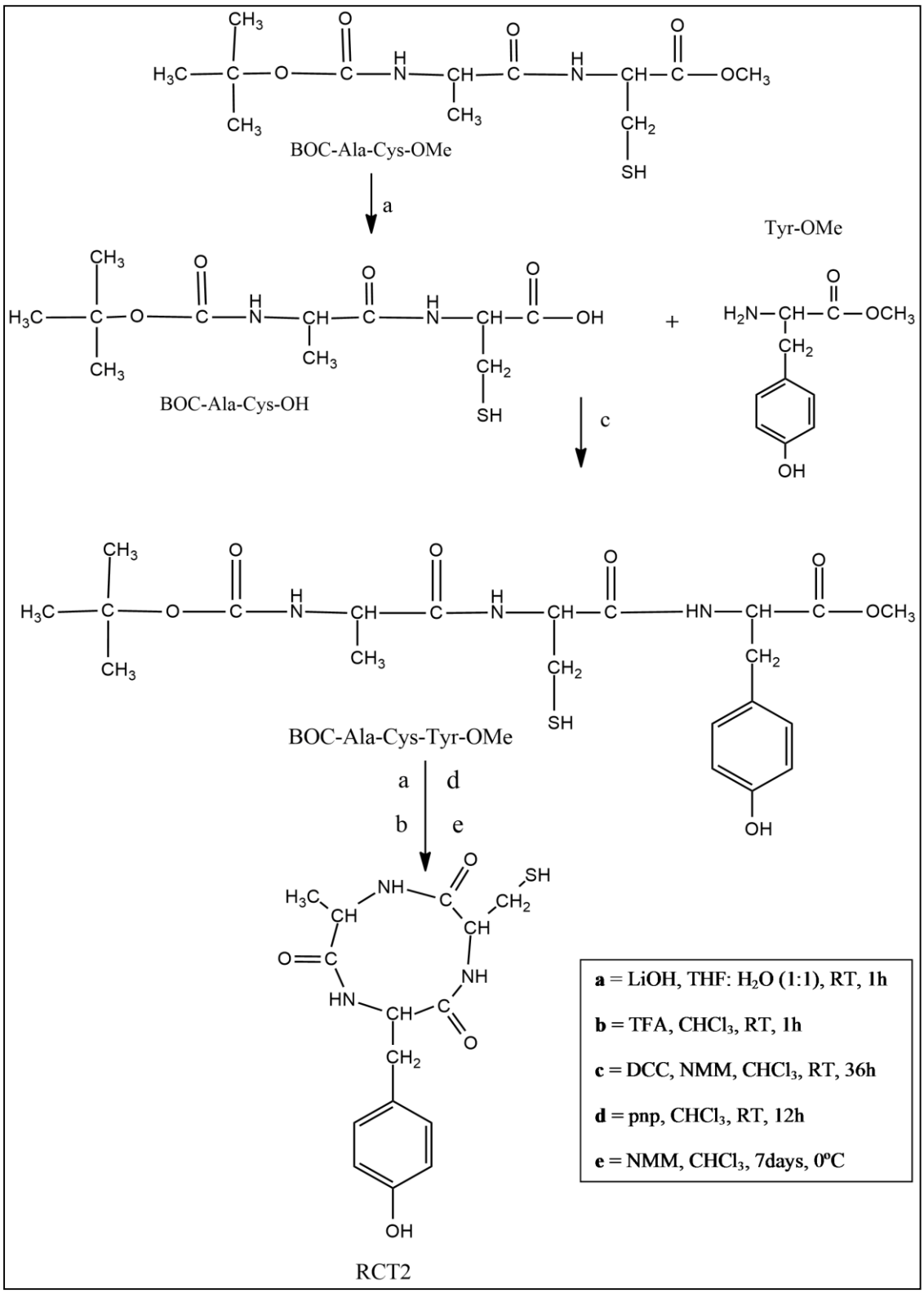


Figure 2. Pathway for synthesis of cyclic tripeptide (CTRIP-2)

Table 1. Physical Characterization of Synthesized Boc-Tripeptide methyl

Sl. No	**Boc-tripeptide methyl ester**	**Rf Value CHCl3: MeOH (9.5:0.5)**	**% age yield**
1.	Boc-Ala-Cys-Phe-OMe	0.80	84.8
2.	Boc-Ala-Cys-Tyr-OMe	0.73	88.5

Evaluation of Pharmacological Activities:

Through a battery of biological tests designed to ascertain the produced peptides' antibacterial, cyclooxygenase (COX) inhibitory, and antioxidant properties, their pharmacological potential was carefully assessed. These investigations were essential to explore the therapeutic relevance of the compounds and their prospective use in managing infectious and inflammatory conditions, as well as oxidative stress-related disorders.

