Synthesis of piperazinylpeptide conjugates containing smaller peptide molecules as a promising avenue for the development of new antimicrobial agents

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Abstract: Novel peptide conjugates with a piperazinyl group were produced by combining shorter peptides with benzylpiperazine using IBCF as the coupling agent, HOBt, and NMM as the base at ±15 ^{0}C. Initially, the process involved stirring the mixture at ±15 ^{0}C for 2 hours, followed by an overnight reaction. The resulting compounds were identified using ^{1}H NMR and R_f values, and then tested for antibacterial effects against Staphylococcus aureus, Escherichia coli, Klebsiella pnemoniae, and Pseudomonas aeruginosa, as well as antifungal effects against Aspergillus Niger, Aspergillus flavus, and Fusarium monoliforme. **XXIV** displayed notable activity among the compounds synthesized, whereas **XXII** and **XXIII** showed moderate activity.

Index Terms - Peptide conjugates, Zone of inhibition, drug delivery, Piperazine and selectivity.

I. INTRODUCTION

There is an increasing demand for new antimicrobial compounds globally, driven by the rapid development of resistance by microbes to traditional antibiotics. This has led to the emergence of bacterial infections that are essentially untreatable with the current arsenal of available treatment options. Numerous initiatives have been undertaken to advance new antimicrobials with unique mechanisms of action that can combat these multi-drug resistant strains. The need for innovative antibiotics to effectively address antibiotic-resistant infections continues to be a pressing concern. As a result, the existing antibiotic arsenal is insufficient to confront the challenges posed by resistance in the present day [1-2].

Peptides and their derivatives display a wide range of biological activities, including antimicrobial [3-5], antiviral, anticancer, anti-glycation properties [5-6]. Moreover, they play a crucial therapeutic role in various fields such as neurology, endocrinology, and haematology [7]. Peptides are highly versatile bioactive molecules. Numerous peptide hormones and shorter peptides exert their effects by binding to membrane receptors [8].

Benzylpiperazine possess a wide range of pharmaceutical properties, 5-HT_{2A} and $5HT_{2C}$ receptor [9], D_2/D_4 antagonists [10], sigma-1-receptors [11], antihistamine property [12], vasodilating activity [13], antinociceptive activity [14], tyrosine kinase inhibitors [15], antibacterial and antifungal activity [16].

In this context, earlier we have synthesized peptide-heterocycles by coupling heterocyclic pioneer quinazolinone with inactive peptide sequences of elastin such as VP, GVP, VGVP and GVGVP peptides [17]. It was observed that the conjugation of quinazolinone heterocycle with VGVP and GVGVP peptides displayed intensification in activity by nearly two-fold comparison with conventional antimicrobials. All the quinazolinone conjugated peptides exhibited improved activity, even though peptides and quinazolinone moiety which when taken in isolation were inactive towards those bacterial strains.

In continuation of our research work peptide-heterocycle conjugation, we have designed and synthesized peptides conjugated heterocycles by coupling heterocyclic precursors such as benzylpiperazine with shorter analogues of Bac7 peptides such as RP, PRP and GPRP.

1.1. Materials and methods:

All tert-butyloxycarbonyl (Boc) amino acid derivatives were used of *L*-configuration and 1-hydroxybenzotriazole (HOBt) were purchased from Advanced Chem. Tech., (Louisville, Kentucky, USA). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI), Isobutyl chloroformate (IBCF), 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: CHCl3-MeOH-HOAc (85:15:3).

Summary of the synthesis of peptide-heterocyclic conjugates:

The peptide synthesis was carried out by the stepwise solution phase method using Boc chemistry. The Boc group was chosen for N^{α}-protection and its removal was achieved with 4*N* HCl in dioxane. The *C*-terminus carboxyl group was protected by the benzyl ester and its removal was achieved by hydrogenolysis using $HCOONH_4$/Pd-C (10%) or by saponification using 1*N* NaOH. The N^g- of Arg was protected by nitro group and its removal was affected by hydrogenolysis using $HCOONH_4$/Pd-C (10%). All the coupling reactions were achieved with IBCF. The protected peptides were characterized by standard physical and analytical techniques.

