Piperidinylpeptide hybrids: An innovative category of antimicrobial agents

Shivakumara K N*

*Assistant professor, Department of chemistry, Maharani's Science College for women, Bangalore-560001, Karnataka, India.

Abstract : Piperidinylpeptide hybrids were prepared by linking smaller Bac7 peptides fragments with 4-benzylpiperidine using IBCF as the coupling agent, HOBt, and NMM as the base at -15 ± 1°C. The reaction mixture was stirred at -15 ± 1°C, followed by warming to room temperature (RT) on overnight reaction. The completion of the reaction was monitored using R_f values. The obtained compounds were identified using spectroscopic technique ^{1}H NMR, and then evaluated for antibacterial activity against Staphylococcus aureus, Escherichia coli, Klebsiella pnemoniae, and Pseudomonas aeruginosa, as well as antifungal activity against Aspergillus Niger, Aspergillus flavus, and Fusarium monoliforme. Among the synthesized compounds, XXIV displayed significant activity, while XXII and XXIII showed moderate activity.

Index Terms - Bac7 (Bactenecin7), toxicity**,** precursors, antibiotic resistant and compatibility.

1.0 INTRODUCTION

The prevalence of antimicrobial resistance is steadily increasing, posing a significant threat of returning to a time before antibiotics were available. As a result, there has been a rise in bacterial infections that cannot be effectively treated using the current arsenal of treatment options. Numerous efforts have been made to develop new antimicrobials with innovative mechanisms of action to combat these strains that are resistant to multiple drugs.[1] However, the need for innovative antibiotics to effectively treat antibiotic-resistant infections remains unmet. Consequently, the existing arsenal of antibiotics is inadequate to address the challenges posed by resistance today. Furthermore, there are very few new agents being developed that can replace existing antibiotics that are becoming less effective due to the growing surge of resistance.[2]

Among the diverse range of bioactive molecules, peptides have emerged as particularly valuable. These innovative compounds present promising opportunities in drug development, offering highly targeted and safe pharmaceuticals. Despite of their versatile bioactivity, peptides are generally poor drugs, because natural peptides are quickly degraded or modified in the body, and are frequently immunogenic. This has stimulated widespread attempts to design peptide mimics or pseudo-peptides for biomedical applications.[3-4] Peptide hormones and shorter peptides exert their effects by binding to membrane receptors, demonstrating their adaptability. Peptides and their derivatives display a broad spectrum of biological activities, such as antimicrobial, antiviral, and anticancer properties. [5-6]

Recently, chemists and biologists have turned their focus to studying peptide-based derivatives due to their minimal toxicity, compatibility with living organisms, and the varied structure of amino acid residues. The practice of combining amino acid/peptide residues with small bioactive heterocyclic motifs in biomedical research is gaining traction.[7-8]

In this particular context, the design and synthesis of heterocyclic conjugated peptides and amino acids involved the coupling of a heterocyclic precursor called quinazolinone with inactive peptide sequences of elastin. These sequences included VP, GVP, VGVP, and GVGVP peptides. The synthesis process resulted in a significant increase in activity, nearly doubling the effectiveness compared to traditional antimicrobials, particularly when VGVP and GVGVP peptides were conjugated with the quinazolinone heterocycle. It is worth noting that all the quinazolinone conjugated peptides exhibited enhanced activity, despite the fact that both the peptides and the quinazolinone moiety were inactive when tested separately against bacterial strains.[9-10]