Antimicrobial Activity:

To assess the antibacterial efficacy of the synthesized peptides, two well-characterized microbial strains were selected—*Escherichia coli* and *Staphylococcus aureus*. In order to provide a wide range for antimicrobial assessment, these organisms were selected to represent both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria. The Microbial Type Culture Collection and Gene Bank (MTCC) provided the bacterial strains, guaranteeing the study's cultures' uniformity and legitimacy. The bacterial strains were cultivated in sterile conditions on nutrient agar slants. These cultures were maintained in optimal conditions by periodic subculturing to retain their viability and purity. Prior to experimentation, the microbial cultures were preserved at 4°C to prevent contamination or overgrowth, ensuring consistency and reproducibility in the antimicrobial assays. The use of both Gram-negative and Gram-positive bacteria allowed for a comprehensive evaluation of the peptides' ability to inhibit or suppress bacterial growth across different cell wall structures and resistance mechanisms.

Table 2. Specifics and growing environments for the investigated microorganisms.

Parameter	***Escherichia coli***	***Staphylococcus aureus***
Antibiotic	Norfloxacin	Norfloxacin
Solvent used	DMSO	DMSO
MTCC No.	1687	737
Strain	Gram -ve	Gram +ve
Incubation time	24 h	24 h
Temperature	30°C	37°C

Antibacterial activity Screening:

The agar well diffusion method, a commonly used and standardised approach for assessing antimicrobial efficacy, was used to evaluate the synthetic peptides' antibacterial activity. The method was carried out in accordance with the procedures outlined by Mukherjee et al. (1995) and Kataki et al. (2010), with slight modifications to suit the laboratory conditions and the nature of the test compounds. In order to create fresh cultures of *Staphylococcus aureus* (MTCC 737) and *Escherichia coli* (MTCC 1687), the bacterial strains were sub-cultured on nutrient agar slants and incubated for 24 hours at their ideal temperatures, which are 37°C for S. aureus and 30°C for E. coli. A consistent inoculum density was then ensured by adjusting the bacterial suspensions to match the turbidity of the 0.5 McFarland standard. Sterile Petri dishes were prepared by pouring molten nutrient agar and allowing it to solidify. The surface of the solidified agar was then evenly inoculated with the prepared bacterial suspensions using sterile cotton swabs to ensure uniform growth. A sterile cork borer was used to precisely punch wells into the agar, each about 6 mm in diameter. A certain concentration of the synthesised peptide dissolved in dimethyl sulfoxide (DMSO) was added to each well. To compare the antibacterial efficacy, DMSO alone was employed as

the negative control and the common antibiotic norfloxacin as the positive control. To ensure that the chemicals properly diffused into the agar, the plates were let to stand at room temperature for an hour. After diffusion, the *E. Coli* and *S. aureus*-inoculated plates were incubated for 24 hours at 30°C and 37°C, respectively. The diameter of the zones of inhibition that developed around each well after incubation was used to gauge the antibacterial activity. A clean zone showed that the test substance effectively inhibited bacterial growth, and the findings were measured in millimetres. To guarantee statistical reliability and reproducibility, each test was run three times (16, 17).

Minimum Inhibitory Concentration (MIC) determination:

With minor adjustments, the broth dilution method, as outlined by Salama and Marraiki (2010) and Kataki (2010), was used to calculate the synthesised peptides' Minimum Inhibitory Concentration (MIC). The goal of this approach was to determine the lowest dose of each peptide that would successfully prevent the test bacteria, Escherichia coli (MTCC 1687) and Staphylococcus aureus (MTCC 737), from growing visibly. A series of test tubes or 96-well microtiter plates were filled with sterile nutritional broth. To get a variety of concentrations, the synthesised peptides were serially diluted twice in the broth. To guarantee a constant bacterial burden in all tubes or wells, a standardised bacterial suspension that was adjusted to match the turbidity of the 0.5 McFarland standard was used to inoculate each dilution. A sterility control (a broth devoid of bacteria or peptide), a growth control (a broth containing bacterial inoculum but no peptide), and a positive control (a broth containing bacterial inoculum and the common antibiotic, norfloxacin) were among the control configurations. Every test tube or plate was incubated for 24 hours at 30°C for E. Coli and 37°C for *S. aureus.* The tubes or wells were checked for turbidity after incubation. The MIC was defined as the lowest peptide concentration at which no discernible bacterial growth was found. This number demonstrated the compound's antibacterial effectiveness by indicating the bare minimum amount needed to stop bacterial growth. To guarantee the precision and repeatability of the findings, every determination was carried out in triplicate (18, 19)**.**

Cyclooxygenase -1 (COX-1) and cyclooxygenase -2 (COX-2) assays:

The COX-1 and COX-2 enzyme inhibitory activities of the synthesized peptides were evaluated using established protocols reported by Redl et al. (1994) and Aguilar et al. (2002), with minor modifications to suit laboratory conditions (20, 21). (20, 21). These assays aimed to determine the ability of the peptides to inhibit prostaglandin synthesis by interfering with cyclooxygenase enzyme activity, thus indicating potential anti-inflammatory properties. For the COX-1 assay, the enzyme source was obtained from ram seminal vesicles, and the enzymatic reaction was carried out in a buffered system containing Tris-HCl (pH 7.4). The reaction mixture included the enzyme, hemoglobin as a cofactor, and the test compound at varying concentrations. The reaction was initiated by adding arachidonic acid as the substrate and was incubated at 37°C for a specific time. The formation of prostaglandins was monitored by measuring the absorbance change at 632 nm, using a spectrophotometer. The percentage inhibition of COX-1 activity by the peptides was calculated in comparison to a control reaction without the test compound. Similarly, the COX-2 assay was conducted using a commercially available COX-2 enzyme preparation. The assay conditions, including buffer composition and temperature, were optimized according to the method of Aguilar et al. (2002). The enzymatic activity was again initiated by the addition of arachidonic acid, and prostaglandin formation was quantified spectrophotometrically. Diclofenac was used as a standard COX inhibitor for comparison in both assays. The most active peptides' IC_{50} values (concentration needed to inhibit 50% of enzyme activity) were calculated, and the degree of enzyme inhibition was reported as a percentage in relation to the control. To guarantee the precision and repeatability of the data, every experiment was carried out three times.

2, 2′-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical decolorization assay:

With minor adjustments to accommodate experimental conditions, the ABTS radical decolorisation assay, as described by Re et al. (1998), was used to evaluate the antioxidant capacity of the synthesised peptides (22). To calculate the IC_{50} value—the concentration needed to scavenge 50% of the ABTS radicals—the percentage inhibition was plotted versus the peptide concentration. The reference standard for comparison was ascorbic acid. To guarantee accuracy and reproducibility of the findings, every experiment was carried out three times.

Statistical analysis:

To guarantee accuracy and reproducibility, all experimental data were presented as mean ± standard deviation (SD) from a minimum of three independent repetitions. To ascertain statistical significance across groups, post hoc tests like Tukey's multiple comparison test were performed after one-way analysis of variance (ANOVA) was used to examine data from antibacterial, COX inhibitory, and antioxidant experiments. At $p < 0.05$, differences were deemed statistically significant. A thorough assessment of the pharmacological actions of the synthesised peptides was ensured by using GraphPad Prism software (version XX) for statistical analysis and data visualisation.

3. RESULTS AND DISCUSSIONS

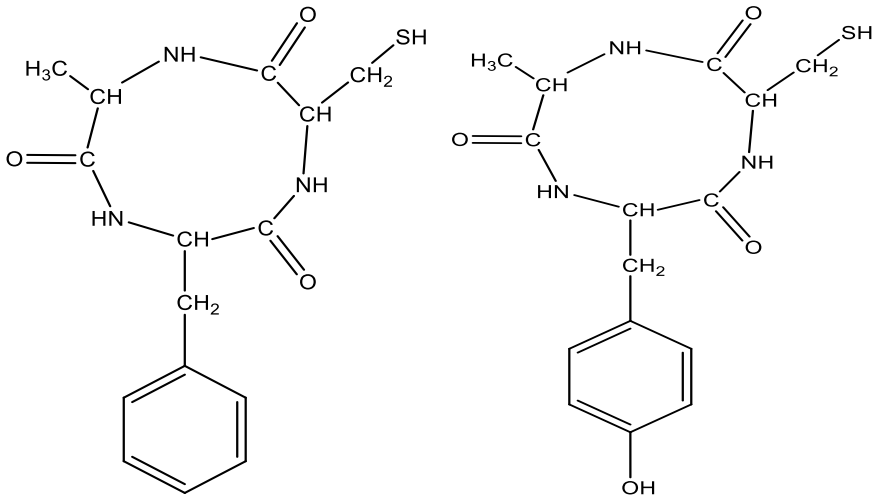
Synthesis and characterization of cyclic tripeptides (CTRIPs):

Melting point, percentage yield and R_f values of the synthetic cyclic tripeptides:

Table 3 presents key physicochemical data for two synthetic cyclic tripeptides, CTRIP-1 and CTRIP-2. The melting points of these compounds were recorded in the range of 170–174°C for CTRIP-1 and 174–179°C for CTRIP-2, indicating their solid and crystalline nature. The slightly higher melting point of CTRIP-2 may suggest increased molecular rigidity or the presence of stronger intermolecular forces compared to CTRIP-1. The percentage yields were found to be moderately high for both compounds, with CTRIP-1 yielding 79.9% and CTRIP-2 yielding 76.8%. These values reflect efficient synthetic processes with minimal loss of product, signifying that the methods employed for the cyclization and purification of these tripeptides were effective. Thin Layer Chromatography (TLC) was used to determine the Rf values using a solvent system of chloroform and methanol ($CHCl_3$:MeOH). The Rf values were closely similar, recorded at 0.79 for CTRIP-1 and 0.80 for CTRIP-2. These comparable Rf values indicate similar polarity and solubility characteristics in the given solvent system, which could suggest structural similarities between the two compounds. Overall, the data confirms successful synthesis of both cyclic tripeptides with good yield, appropriate purity (as indicated by consistent TLC values), and distinct melting points, supporting the stability and potential for further biological or analytical evaluations.