The synthesis of the tetrapeptide Gly-Pro-Arg-Pro was carried out using a stepwise approach, as illustrated in **Scheme-1.** In this method, Boc-Arg (NO_2)-Pro-OBzl was first synthesized through the mixed anhydride method in the presence of HOBt. The Boc protecting group was then removed, and the resulting compound was coupled with Boc-Pro to yield Boc-Pro-Arg (NO_2)-Pro-OBzl. Subsequently, the Boc protecting group was removed again, and the compound was coupled with Boc-Gly to obtain Boc-Gly-Pro-Arg (NO_2)-Pro-OBzl.

In the present study, the peptides Boc-Arg (NO_2)-Pro-OH, Boc-Pro-Arg (NO_2)-Pro-OH and Boc-Gly-Pro-Arg (NO_2)-Pro-OH are coupled to heterocycle, 4-benzylpiperizine. The synthesized Boc-protected peptide conjugated heterocyclic conjugates have been characterized by standard physical and analytical techniques. The synthesized peptide conjugated heterocyclic conjugates were deblocked with 4*N* HCl in dioxane to remove Boc group and the nitro group protection of N^g of Arg was removed by hydrogenolysis using $HCOONH_4$/Pd-C (10%). The deblocked compounds were used for antibacterial and antifungal activities. The synthesis of piperazinyl shorter peptide conjugates are presented below in **scheme-1**.

Peptide synthesis:

Synthesis of peptides Boc-Arg-(NO_2)-Pro-OBzl or Boc-RP-OBzl (IV), Boc-Pro-Arg (NO_2)-Pro-OBzl or Boc-PRP-OBzl(V) and Boc-Gly-Pro-Arg (NO_2)-Pro-OBzl or Boc-GPRP-OBzl (VI) done using solution phase approach via Boc chemistry [22] using IBCF and HOBt as coupling agent, NMM as base in DMF by stirring the reaction mixture over night at -15 ^{0}C ± 1 ^{0}C to room temperature (RT) [18]. The solvent was removed under reduced pressure, and the remaining DMF solution was poured into approximately 600 mL of ice-cold 90% saturated $KHCO_3$ solution and stirred for 30 minutes. The resulting peptide precipitate was filtered, washed with water, 0.1N cold HCl, water, and dried. The crude peptide was recrystallized from ether and petroleum ether to obtain the desired product, with a yield of 88% and a melting point of 44-45°C (literature: 45-46°C) for IV, Yield (87%), M. P. 46-47 0C (lit. 47 ^{0}C) for V and Yield (87%), M. P. 46-47 ^{0}C (lit. 47 ^{0}C).

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 1*N* 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 1*N* HCl (cold) [18]. Then, extract with chloroform (3 X 25 mL) and washed with 1*N* HCl (1 X 25 mL), water (1 X 25 mL) and dried over Na_2SO_4. The solvent was removed under reduced pressure and dried over vacuum to get debenzylated peptide {**VII** yield 85, **VIII**, yield (88%) & **IX,** yield (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 [18]. 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_2SO_4. 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**) **by deblocking of Boc-group of peptides.**

0.5 g of **X, XI and XII** was deblocked by stirring with 5 mL of 4*N* HCl/dioxane for 1.5 hr. Excess HCl and dioxane were removed under reduced pressure, triturated with ether, filtered, washed with ether and dried [22], Yield (**XIII**, **XIV & XV** 100%).

Synthesis of Peptide Conjugated Heterocycles:

General procedure for the synthesis of Boc-protected piperazinylpeptide 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 ^{0}C ± 1 ^{0}C and added EDCI (0.39 g, 2 mmol) and 4-benzylpiperazine (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 [19]. 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% $NaHCO_3$ (3 x 20 mL), H_2O (1 x 20 mL) followed by 0.1*N* cold HCl (3 x 20 mL) and brine solution (3 x 20 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 presented in **Table-1**.

