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IN VITRO ANTIMICROBIAL ACTIVITY OF SOME NEWLY SYNTHESIZED POLYPEPTIDE CANDIDATES

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Antimicrobial peptides (AMPs) have been tested with respect to their possible application as chemotherapeutic agents. The current study aimed to synthesize and evaluate the potential antimicrobial activity of new peptide chains.These compounds were prepared by the liquid phase method loaded on polyethylene glycol as apolymeric support. The compounds were fully characterized by spectral data. The *in vitro* antimicrobial activity of the synthesized peptides was screened against Gram-positive (*Bacillus subtilits*) and Gram-negative (*Escherichia coli*) bacteria, yeast (*Candida albicans*) and filamentous fungi (*Aspergillusniger*). The results of biological activity were interpreted and discussed.

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1. Introduction

Since the introduction of the *Merrifield* method for peptide synthesis [1]**,** insoluble polymer supports have been incorporated into numerous synthetic methodologies to facilitate product purification [2]. Although highly successful, solid-phase synthesis still exhibits several shortcomings due to the nature of heterogeneous reaction conditions [3]. By replacing insoluble cross-linked resins with *soluble* polymer supports, the familiar reaction conditions of classical organic chemistry are reinstated, and yet product purification is still facilitated through application of macromolecular properties [4]. This methodology, termed liquid-phase peptide synthesis, in essence avoids the difficulties of solid-phase synthesis while preserving its positive aspects [5].

Soluble polymer that has been used in liquid phase synthesis is polyethylene glycol that is an uncharged, hydrophilic polymer, which is soluble in water as well as in most organic solvents and insoluble in hexane, diethyl ether [6]. Furthermore, the solubilizing power of PEG not only allows homogeneous reactions under numerous reaction conditions, but these solubility properties permit individual reactions steps to be monitored without requiring cleavage of product from the polymer support [7]. The characterization of PEG-bound organic moieties is often straightforward as the polymer does not interfere with spectroscopic or chemical methods of analysis; additionally [8]. Due to its low toxicity and immunogenicity, PEG is highly suitable for biomedical

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applications [9, 10]. Also,the physicochemical properties of polyethylene glycol permits its penetration through the cell membrane, so antimicrobial measurements could be carried out on PEG-bounded peptides [11].

In recent years, a large number of natural antimicrobial peptides (AMPs) as well as several synthetic analogues have been tested with respect to their possible application as chemotherapeutic agents [12]. Due to the low toxicity of peptide antibiotics particularly ardent hopes are put on the possibility of the application of endogenous ones as biopharmaceuticals in modern chemotherapy of infectious diseases [13]. Antimicrobial peptides have been identified in various species ranging from bacteria, frogs to mammals, including humans. They form the first line of host defense against pathogenic infections and are a key component of the ancient innate immune system [14]. Most AMPs possess 6-50 amino acid residues with net positive charges [15]. It is generally accepted that these cationic peptides selectively interact with anionic bacterial membranes [16, 17] although different mechanisms may be used by different peptides under different conditions for killing [18].

Based on the results presented here and in view of these observations, a set of new derived peptides for systemic antimicrobial use are proposed loaded on polyethylene glycol by use of liquid phase method.

2. Experimental

Chemistry

The peptides were prepared by step-wise manual liquid phase synthesis using *tert*. butyloxycarbonyl (*tert*. Boc) chemistry with polyethylene glycol (PEG) polymer.The organic solvents and the chemicals used in this part were obtained from Sigma (USA) and Fluka (Switzerland) Chemical Companies. All amino acids were purchased from Novabiochem; the used amino acids are of L- configuration (Glu: Glutamic Acid, Ala: Alanine, Gly: Glycine, Pro: Proline, Tyr: Tyrosine). Polyethylene glycol (PEG) polymer with molecular weight 6000 was obtained from Fluka (Switzerland) chemical company. All solvents were analytically pure and dried, when necessary, over molecular sieves (4Å) or sodium metal followed by distillation.

Synthesis of Boc-Amino Acid

A solution of the amino acid (10 mmol) in a mixture of dioxane (20 mL), water (10 mL) and 1 N NaOH (10mL) is stirred and cooled in an ice-water bath. Di-*tert*. butyl pyrocarbonate, (2.4 gm, 11 mmol) is added and stirring is continued at room temperature for half an hour. the solute on is concentrated in vacuo to about 10 to 15 mL, cooled in an ice water bath, covered with a layer of ethyl acetate (30 mL) and acidified with a dilute solution of $KHSO_4$ to pH ~ 2-3. The aqueous phase is extracted with ethyl acetate 15 mL and the extraction repeated the ethyl acetate extracts are pooled washed with water (twice, 30 mL each time), dried over anhydrous Na_2SO_4 and evaporated in vacuo. The residue is recrystallized with a suitable solvent.