Table 3. Rf values, percentage yield, and melting point of the synthesised cyclic tripeptides

Serial No.	Compound Code	Melting Point	% Yield	TLC (R_f value) $CHCl_3$: MeOH
1	CTRIP-1	170-174	79.9	0.79
2	CTRIP-2	174-179	76.8	0.80



Cyclo (Ala-Cys-Phe) **Cyclo (Ala-Cys-Phe)**

Figure 3. Cyclic tripeptides structures (CTRIP-1 and CTRIP-2)

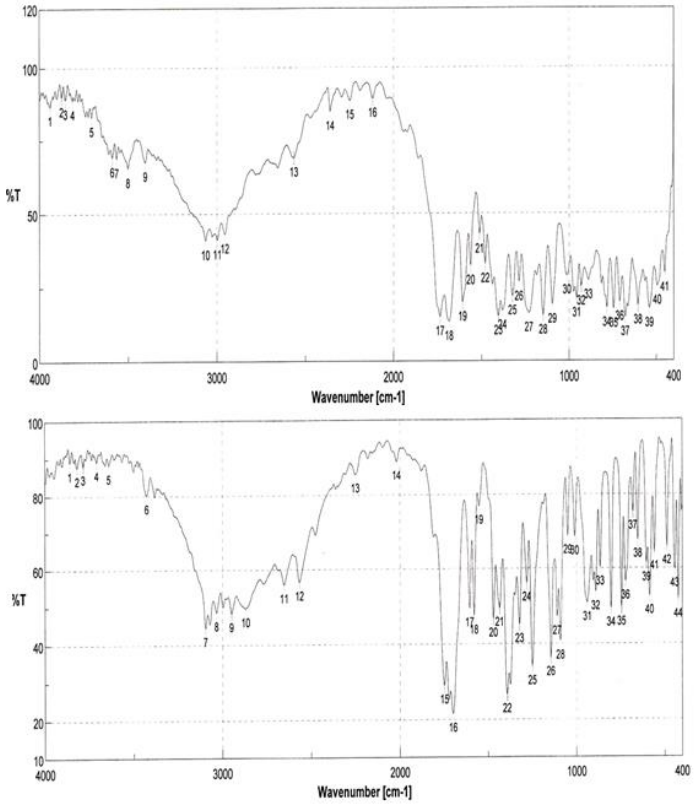


Figure 4. FTIR Spectra of CTRIP-1 and CTRIP-2

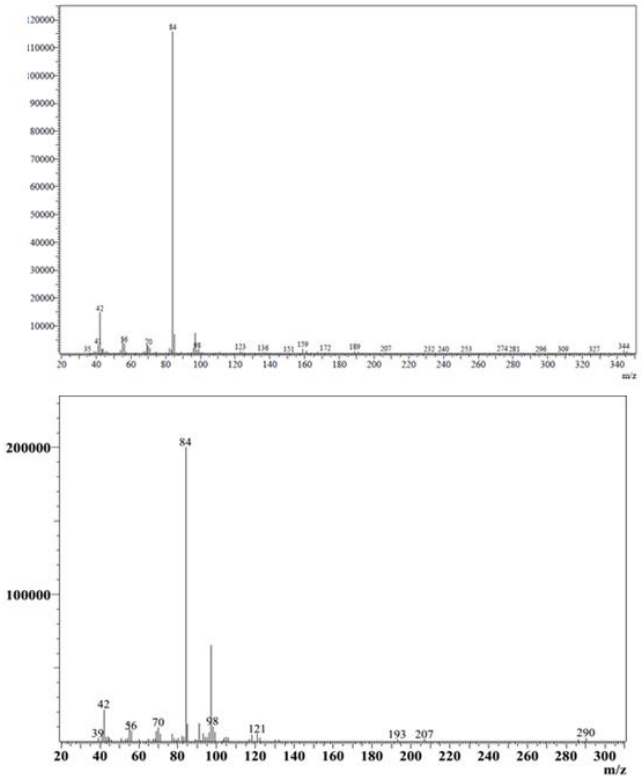


Figure 5. Mass Spectra of CTRIP-1 and CTRIP-2

Spectral interpretations and data for the cyclic tripeptides:

The spectral data for CTRIP-1 and CTRIP-2 provides detailed insights into the structural features and successful synthesis of these cyclic tripeptides. The existence of predicted functional groups and the integrity of the molecular frameworks are confirmed by nuclear magnetic resonance (^1H NMR) data, infrared (IR) spectroscopy, and mass spectrometry (ESI-MS). The peptide bond structure was confirmed by CTRIP-1's characteristic IR absorption bands, which were particularly visible at 3318 cm^{-1} for N–H stretching (amide) and at 1665 cm^{-1} for C=O stretching (secondary amide). Several C–H stretching bands in the 3055–2856 cm^{-1} range are indicative of cyclic and aromatic methylene groups. A thiol (-SH) group was indicated by a strong peak at 2583 cm^{-1}, which would point to the involvement of cysteine or other sulfur-containing amino acids. The band at 1050 cm^{-1} was ascribed to C–N stretching in amide bonds, but the C=C stretching vibrations at 1585 and 1463 cm^{-1} verified the aromaticity. The identity of the molecule was confirmed by the ESI-MS spectrum, which displayed a molecular ion peak at m/z 321.40 [M+H]^+ that matched the predicted molecular weight. CTRIP-2 presented a broader IR profile, including a strong peak at 3507 cm^{-1}, attributed to O–H stretching from tyrosine residues, along with 3307 cm^{-1} for N–H stretching (amide), confirming peptide linkages. Aromatic and cyclic C–H stretching bands were observed at 3048–2854 cm^{-1}, and the C=O stretching at 1665 cm^{-1} was consistent with secondary amides. The C–O stretching band at 1211 cm^{-1} further supported the presence of tyrosine. The molecular ion detected by ESI-MS analysis at m/z 337.39 confirmed that CTRIP-2 has a larger molecular weight than CTRIP-1, most likely as a result of the presence of tyrosine. Additionally, distinctive chemical shifts were detected in the ~1H NMR spectra of CTRIP-2 in $CDCl_3$. A carboxylic proton (COOH) was identified by the singlet at δ 9.82 ppm, whereas amide NH protons were indicated by wide signals at δ 8.40 ppm. Signals between δ 7.51–7.69 ppm were attributed to imidazole ring protons, suggesting the presence of histidine. The multiplet at δ 4.55–4.72 ppm was assigned to α-protons of histidine, while signals at δ 3.90 and δ 3.77–3.89 ppm indicated glycine and proline α-protons, respectively. Additional multiplets between δ 1.49–2.98 ppm corresponded to proline β- and γ-protons and histidine side chain protons. These data confirmed the presence of histidine, glycine, and proline residues within CTRIP-2, validating the peptide's proposed structure. In summary, the spectral analyses confirm the successful synthesis and structural integrity of CTRIP-1 and CTRIP-2. The presence of specific amino acid residues like histidine, proline, glycine, and tyrosine was strongly supported by the IR, MS, and NMR data, demonstrating the reliability of the synthetic method and the purity of the products.

Table 4. Data and interpretations of the cyclic tripeptides' spectra

Compound	**IR Value (υ cm^{-1})**	**Mass**
CTRIP-1	3318 (N–H stretching, amide), 3055 (C–H stretching, aromatic ring), 3007 , 2998 (C–H stretching, cyclic ,CH2 and CH), 2933 (C–H stretching, asymmetric, CH2), 2856 (C–H stretching, symmetric, CH2), 2583 (S-H streching,SH), 1665 (C=O stretching, 2° amide), 1585, 1463 (C=C ring stretching), 1520 (N–H bend, 2° amide), 1050(C-N stretching, amide).	ESI-MS: m/z 321.40 [M+H]+
CTRIP-2	3507 (OH stretching, Tyrosine), 3307 (N–H stretching, amide), 3048 (C–H stretching, aromatic ring), 2992(C–H stretching, cyclic CH2 and CH), 2932 (C–H stretching, asymmetric, CH2), 2854 (C–H stretching, symmetric, CH2), 1665 (C=O stretching, 2° amide), 1589, 1468 (C=C ring stretching), 1515 (N–H bend, 2º amide), 1211 cm-1 (C–O stretching, Tyrosine).	ESI-MS: m/z 337.39
CTRIP-2	**^{1}H NMR ($CDCl_3$) δ**	
	9.82 (s, 1H, COOH), 8.40 (br. s, 3H, NH, amide), 7.69 (s, 1H, NH's, imz ring), 7.51 (s, 1H, imz ring), 7.62 (s, 1H, imz ring), 4.55-4.72 (m, 1H, α-H, His), 3.90n (s, 2H, Gly), 3.77-3.89 (m, 1H, α-H, Pro), 3.55-3.62 (m, 2H, Pro), 2.92-2.98 (d, 2H, His), 1.49-1.72 (m, 4H, β-H's, and γ -H's, Pro).	

Biological and Pharmacological activity:

Antimicrobial activity:

The data presented in Tables 5 and 6 reflect the antibacterial efficacy of the synthesized cyclic tripeptides (CTRIP-1 and CTRIP-2) against *Staphylococcus aureus* and *Escherichia coli*, measured in terms of zone of inhibition (ZOI) at varying concentrations (25–400 µg/mL). Against *Escherichia coli* (Table 5), both peptides demonstrated a concentration-dependent increase in antibacterial activity. CTRIP-2 consistently exhibited greater zones of inhibition compared to CTRIP-1 across all concentrations, with maximum inhibition observed at 400 µg/mL (CTRIP-1: 24.69 mm; CTRIP-2: 26.21 mm). Notably, CTRIP-2 at 400 µg/mL approached the efficacy of the standard antibiotic Norfloxacin (25.65 mm ZOI at 60 µg/mL), highlighting its strong antibacterial potential. DMSO, the solvent control, showed no inhibitory activity, confirming the validity of the results. Similarly, against *Staphylococcus aureus* (Table 6), both peptides again showed dose-dependent inhibition, with CTRIP-2 displaying superior antibacterial activity compared to CTRIP-1. At 400 µg/mL, CTRIP-2 exhibited a ZOI of 27.89 mm, closely matching Norfloxacin's inhibition of 27.96 mm at 60 µg/mL. This suggests that CTRIP-2 is not only effective against Gram-negative *E. coli* but also highly potent against Gram-positive *S. aureus*, with its antibacterial activity nearly equating that of a standard drug.