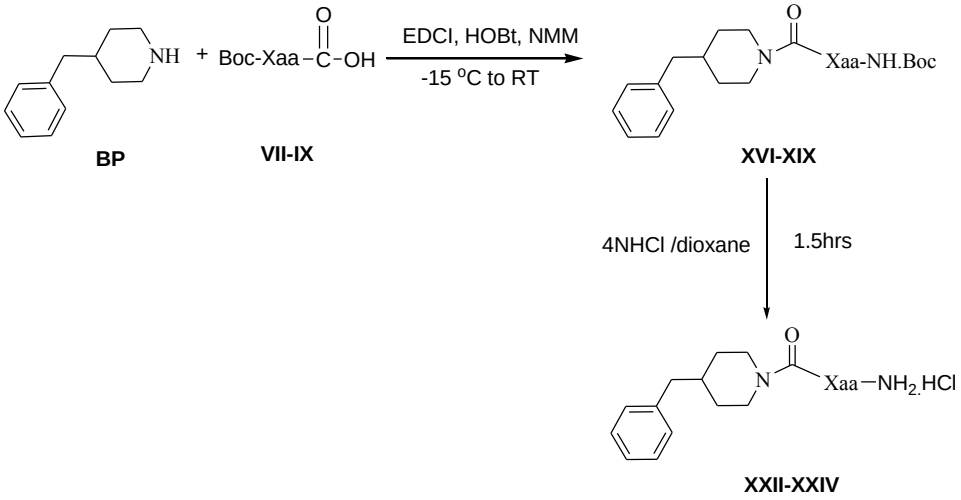
Based on the information provided, we have decided to further explore the synthesis of small peptide-heterocyclic conjugated molecules. To achieve this, we have designed a method to synthesize peptides linked with heterocycles by combining small heterocyclic precursors, specifically 4-benzylpiperidine (BP), with Bac7 small peptide fragments, RP, PRP, and GPRP.

Peptide Synthesis:

The Bac7 peptide's shorter analogues were synthesized using the solution phase method, following a step-by-step approach.[11-13] The N^{α}-protection was carried out with the Boc group, which was subsequently removed by treating the compound with 4N HCl in dioxane. The benzyl ester was used to protect the carboxyl group at the C-terminus, and it was subsequently removed either through hydrogenolysis using $HCOONH_4$/Pd-C (10%) or through saponification using 1N NaOH. The protection of the N^{g}- of Arg was achieved through the presence of a nitro group, and its elimination was accomplished by employing hydrogenolysis with $HCOONH_4$/Pd-C (10%). The catalyst IBCF was effectively utilized to achieve all the coupling reactions. The protected peptides were subjected to characterization using standard physical and analytical techniques, resulting in successful outcomes.

The synthesis of the tetrapeptide Gly-Pro-Arg-Pro was carried out using a stepwise approach. In this method, Boc-Arg(NO_2)-Pro-OBzl was first synthesized through the mixed anhydride technique in the presence of HOBt. The Boc protecting group was then removed and coupled with Boc-Pro to yield Boc-Pro-Arg(NO_2)-Pro-OBzl. Subsequently, the Boc group was deblocked and coupled with Boc-Gly to obtain Boc-Gly-Pro-Arg(NO_2)-Pro-OBzl.

The peptides Boc-Arg(NO_2)-Pro-OH, Boc-Pro-Arg(NO_2)-Pro-OH, and Boc-Gly-Pro-Arg(NO_2)-Pro-OH were linked to heterocycle 4-benzylpiperidine(BP) in the current investigation. The Boc-protected peptide conjugated heterocycles that were synthesized have been analysed using standard physical and analytical methods. The Boc group of the synthesized peptide conjugated heterocycles was eliminated by treating them with 4N HCl in dioxane, while the nitro group protection of N^{α} of Arg was removed through hydrogenolysis using $HCOONH_4$/Pd-C (10%). These deblocked compounds were then utilized for their antibacterial and antifungal properties. The synthetic approach of piperidine-peptide conjugates is given in **scheme-1**.



BP VII-IX XVI-XIX

XXII-XXIV

Xaa = RP for VI, PRP for VII & GPRP for VIII.

Scheme- 1: Synthesis of piperidinyl-peptide hybrids.