Coupling of PEG_{6000} with the first amino acid

PEG_{6000} (7.92 g, 1.58 mmol) was dissolved in 10 mL CH_2Cl_2. Symmetrical anhydride of the N^{α}-*tert*.Boc-amino acid was prepared in a separate vessel by adding (5 mmol) of 1,3-dicyclohexylcarbodiimide (DCC) to (10 mmol) N^{α}-*tert.*Boc-amino acid in CH_2Cl_2. Symmetrical anhydride was filtered upon the PEG_{6000} solution. Then 5 mL pyridine was added to the reaction mixture, the volume was reduced to 30 mL under vacuum and the reaction mixture was stirred for 8-24 hours. The product was precipitated by dropwise addition of dry ether to the concentrated solution under vigorous stirring at $0^{o}C$; the precipitate was filtered off, washed with dry ether. The produced powder was dissolved in least amount of proper solvent (DMF, CH_2Cl_2 or MeOH) and precipitated by dropwise addition of dry ether while cooling several times till chromatographically pure product was obtained. TLC: $R_f = 0$, solvent system:1-Butanol (30), acetic acid (10) water (10).

Removal of the (tert. Boc-group) N^{α}-Protected group

For cleavage of *tert*. Boc-group, (2 mmol) of PEG_{6000} amino acid-Boc was dissolved in (20 ml) TFA/CH_2Cl_2 (1:1) and the solution was stirred for 30 minutes at room temperature, then the reaction mixture was reduced under vacuum, the produced oil was dissolved in CH_2Cl_2 and the product was precipitated by dropwise addition of dry ether while cooling with vigorous stirring till *Kaiser* test showed a positive result. The pure crystals was filtered off and dried under vacuum. TLC: R_f= 0, solvent system: 1-Butanol (30), acetic acid (10) water (10).

Coupling reaction of PEG_{6000} amino acid-H with next tert. Boc-amino acid

(2 mmol) PEG_{6000}- amino acid-H was dissolved in (20 mL) CH_2Cl_2, in another flask (10 mmol) *tert*. Boc-amino acid was dissolved in (10 mL) CH_2Cl_2 and cooled to zero ^oC, (5 mmol) of DCC dissolved in CH_2Cl_2 was added and the reaction mixture was allowed to stand with stirring for 30 minutes at zero ^oC. The precipitate of dicyclohexylurea was removed by filtering the anhydride solution directly into the flask containing the deprotected amino component in CH_2Cl_2. Then the solution was neutralized by N-methyl morpholine till pH=7 and concentrated to about (10 mL) and stirred overnight at room temperature. The product was precipitated by dropwise addition of dry ether while vigorous stirring under cooling, then the product was recrystallized twice till pure crystals were obtained the coupling was controlled by quantitative ninhydrin test. TLC: R_f= 0, solvent system: 1-Butanol (30), acetic acid (10) water (10).The rest peptide sequences were synthesized according to the above steps.

Amino acid analysis

The amino acid composition of peptides was determined by amino acid analysis using a LC3000 Eppendrof with an integrator system 1. Prior to analysis samples were hydrolysed in 6 N HCl in sealed and evacuated tubes at 110 ^oC for 24 hours.

Fourier-transform infrared (FT-IR)spectrometry

Infra-red spectra were recorded on FT-IR Spectrum BX Perkin-Elmer spectrometer. 5 mg of sample was mixed with 100 mg of Potassium bromide (KBr) by trituration. This triturated mixture was filled in dye press and then compressed to prepare a disc. The prepared disc was put in sample holder and scanned from wave no. 4000 to 400 cm^{-1} using spectrum software of Perkin-Elmer spectrometer.

Ultra violet (UV) Spectrometry

Ultra violet spectra were recorded on UV 1601PC Shimadzu spectrophotometer; 5 mg of sample was weighed accurately and dissolved in 10 mL of HPLC grade ethanol. From this stock solution different dilutions were prepared using serial dilution method. UV absorbance was scanned from 190 nm to 600 nm using UV Probe (Version 1.11) software.

High Performance Liquid Chromatography (HPLC)

Purification of crude peptides using HPLC Chromatogram; Sykam S2000, 52110 Injector: Rheodyne 7125. UV Detector: Shimadzu, SPD, GAV. Detection at 220 nm, Integrator: Sykam, C-RGA Chromatopac. Column: Nucleosil 120 C18, 25 cm x 4.6 mm. Solvent: A) 0.1% TFA in H_2O, B) 0.1% TFA in acetonitrile. Gradient: 0-50 % B in 50 minutes.