General procedure for the catalytic transfer hydrogenation of Boc-protected piperazinylpeptide conjugates:

The guanidine nitro group of each peptide conjugated heterocycles (XVI - XVIII, 1.5 mmol) underwent hydrogenolysis in methanol (10 mL/ g of peptide) using ammonium formate (2 eq.) and 10% Pd/C (0.1 g/1.0 g of compound) for a duration of 30 minutes at room temperature [19]. The catalyst was then filtered and rinsed with methanol. The combined filtrate was evaporated under vacuum, and the resulting residue was dissolved in chloroform, washed with water, and dried using Na_2SO_4. The solvent was subsequently removed under reduced pressure, and the obtained 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 piperazinylpeptide conjugates:

0.5 g of each peptide conjugated heterocycles (**XIX-XXI**) was deblocked by stirring with 5 mL of 4*N* HCl/dioxane for 1.5 hr [19]. 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-XIV**), Yield (100%). These compounds were used for antimicrobial study and given in **Table 1& 2**.

1.1.1. Results and discussion

Boc-RP-BZP

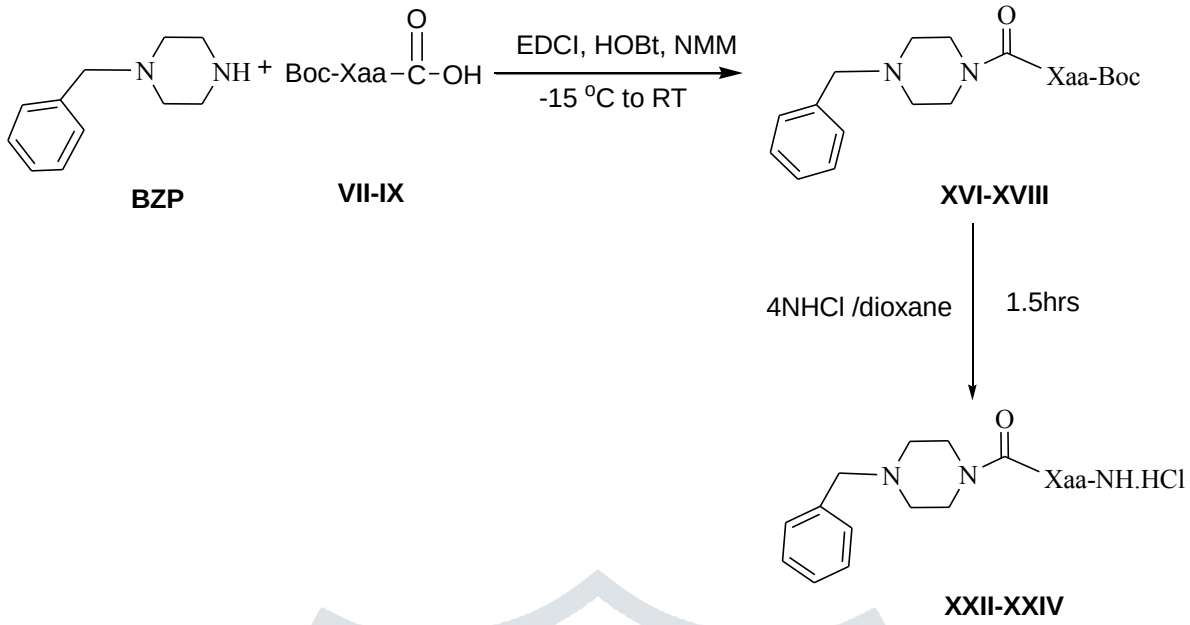
Yield (%): 90, Arg^{2}4.46[1H, t, $^{\alpha}$CH), 2.12[2H, m, $^{\beta}CH_2$], 1.90[2H, m, $^{\gamma}CH_2$], 3.47[2H, m, $^{\delta}CH_2$], 8.16-8.28 [2H, s, NH], 1.45[9H, s, Boc]; Pro^1 4.30[1H, t,$^{\alpha}$CH], 3.17[2H, m, $^{\beta}CH_2$], 2.62[2H, m, $^{\gamma}CH_2$], 3.55[2H, t, $^{\delta}CH_2$] **;** BZP 7.25-7.40[5H, m, ArH], 3.15[4H, t, -CH_2-piperazine], 2.25[4H, t, -CH_2-piperazine], 4.1[2H, s, ArCH_2].