2.0 Materials and methods:

All the amino All tert-butyloxycarbonyl (Boc) amino acid derivatives used were of *L*-configuration and 1-hydroxybenzotriazole (HOBt) were purchased from Advanced Chem. Tech., (Louisville, Kentucky, USA). Isobutyl chloroformate (IBCF), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) and *N*-methyl morpholine (NMM) were purchased from Sigma Chemicals Co. (St. Louis, USA). All solvents and reagents were of analytical grade or were purified according to standard procedure recommended for peptide synthesis. The melting points were determined with open capillary and are uncorrected. ^{1}H NMR spectra were obtained on a 300 MHz Bruker FT-NMR Spectrometer instrument by using $CDCl_3$/DMSO as solvent and TMS as an internal standard. The thin layer chromatography (TLC) was carried out on precoated silica gel plates prepared in laboratory with the following solvent systems: R_f^1:$CHCl_3$-MeOH-HOAc (95:5:3), R_f^2: $CHCl_3$-MeOH-HOAc (90:10:3), Rf^3: $CHCl_3$-MeOH-HOAc (85:15:3).

Synthesis of peptides, Boc-Arg (NO_2)-Pro-OH(VII) and Boc-Xaa-Arg (NO_2)-Pro-OH (Xaa is Pro for VIII, Gly-Pro for IX) by debenzylation of peptides IV, V &VI:

Each peptide (IV, V & VI, 10 mmol) was dissolved in methanol (10 mL/g of peptide) and cooled to 0 ^{0}C. Then, added a cooled solution of 1N NaOH (20 mL, 20 mmol) slowly and stirred the solution for about 1.5 hours. When the reaction gets completed (monitored by TLC), evaporated the methanol, cooled and neutralized with 1N HCl (cold).[13] Then, extract with chloroform (3 X 25 mL) and washed with 1N HCl (1X25 mL), water (1X25 mL) and dried over Na_2SO_4. The solvent was removed under reduced pressure and dried over vacuum to get debenzylated peptides [yield; VII (85%), VIII (88%) & IX (88%)]. The above synthesized peptidic precursors (VII to IX) were divided into two parts. One part of which is used for further coupling with heterocycles and other part was used for the antimicrobial studies after removing side chain protections completely.

Removal of guanidine nitro group of peptides, Boc-Arg-Pro-OH (X) and Boc-Xaa-Arg-Pro-OH (Xaa is Pro for XI, Gly-Pro for XII):

The guanidine nitro group of compounds VII (0.6g, 1.4 mmol, VII, VIII & IX, 1.5 mmol) underwent hydrogenolysis in methanol (5 mL) with the use of ammonium formate (2 equivalents) and 10% Pd/C (0.1 g) for a duration of 30 minutes at room temperature [13]. The catalyst was then filtered and rinsed with methanol. The combined filtrate was concentrated under reduced pressure, and the resulting residue was dissolved in chloroform, followed by washing with water and drying over Na_2SO4. The solvent was evaporated under reduced pressure, and the product was triturated with ether, filtered, washed with ether, and dried to yield compound X (85%), XI (90 % & XII, 90%).

Synthesis of HCl.NH_2-Arg-Pro-OH (XIII): HCl.NH_2-Xaa-Arg-Pro-OH (Xaa is Pro for XIV, Gly-Pro for XV):deblocking of Boc-group of peptides.

0.5 g of X, XI and XII was deblocked by stirring with 5 mL of 4N HCl/dioxane for 1.5 hr. Excess HCl and dioxane were removed under reduced pressure, triturated with ether, filtered, washed with ether and dried [12], Yield; (XIII, XIV & XV 100%).