Biological study

Samples Preparation

All samples were dissolved in dimethylsulfoxide at a concentrations as shown in the **Table 4**, in comparing with different standard antibiotics. Antibiotic discs of Streptomycin (10 µg/mL) and Tetracyciline (30 µg/mL) were used as positive control for bacteria, Neomycin (30 µg/mL) and Nystatin (100 µg/mL) were used for fungi and sterilized paper discs without extracts or antibiotics were used as negative controls for both the bacteria and fungi. The experiment was performed in triplicate.

Antimicrobial activity

The ability to inhibit the growth of Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi were observed using an overlay method [19].

Antimicrobial assay

Strains used

The common pathogenic and food spoilage microorganisms were selected for their relevance in bakery products and other food: the gram-positive bacteria; *Bacillus cereus* and the gram negative bacteria; *Escherichia coli,* yeasts *such as Candida albicans* and fungi *(Asperagillusniger).*

Media used

The bacteria were slanted on nutrient agar (Merck, Darmstadt, Germany), Yeast was slanted and mentioned on Sabaroud's agar medium (Lab M., Bury, Lancashire, UK) and the fungi was slanted and mentioned on the potato Dextrose Agar medium (Lab M Limited, Bury, Lancashire, UK). Mueller-Hinton agar (Lab M., Bury, Lancashire, UK) following the manufacturer's instructions were used for the assay.

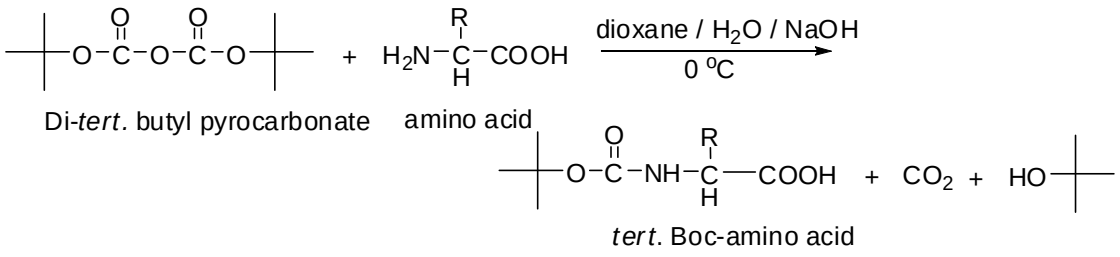
Bioassay

The antibacterial screening was essentially by the disk diffusion, agar method described by Moosdeen [20]. The organisms will be streaked in radial patterns on the agar plates. Plates will be incubated under aerobic conditions at 37°C and 28 °C for 24 hours and 48 hours for bacteria and fungi. In order to obtain comparable results, all prepared solutions were treated under the same conditions under the same incubated plates. All tests were performed for three replicates. Plates were examined for evidence of antimicrobial activities, represented by a zone of inhibition of microorganism's growth around the paper disk and diameters of clear zones were expressed in millimeters [21].