In conclusion, both CTRIP-1 and CTRIP-2 demonstrated promising antibacterial activity, with CTRIP-2 being more effective against both bacterial strains. Their efficacy at higher concentrations, particularly that of CTRIP-2, underscores their potential as alternative antimicrobial agents, warranting further investigation into their mechanism of action and potential clinical applications.

Table 5. The following compounds demonstrate antibacterial activity against *Escherichia coli* in terms of ZOI.

E coli		
Conc. (µg/mL)	**Zone of inhibition (mm)**	
	Cyclic Tripeptides	
	CTRIP-1	**CTRIP-2**
25	17.93	18.62
50	19.82	21.55
100	20.39	23.29
200	22.33	24.84
400	24.69	26.21
DMSO	0	0
Norfloxacin (60 µg/mL)	25.84	25.65

Table 6. Shows antibacterial activity of synthesized compounds against *Staphylococcus aureus* terms of ZOI are as follow:

S aureus		
Conc. (µg/mL)	**Zone of inhibition (mm)**	
	Cyclic Tripeptides	
	CTRIP-1	**CTRIP-2**
25	17.84	18.94
50	19.67	22.88
100	22.39	23.67
200	23.56	25.86

400	26.45	27.89
DMSO	0	0
Norfloxacin (60 µg/mL)	27.77	27.96

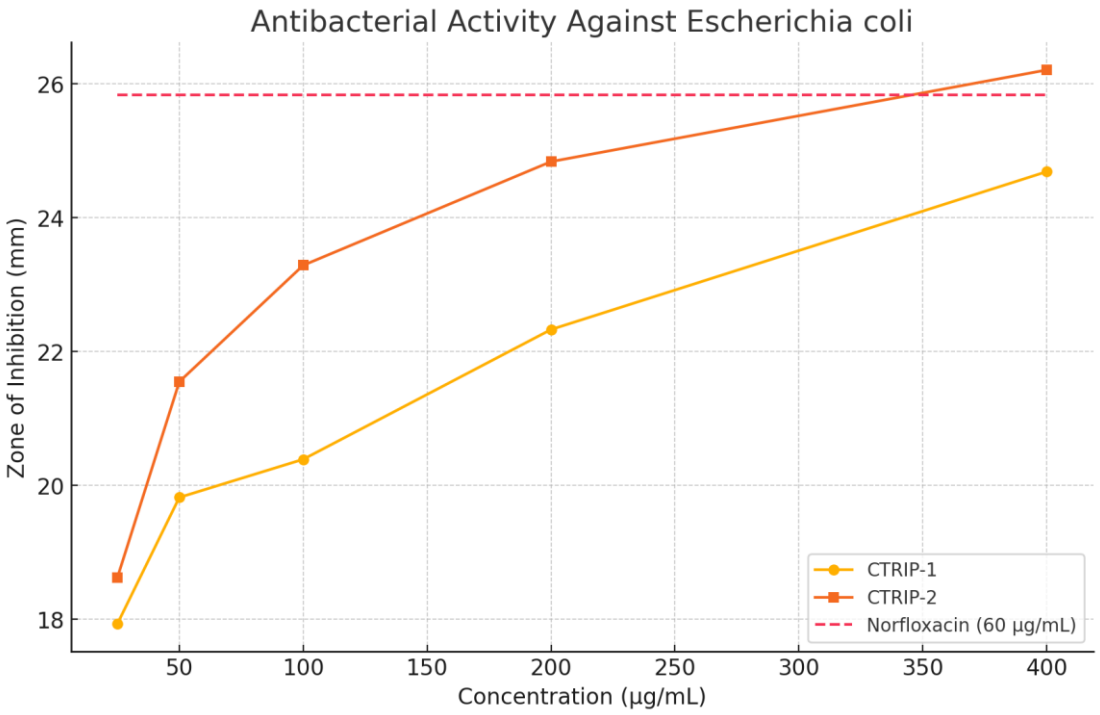


Figure 6. Antibacterial activity of synthesized peptides (CTRIP-1 and CTRIP-2) against *Escherichia coli*.