Synthesis of Peptide Conjugated Heterocycles:

General procedure for synthesis of Boc protected piperidinyl shorter peptide conjugates:

To the stirred solution of Boc-peptide (VII-IX, 2 mmol) and HOBt (0.31 g, 2 mmol) in DMF (10 mL) cooled to 0 ^{0}C, added NMM (0.22 mL, 2 mmol). The reaction mixture was further cooled to -15 0C ± 1 ^{0}C and added EDCI (0.39 g, 2 mmol) and 4-benzylpiperidine (I) (2 mmol). After 20 minutes, the pH of solution was adjusted to 8 by the addition of NMM and the reaction mixture was stirred overnight while slowly warming to RT.[10] The reaction mixture was quenched with water (2 mL) and the solvent was condensed. The residue was dissolved in chloroform (25 mL), washed with 5% $NaHCO_3$ (3x20 mL), H_2O (1x20mL) followed by 0.1N cold HCl (3x20 mL) and brine solution (3x20 mL), dried over anhydrous Na_2SO_4. The chloroform was removed under reduced pressure to obtain the desired products (XVI -XVIII). Yield, melting point and ^{1}H NMR data of these compounds are given below.

General procedure for the catalytic transfer hydrogenation of Boc-protected piperidinyl shorter peptide conjugates:

The guanidine nitro group of each peptide conjugated heterocycles (XVI-XVIII, 1.5 mmol) was subjected to hydrogenolysis in methanol (10 mL/ g of peptide) with the use of ammonium formate (2 eq.) and 10% Pd/C (0.1 g/1.0 g of compound) for a period of 30 minutes at room temperature.[13] Following this, the catalyst was filtered and washed with methanol. The combined filtrate was then evaporated under vacuum, and the resulting residue was dissolved in chloroform, washed with water, and dried using Na2SO4. Subsequently, the solvent was removed under reduced pressure, and the resulting compounds (XIX- XXI) were further purified by triturating with ether. The purified compounds were filtered, washed with ether, and dried, resulting in a yield of 90%.

General procedure for the deprotection of Boc protected piperidinyl shorter peptide conjugates:

0.5 g of each peptide conjugated heterocycles (XIX-XXI) was deblocked by stirring with 5 mL of 4N HCl/dioxane for 1.5 hr.[12] Excess HCl and dioxane were removed under reduced pressure, triturated with ether, filtered, washed with ether and dried get hydrochloride salts of peptide conjugated heterocycles (XXII-XXIV), Yield (100%). These compounds were used for antimicrobial study and given in **table 1 & 2**.

3.0 RESULTS AND DISCUSSION

Boc-RP-BP

Yield (%): 90; Arg^{2}4.50[1H, t, $^{\alpha}$CH), 2.00[2H, m, $^{\beta}$CH$_2$], 1.82[2H, m, $^{\gamma}$CH$_2$], 3.29[2H, m, $^{\delta}$CH$_2$], 8.20-8.26 [2H, s, NH], 1.45[9H, s, Boc];Pro^1 4.26[1H, t,$^{\alpha}$CH], 3.16[2H, m, $^{\beta}$CH$_2$], 2.62[2H, m, $^{\gamma}$CH$_2$], 3.55[2H, t, $^{\delta}$CH$_2$] ;BP 7.13-7.29[5H, m, ArH], 2.45[2H, s, -CH$_2$-piperidine], 2.85[4H, t, -CH$_2$-piperidine], 1.75[4H, t, -CH$_2$-piperidine], 1.90[1H, s, CH].

Boc-PRP-BP

Yield (%): 85; Pro^3 4.24[1H, t, $^{\alpha}$CH], 3.13[2H, m, $^{\beta}$CH$_2$], 2.62[2H, m, $^{\gamma}$CH$_2$], 3.54[2H, t, $^{\delta}$CH$_2$], 1.45[9H, s, Boc]; Arg^2 4.49[1H, t, $^{\alpha}$CH], 1.97[2H, m, $^{\beta}$CH$_2$], 1.80[2H, m, $^{\gamma}$CH$_2$], 3.31[2H, m, $^{\delta}$CH$_2$], 8.15-8.24 [2H, s, NH]; Pro^1 4.26[1H, t, $^{\alpha}$CH], 3.17[2H, m, $^{\beta}$CH$_2$], 2.64[2H, m, $^{\gamma}$CH$_2$], 3.56 [2H, t, $^{\delta}$CH$_2$]; BP 7.20-7.32 [5H, m, ArH], 2.5 [4H, s, ArCH$_2$], 2.80[4H, t, -CH$_2$-piperidine], 1.75[4H, t, -CH$_2$-piperidine], 1.94 [1H, s, -CH-piperidine].