Boc-PRP-BZP

Yield (%): 85, Pro^3 4.23[1H, t, $^{\alpha}$CH], 3.12[2H, m, $^{\beta}CH_2$], 2.58[2H, m, $^{\gamma}CH_2$], 3.50[2H, t, $^{\delta}CH_2$], 1.45[9H, s, Boc]; Arg^2 4.47[1H, t, $^{\alpha}$CH], 2.11[2H, m, $^{\beta}CH_2$], 1.89[2H, m, $^{\gamma}CH_2$], 3.54[2H, m, $^{\delta}CH_2$], 8.14-8.28 [2H, s, NH]; Pro^1 4.33[1H, t, $^{\alpha}$CH], 3.17[2H, m, $^{\beta}CH_2$], 2.66[2H, m, $^{\gamma}CH_2$], 3.55 [2H, t, $^{\delta}CH_2$]; BZP 7.30-7.47 [5H, m, ArH], 3.12 [4H, t, -CH_2- piperazine], 2.17 [4H, t, -CH_2-piperazine], 4.1 [1H, s, ArCH_2].

Boc-GPRP-BZP

Yield (%): 89; Gly^4 4.10[2H, s, $^{\alpha}$CH], 8.1[1H, s, NH], 1.43 [9H, s, Boc]; Pro^3 4.33[1H, t, $^{\alpha}$CH], 3.15 [2H, m, $^{\beta}CH_2$], 2.64[2H, m, $^{\gamma}CH_2$], 3.55[2H, t, $^{\delta}CH_2$]; Arg^2 4.49[1H, t, $^{\alpha}$CH], 1.98 [2H, m, $^{\beta}CH_2$], 1.89 [2H, m, $^{\gamma}CH_2$], 3.43[2H, m, $^{\delta}CH_2$], 8.12-8.22 [2H, s, NH]; Pro^1 4.33[1H, t, $^{\alpha}$CH], 3.17 [2H, m, $^{\beta}CH_2$], 2.66[2H, m, $^{\gamma}CH_2$], 3.56[2H, t, $^{\delta}CH_2$]; BZP 7.2-7.3 [5H, m, ArH], 3.12 [4H, t, -CH_2-piperazine], 2.31[4H, t, -CH_2-piperazine], 4.00[2H, s, ArCH_2].



BZP **VII-IX** **XVI-XVIII** **XXII-XXIV**

Xaa = RP for VII, PRP of VIII & GPRP of IX

Scheme 1. Synthesis of piperazinyl shorter peptide conjugates.

a) General method for antibacterial assay:

In vitro antibacterial activity of piperazinyl peptide conjugates was carried out against various bacteria, Staphylococcus aureus, Escherichia coli, Klebsiella pnemoniae, and Pseudomonas aeruginosa using the agar well diffusion system [20]. 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 Fig.1**.

Table 1 Antibacterial Activity of piperazinylpeptide conjugates.

Entry	Compounds [a]	Inhibitory Zone (diameter) mm[b]			
		Escherichia coli	Klebesiella pneumoniae	Pseudomonas auregenosa	Staphylococcus aureus
XIII	RP	00	00	00	00
XIV	PRP	01	01	00	00
XV	GPRP	02	02	02	01
XXII	BZP-RP	05	05	05	05
XXIII	BZP-PRP	06	06	05	05
XXIV	BZP-GPRP	10	08	09	07
BZP	BZP	02	02	03	03
Std.	Streptomycin	12	10	11	12

[a] The compounds and reference drug were concentrated at 10 µg/ml.

[b] The values presented are the averages of three determinations, with the ranges of these determinations being less than 5% of the mean in every instance.