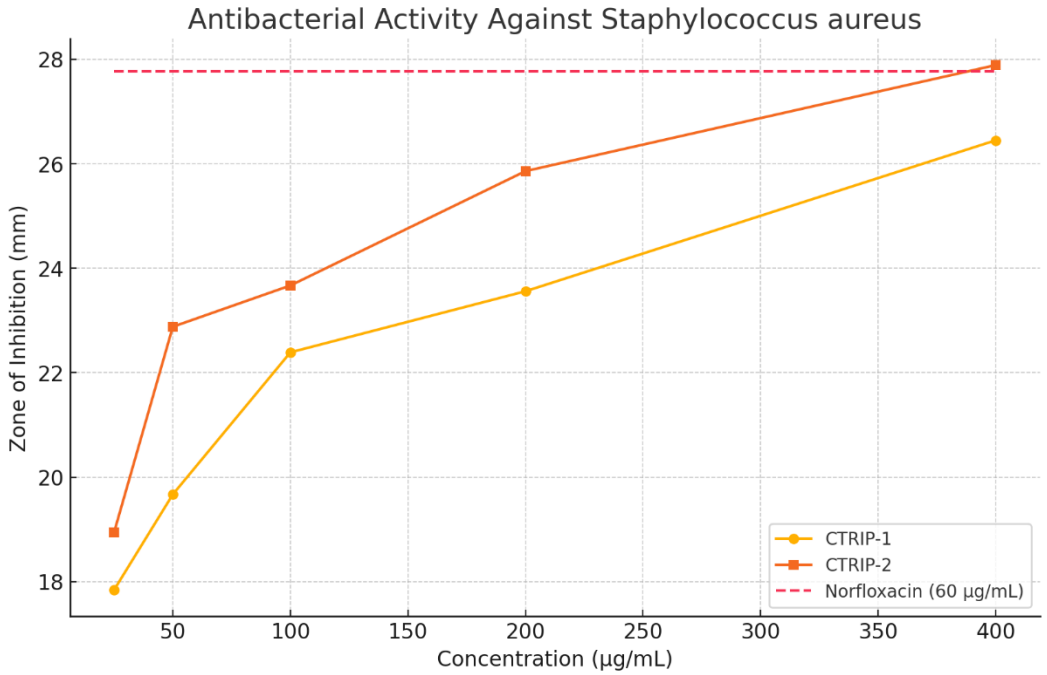


Figure 7. Antibacterial activity of synthesized peptides (CTRIP-1 and CTRIP-2) against *Staphylococcus aureus*.

Cyclooxygenase -1 (COX-1) and cyclooxygenase -2 (COX-2) assays:

The effects of the synthesised cyclic tripeptides, CTRIP-1 and CTRIP-2, at a concentration of 250 µg/mL on the enzymes cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) are shown in Table 7. Based on triplicate trials, the data are shown as mean % inhibition ± standard deviation (SD). CTRIP-1 exhibited substantial inhibitory activity, with 76.76 ± 1.21% inhibition of COX-1 and 81.27 ± 1.19% inhibition of COX-2. CTRIP-2 demonstrated even stronger activity, showing 82.85 ± 1.24% inhibition of COX-1 and 85.76 ± 1.21% inhibition of COX-2. These findings indicate that both peptides possess significant COX-inhibitory potential, with CTRIP-2 being more potent than CTRIP-1 against both isoforms. In comparison, the standard anti-inflammatory drug Indomethacin showed the highest inhibition, with 90.17 ± 1.19% for COX-1 and 92.84 ± 1.27% for COX-2. While the synthesized peptides did not exceed Indomethacin in inhibitory strength, CTRIP-2's activity closely approached it, especially against COX-2, suggesting a promising anti-inflammatory profile. Overall, the results highlight the potential of CTRIP-2 as a potent dual COX inhibitor, with a slightly higher preference for COX-2. Compared to non-selective COX inhibitors, these characteristics make it a promising option for additional research as an anti-inflammatory drug with perhaps less gastrointestinal side effects.

Table 7. Cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) inhibitory activity of CTRIP-1 and CTRIP-2 at 250 µg/ml concentration (mean ± SD; n=3)

Drugs	**% Inhibition**	
	COX-1	**COX-2**
CTRIP-1	76.76 ± 1.21	81.27 ± 1.19
CTRIP-2	82.85 ± 1.24	85.76 ± 1.21
Indomethacin	90.17± 1.19	92.84v± 1.27

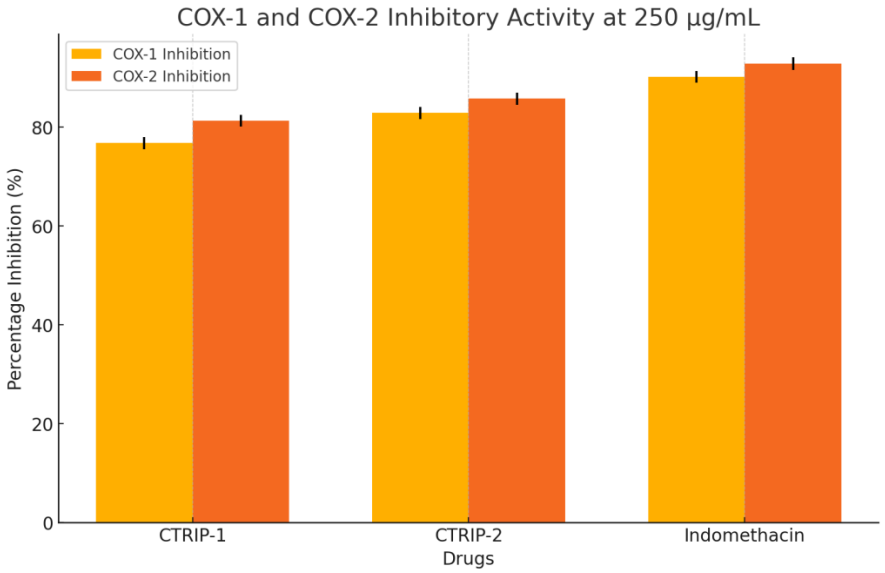


Figure 8. CTRIP-1 and CTRIP-2 inhibitory action of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) at 250 µg/ml concentration

2, 2'-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical decolorization assay

The antioxidant capacity of the produced cyclic tripeptides, CTRIP-1 and CTRIP-2, as assessed by the ABTS radical scavenging assay, is listed in Table 8. Each compound's IC_{50} value (µg/mL), which shows the concentration needed to block 50% of the ABTS radicals, serves as a representation of its efficacy. Stronger antioxidant activity is reflected in lower IC_{50} values.