Boc-GPRP-BP

Yield (%): 83; Gly^4 4.16[2H, d, $^{\alpha}$CH], 8.1[1H, s, NH], 1.43 [9H, s, Boc]; Pro^3 4.28[1H, m, $^{\alpha}$CH], 3.17 [2H, m, $^{\beta}$CH$_2$], 2.68[2H, m, $^{\gamma}$CH], 3.56[2H, t, $^{\delta}$CH]; Arg^2 4.51[1H, m, $^{\alpha}$CH], 1.98 [2H, m, $^{\beta}$CH], 1.82 [2H, m, $^{\gamma}$CH$_2$], 3.33[2H, m, $^{\delta}$CH], 8.17-8.30 [2H, s, NH] Pro^1 4.28[1H, m, $^{\alpha}$CH], 3.17 [2H, m, $^{\beta}$CH], 2.69[2H, m, $^{\gamma}$CH], 3.57[2H, t, $^{\delta}$CH]; BP 7.2-7.3 [5H, m, ArH], 2.52 [2H, s, ArCH$_2$], 2.82[4H, t, -CH$_2$-piperidine], 1.75[4H, t, -CH$_2$-piperidine], 1.88[2H, s, -CH-piperidine].

a) General method for antibacterial assay:

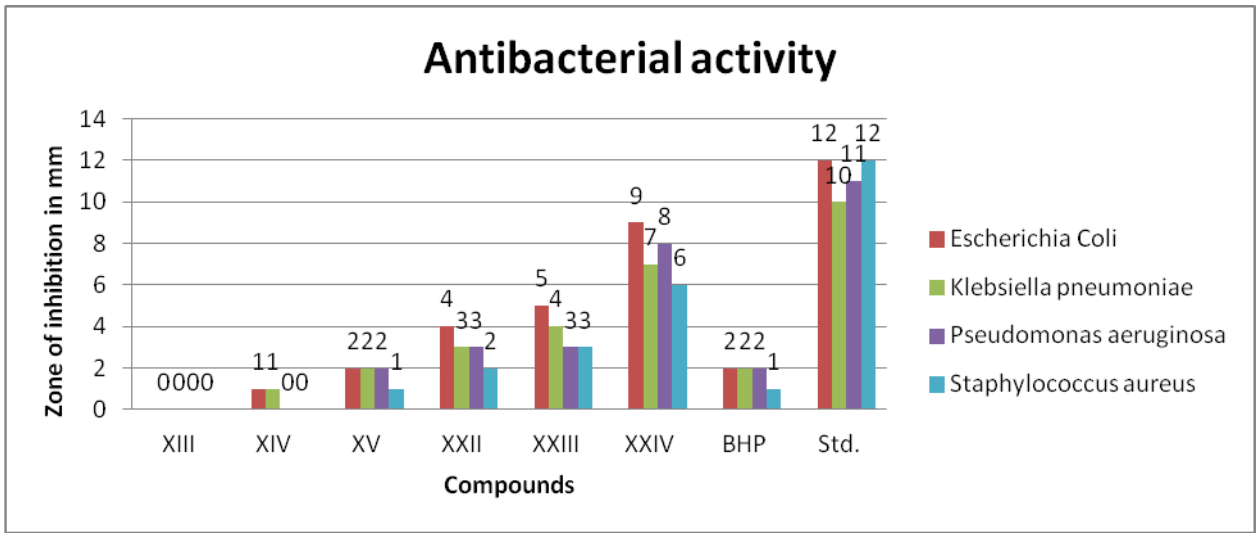
In vitro antibacterial activity of piperidinylpeptide conjugates was carried out against various bacteria, Staphylococcus aureus, Escherichia coli, Klebsiella pnemoniae, and Pseudomonas aeruginosa using the agar well diffusion system.[14] The bacterial strains were cultivated in Muller-Hinton broth. The inoculum concentration was calibrated using the mid-logarithmic phase system (OD 600 = 0.5). Agar media is prepared using Muller-Hinton agar with sterile distilled water and autoclaved for one hour. The autoclaved media was then poured into pre-sterilized 90 mm petriplates and allowed to solidify. Using an 8 mm sterile cork borer, the media was removed from the centre, creating a well for the assay. The inoculum was spread evenly over the media. Additionally, 50 µL of a stock solution of compounds (10 µg/well) was added to the wells created in the petriplates. The petriplates were then incubated at 37 °C for 3-4 days. All piperazinyl shorter peptide conjugates were tested in triplicate, with streptomycin serving as the positive control and water as the negative control. The zone of inhibition, measured in millimetres, was recorded and presented in **table-1** and **graph-1**.