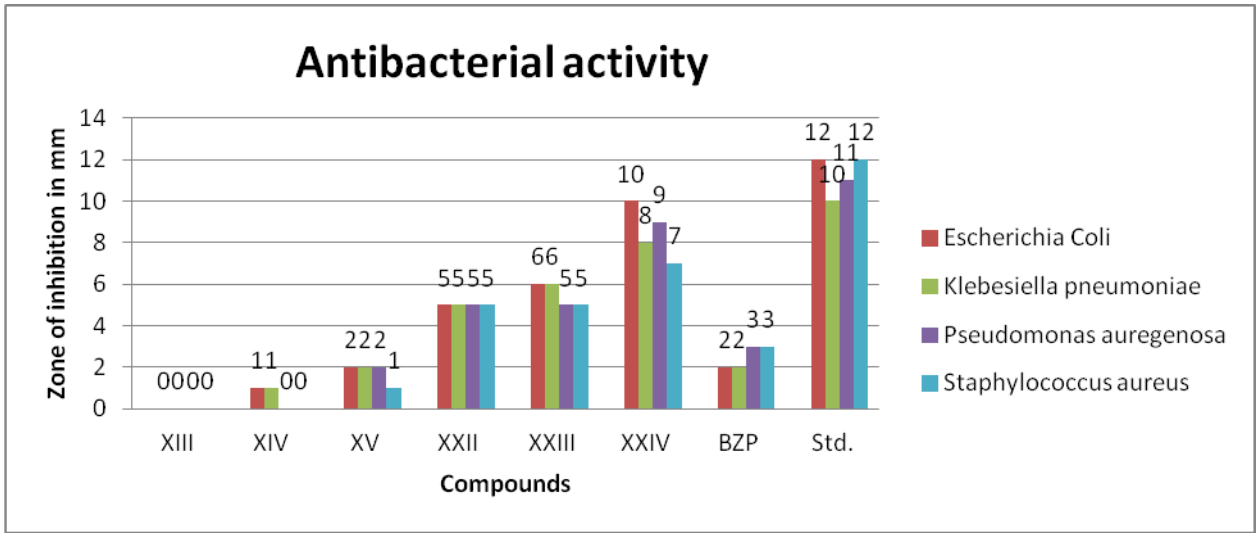


Fig. 1 Graphical representation of antibacterial Activity of piperazinylpeptide conjugates.

b) General method of antifungal assay:

In vitro antifungal activity piperazinylpeptide conjugates was carried out against Aspergillus Niger, Aspergillus flavus, and Fusarium monoliforme using the agar well diffusion technique [21]. 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 $1x10^6$/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 centre 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 Fig. 2.

Table 2 Antifungal Activity of piperazinylpeptide conjugates.

Entry	Compounds [a]	Inhibitory zone (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	BZP-RP	04	05	06
XXIII	BZP-PRP	05	05	06
XXIV	BZP-GPGP	08	08	09
BZP	BZP	02	03	04
Std.	Bavistin	09	10	09

[a] The compounds and reference drug were concentrated at 10 µg/ml.

[b] The values presented are the averages of three determinations, with the ranges of these determinations being less than 5% of the mean in every instance.