CTRIP-1 exhibited an IC_{50} of 122.78 ± 1.47 µg/mL, while CTRIP-2 demonstrated slightly greater antioxidant efficacy with an IC_{50} of 118.92 ± 1.28 µg/mL. Quercetin, a potent antioxidant standard, has the best radical scavenging activity among the investigated substances with the lowest IC_{50} value of 85.86 ± 1.31 µg/mL. Vitamin C, a well-known antioxidant, showed an

IC_{50} of 116.68 ± 1.27 µg/mL, which was slightly better than both synthesized peptides but comparable to CTRIP-2. These findings suggest that CTRIP-2 possesses better antioxidant capacity than CTRIP-1 and is nearly as effective as Vitamin C. The close proximity of CTRIP-2's IC_{50} value to Vitamin C highlights its potential as a natural antioxidant agent. Overall, both cyclic tripeptides showed promising free radical scavenging properties, with CTRIP-2 being the more potent of the two, warranting further investigation for therapeutic use in oxidative stress-related conditions.

Table 8. CTRIP-1 and CTRIP-2's ability to scavenge ABTS radicals (values are given as mean ± SD; n=3)

Drugs	**IC_{50} (µg/ml)**
	ABTS radical
CTRIP-1	122.78 ± 1.47
CTRIP-2	118.92 ± 1.28
Quercetin	85.86 ± 1.31
Vitamin C	116.68 ± 1.27

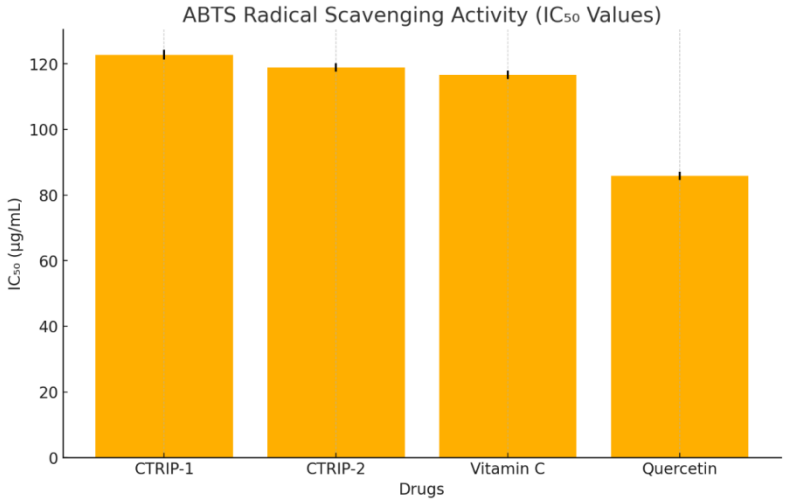


Figure 9. CTRIP-1 and CTRIP-2's ability to scavenge ABTS radicals (values are given as mean ± SD; n=3)

4. CONCLUSIONS

The findings of this study demonstrate that the synthesized cyclic tripeptides, CTRIP-1 and CTRIP-2, possess significant pharmacological activities, notably in the domains of antimicrobial, anti-inflammatory, and antioxidant potential. Both peptides were effectively characterized by physicochemical and spectral methods, confirming their structural integrity and purity. The antimicrobial evaluation against Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus* revealed that both peptides inhibit bacterial growth in a concentration-dependent manner. CTRIP-2 consistently outperformed CTRIP-1, achieving inhibition zones comparable to the standard antibiotic Norfloxacin at higher concentrations, indicating a broad-spectrum antibacterial effect. The anti-inflammatory potential was assessed via COX-1 and COX-2 inhibition assays. CTRIP-2 exhibited notably higher inhibitory activity than CTRIP-1, suggesting its enhanced potential in modulating inflammatory responses, especially given its preferential COX-2 inhibition, which may offer therapeutic advantages with reduced gastrointestinal side effects typically associated with non-selective COX inhibitors. Antioxidant activity assessed by the ABTS assay further supported the superior efficacy of CTRIP-2 over CTRIP-1, with CTRIP-2's IC_{50} value being close to that of Vitamin C, a known antioxidant. These findings suggest that CTRIP-2 could play a role in mitigating oxidative stress, which is implicated in a variety of chronic diseases. In summary, CTRIP-2, due to its superior antibacterial, anti-inflammatory, and antioxidant activities, represents a promising candidate for further preclinical investigations. Its potential application in treating infections, inflammatory disorders, and oxidative stress-related conditions merits continued exploration. These results pave the way for advancing cyclic peptides as multifunctional therapeutic agents with potential clinical relevance.

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