Table-1:**Antibacterial Activity of piperidinylpeptide hybrids:**

Entry	Compounds[a]	Zone of Inhibiton (diameter) mm[b]			
		Escherichia Coli	Klebsiella pneumoniae	Pseudomonas aeruginosa	Staphylococcus aureus
XIII	RP	00	00	00	00
XIV	PRP	01	01	00	00
XV	GPRP	02	02	02	01
XXII	BP-RP	04	03	03	02
XXIII	BP-PRP	05	04	03	03
XXIV	BP-GPRP	09	07	08	06
BHP	BP	02	02	02	01
Std.	Streptomycin	12	10	11	12

[a] Concentration of compounds and reference drug:10 µg/ml.

[b] Values are means of three determinations, the ranges of which are less than 5% of the mean in all cases.



Graph-1: Graphical representation of antibacterial activity of piperidinylpeptide hybrids.

b) General method of antifungal assay:

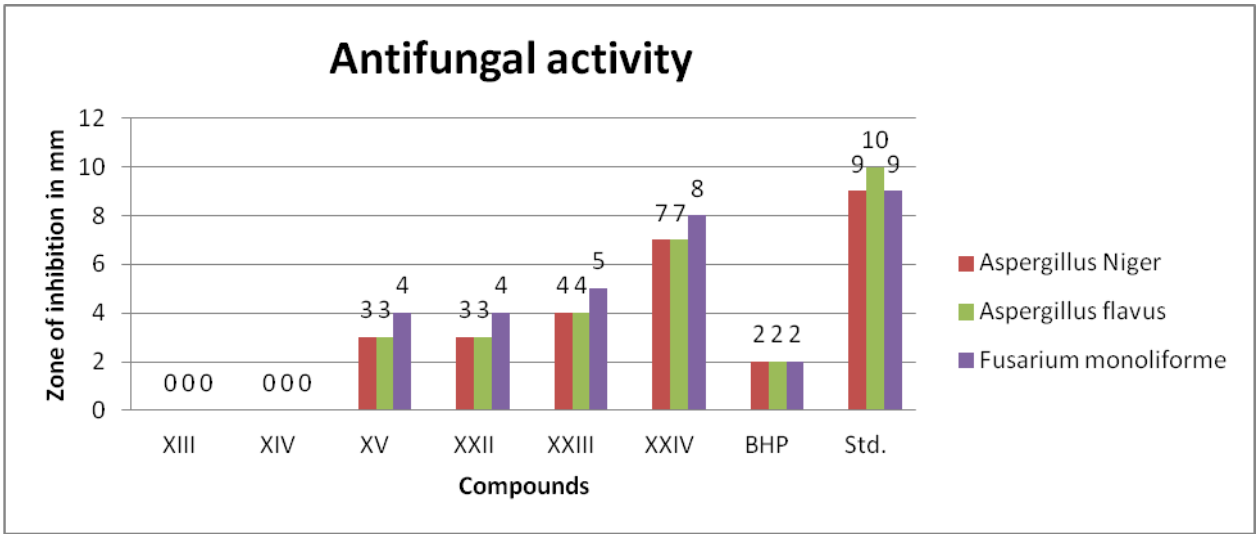
In vitro antifungal activity piperazinyl shorter peptide conjugates were carried out against Aspergillus niger, Aspergillus flavus, and Fusarium monoliforme using the agar well diffusion technique. [15] The fungal cultures were grown on PDA media with a pH of 7.4 for six days at a temperature of 25°C. The spores were collected in sterilized normal saline solution and adjusted to a concentration of 1x106/ml using a Haemocytometer. Each sterilized 90 mm petriplate was filled with 20mL of autoclaved molten media and allowed to solidify. To evaluate the growth response of the fungal species, 0.4 mL of the synthesized compounds (10 µg/mL) was evenly spread over the agar media in each plate. Then, 10µL of spore suspension was added to the small depression created at the center of each plate, and the plates were incubated for six days at 25°C. After the incubation period, the plates were examined and compared to their respective control plates. The control plates contained only distilled water, representing 100% fungal growth with no inhibition. The fungicidal activity of the synthesized compounds was determined by comparing the zone of fungal growth in the treated plates to that of the control plates in millimeters. The results can be found in **table-2 and graph-2**.