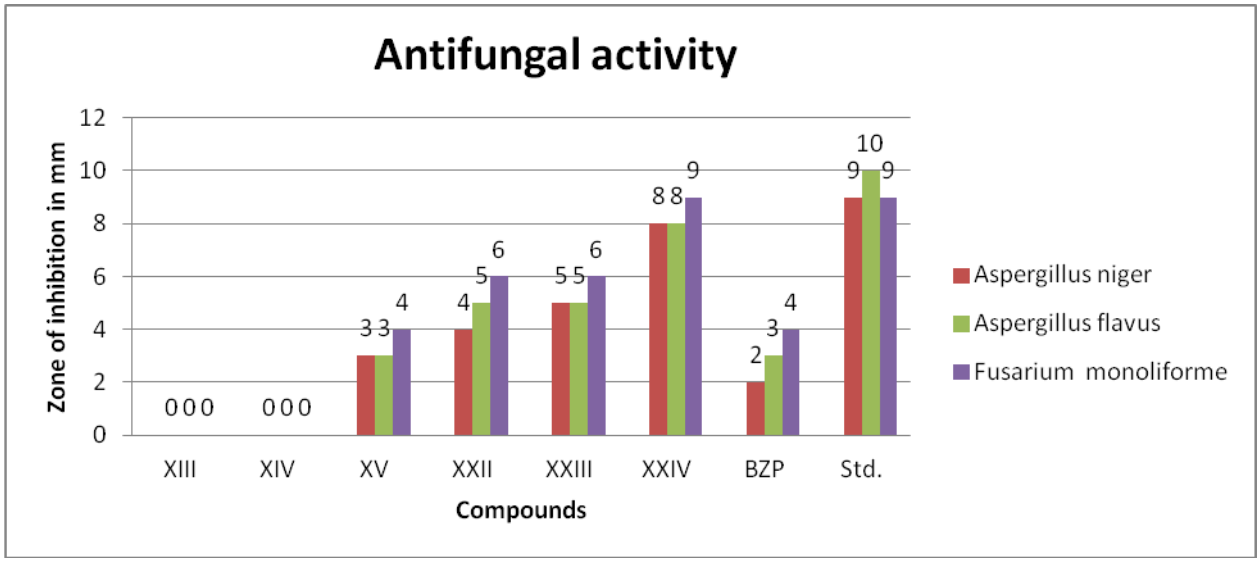


Fig. 2 Graphical representation antifungal Activity of piperazinylpeptide conjugates.

Antibacterial studies

The antibacterial efficacy of the above said compounds were tested against both gram + ve and gram - ve bacteria such as Staphylococcus aureus, Escherichia coli, Klebesiella pneumoniae and Pseudomonas auregenosa at the same concentration as that of the standard, streptomycin. The results are expressed as zone of inhibition measured in mm. The results are summarized in Table 1.

Among all the synthesized compounds, GPRP-BZP (XXIV) showed highest activity against all the bacterial strains tested which are equipotent with the standard drug. In the remaining two analogues, PRP-BZP (XXIII) and RP-BP (XXII) also showed moderate activity. On the whole, all the compounds showed good activity against the gram -ve bacteria when compared to gram +ve bacteria. This may be due to the easy passage of tested compounds through thin peptidoglycan layer of gram-negative bacteria cell wall when compared to the gram-positive bacteria, which is very much thicker. In view of this, antibacterial activity of the synthesized compounds reveals that, in both heterocyclic conjugated peptides and peptides alone, activity increases from dipeptide to tetrapeptide i.e., as the chain length of the peptide increases the activity also increases. Whereas peptides tested alone, the dipeptide does not show any activity, but, tri and tetrapeptides showed some activity against all the bacterial strains tested. On the contrary, all the heterocycles taken in isolation have also been subjected to antibacterial studies which fetched the negligible results. Thus, it can be emphasized that the conjugation of peptides with that of heterocycles resulted in marked increase in the activity which can be viewed as lead forefront molecules for further investigations.

Antifungal activity:

The antifungal efficacy of the aforementioned compounds was assessed against different fungal strains including Aspergillus niger, Aspergillus flavus, and Fusarium monoliforme at an equivalent concentration to the standard, Bavistin. The results, presented as the zone of inhibition measured in millimetres, are detailed in Table 2. Among the compounds tested, the piperazinylpeptide conjugates (XXIV) exhibited the highest level of activity in the series, showing equal potency to Bavistin. PRP-BZP (XXIII) and RP-BP (XXII) also displayed moderate effectiveness. The characteristics observed in terms of antibacterial activity were similarly applicable to the antifungal investigations.

1.1.2 Conclusion

In an effort to explore peptide conjugated heterocycles as a new class of antimicrobial drugs, we found that shorter peptides benzyl piperazinyl conjugates containing tetrapeptide fragments showed good antibacterial activity as well as good antifungal activity and all other compounds showed a moderate activity. Hence, it is possible to conclude that the combination of peptides with different chain lengths with heterocyclic motifs has improved both the antibacterial and antifungal activities when compared to the original molecules. The outcomes of this study suggest that compounds XXII, XXIII, and XXIV could be valuable in creating new analogues with enhanced antimicrobial efficacy.

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