Table-2: Antifungal activity of piperidinyl-peptide hybrids:

Entry	Compounds[a]	Zone of Inhibiton (diameter) mm [b]		
		Aspergillus Niger	*Aspergillus flavus*	*Fusarium monoliforme*
XIII	RP	00	00	00
XIV	PRP	00	00	00
XV	GPRP	03	03	04
XXII	BP-RP	03	03	04
XXIII	BP-PRP	04	04	05
XXIV	BP-GPGP	07	07	08
BHP	BP	02	02	02
Std.	Bavistin	09	10	09

[a] Concentration of compounds and reference drug:10 µg/mL

[b] Values are means of three determinations, the ranges of which are less than 5% of the mean in all cases.



Graph-2: Graphical representation of antifungal activity of piperidinyl-peptide hybrids.

Antibacterial studies

The antibacterial effectiveness of the aforementioned compounds was assessed against both gram-positive and gram-negative bacteria including Staphylococcus aureus, Escherichia coli, Klebesiella pneumoniae, and Pseudomonas auregenosa, using the same concentration as the standard, streptomycin. The results, expressed as the zone of inhibition measured in millimeters, are summarized in **table-1**.

Among the synthesized 4-benzylpiperidine conjugated peptides, GPRP-BP (XXIV) exhibited significant activity. This could be attributed to the compounds' ability to easily penetrate the thin peptidoglycan layer of gram-negative bacterial cell walls compared to gram-positive bacteria, which have much thicker walls. The antibacterial activity of the synthesized compounds indicates that both heterocyclic conjugated peptides and peptides alone show increased activity as the peptide chain length increases from dipeptide to tetrapeptide. While the dipeptide alone does not exhibit any activity, the tripeptide and tetrapeptides show some activity against all tested bacterial strains.

In contrast, when the heterocycles were tested in isolation, they showed negligible antibacterial activity. Therefore, it can be concluded that the conjugation of peptides with heterocycles significantly enhances their activity, making them promising molecules for further investigation.

Antifungal activity:

Various fungal strains, including Aspergillus niger, Aspergillus flavus, and Fusarium monoliforme were subjected to antifungal testing using the compounds mentioned above. The concentration used was the same as that of the standard, Bavistin, and the results were expressed as the zone of inhibition measured in mm. The findings are presented in **table-2.** Out of all the compounds tested, tetrapeptide conjugated 4-benzylpiperidine (**XXIV**) exhibited good activity in the series, with potency equivalent to Bavistin. The attributes discussed in relation to antibacterial activity also apply to antifungal studies. The remaining di and tripeptide conjugated 4-benzylpiperidine showed moderate to good activity compared to the peptide and heterocycles alone.

4.0 Conclusion

In an effort to explore peptide-conjugated heterocycles as a new class of antimicrobial drugs, we have made an interesting discovery. It has been found that compounds containing tetrapeptide fragments of 4-benzylpiperidine possess remarkable antibacterial and antifungal properties. On the other hand, all other compounds exhibited only a moderate level of activity. Therefore, it can be inferred that the combination of peptides with different chain lengths and heterocyclic motifs greatly enhances both antibacterial and antifungal activities compared to their original molecules. The results of this study indicate that compounds **XXIV** have significant potential for the development of improved analogues with enhanced antimicrobial efficacy.

References

[1] William Stubbings and Harald Labischinski., 2009. F1000 Biology Reports 1: 40.

[2] Rai, J. Randhawa, G.K. Kaur, M. 2013. Recent advances in antibacterial drugs. Int. J. Appl. Basic. Med. Res, 3(1): 3-10.

[3] Nielsen, PE. 2000. Pseudo-peptides in Drug Discovery, Wiley, New York.

[4] Leader, B. Baca, QJ. Golan, DE. 2008. Protein therapeutics: a summary and pharmacological classification. Nat. Rev. Drug Discov., 7(1): 21-39.

[5] Hancock, RE. Scott, MG. 2000. The role of antimicrobial peptides in animal defenses. Proc. Natl. Acad. Sci. USA. 97(16): 8856-61.

[6] Kannan, A. Hettiarachchy, N. Johnson, MG. Nannapaneni, R. 2008. Human colon and liver cancer cell proliferation inhibition by peptide hydrolysates derived from heat-stabilized defatted rice bran." J. Agric. Food Chem. 56(24), 11643-7.

[7] Kim, MK. Park, K.S, Yeo, WS, Choo, H. Chong, Y. 2009. In vitro solubility, stability and permeability of novel quercetin-amino acid conjugates." Bioorg. Med. Chem. 17(3): 1164-71.

[8] Chou, PY. Fasman, GD. 1977. Beta-turns in proteins Interaction of the fragments characteristic of bactenecin 7 with phospholipid bilayers and their antimicrobial activity. J. Mol. Biol. 115(2): 135-175.

[9] Suresha, GP. Prakasha, KC, Shiva Kumara, K.N. Gowda, D.C. 2009. Design and Synthesis of Heterocyclic Conjugated Peptides as Novel Antimicrobial Agents. Int. J. Pep.t Res. Ther. 15: 25–30.

[10] Shivakumara, K.N. Prakasha, K.C. Suresha, GP. Suhas, R. Gowda, D. C. 2007. Synthesis and antimicrobial study of amino acids conjugated benzylpiperazine derivatives. my Science., II(2): 100-106.

[11] Abiraj, K. Prakasha Gowda, AS. & Gowda, DC. 2002. Synthesis of shorter active analogues of Bactenecin7: The effect of change of N-terminal configuration on antimicrobial activity." Lett. pept. sci. 9(6): 283-290,

[12] Abiraj, K. Prasad, SH.. Prakasha Gowda, AS and Gowda, DC. 2004. Design, Synthesis and Antibacterial Activity Studies of Model Peptides of Proline / Arginine-Rich Region in Bactenecin7," Protein Pept. Lett., 11(4).

[13] Anwer, MK.. Arno, F. Spatola, A. 1981. Rapid non-acidolytic method for deprotection and removal of peptides from solid phase resins - applications of ammonium formate catalytic transfer hydrogenation, Tetrahedron Lett. 22(44): 4369-4372,

[14]. Perez, C. Pauli, M. and Bazerque, P. 1990. An Antibiotic Assay by Agar Well Diffusion Method. Acta Biologiae et Medicinae Experimentalis, 15: 113-115.

[15] Singh, I. Singh, VP. 2000. Antifungal properties of aqueous and organic solution extracts of seed plants against Aspergillusflavus and A. niger." Phytomorphology. 50(2): 151-157.