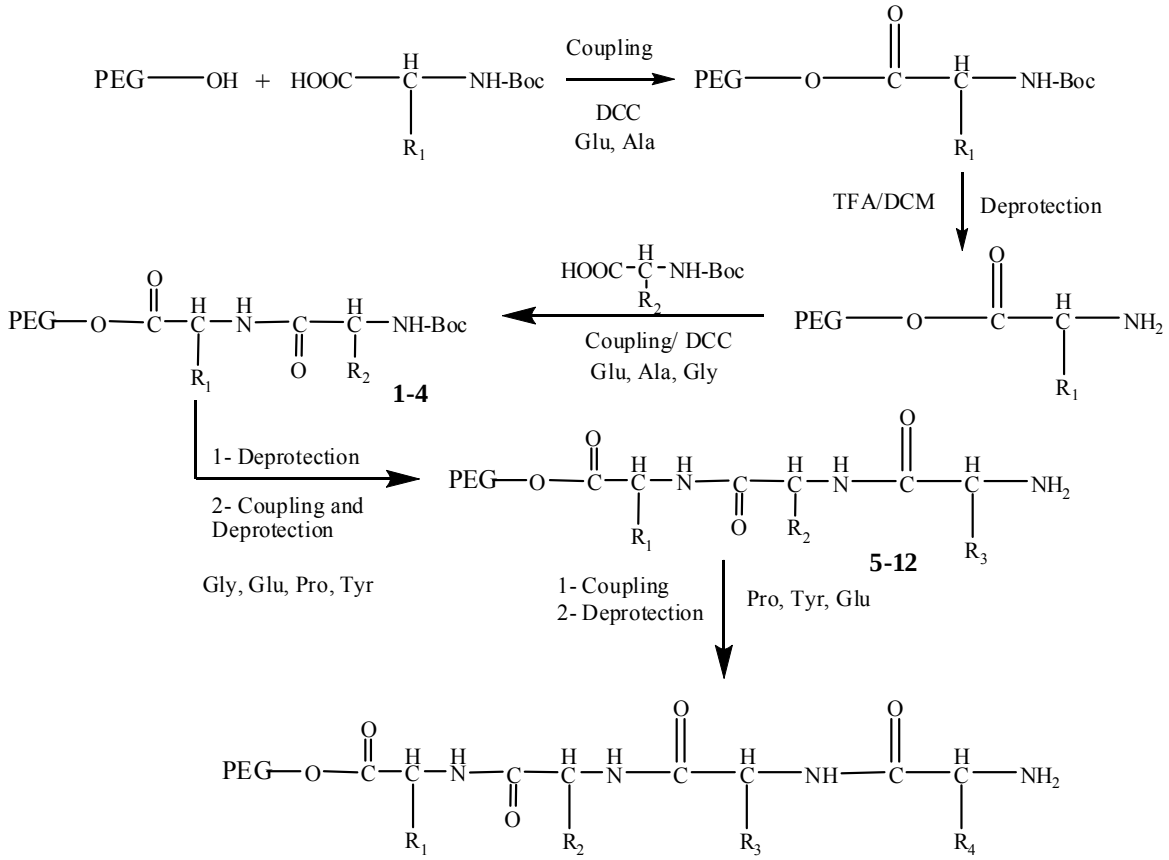
3. Results and Discussion

Chemistry

In an attempt to investigate the polymer bounded peptides which possess antimicrobial activity, the new peptide chains were synthesized using liquid phase method. The peptides are covalently bounded to polyethylene glycol of molecular weight 6000 Daltons. The acid labile *tert*. butyloxycarbonyl (*tert*. Boc) group was employed as N-terminal protecting group, **Scheme 1**. The first amino acid was covalently bounded to PEG; coupling reactions were carried out via symmetrical anhydride separately by the reaction of two moles of (*tert*. Boc-amino acid) with one mole of *N, N*-dicyclohexylcarbodiimide (DCC) and were monitored by ninhydrin and *Kaiser* test [22], **Scheme 2**. Initially, PEG-peptides were purified by ultrafiltration, but it was soon realized that purification by precipitation/crystallization was faster and more efficient; polymer recovery was achieved by simple filtration after addition of greater than 5-fold excess diethyl ether to PEG solutions in methylene chloride.



Scheme 1.Coupling of tert.butyloxycarbonyl group with tert.butylPyrocarbonate [23]



Comp. No.	Peptide chains	Comp. No.	Peptide chains
1	PEG-Glu-Ala-Boc	11	PEG-Ala-Gly-Pro- NH_2
2	PEG-Ala-Gly-Boc	12	PEG-Ala-Gly-Tyr- NH_2
3	PEG-Glu-Ala-NH_2	13	PEG-Glu-Ala-Gly-Pro-Boc
4	PEG-Ala-Gly- NH_2	14	PEG-Glu-Ala-Gly-Tyr-Boc
5	PEG-Glu-Ala-Gly-Boc	15	PEG-Glu-Ala-Glu-Glu-Boc
6	PEG-Glu-Ala-Glu-Boc	16	PEG-Glu-Ala-Glu-Tyr-Boc
7	PEG-Ala-Gly-Pro-Boc	17	PEG-Glu-Ala-Gly-Pro-NH_2
8	PEG-Ala-Gly-Tyr-Boc	18	PEG-Glu-Ala-Gly-Tyr- NH_2
9	PEG-Glu-Ala-Gly-NH_2	19	PEG-Glu-Ala-Glu-Glu- NH_2
10	PEG-Glu-Ala-Glu-NH_2	20	PEG-Glu-Ala-Glu-Tyr- NH_2

Scheme 2. The synthetic routes for PEG-peptides

The N-protecting group was cleaved by dissolving the PEG-peptides in the mixture of TFA/CH_2Cl_2 (1:1) with stirring for half an hour at room temperature. The crude PEG-peptides were obtained by dissolving in distilled water, lyophilized, subjected to HPLC and identified by amino acid analysis and were illustrated in **Table 1**.

Table 1. The amino acid analysis for the synthesized peptide chains

Comp. no.		Amino Acids				
		Glu	Ala	Gly	Pro	Tyr
3	Found	-	2	1.8	-	1.59
	Calculated	2	2	-	-	-
4	Calculated	-	2	2	-	-
	Found	-	1.95	2.1	-	-
7	Calculated	-	2	2	2	-
	Found	-	1.9	1.9	1.8	-
8	Calculated	-	2	2	-	2
	Found	1.9	2.1	-	-	-
9	Calculated	2	2	2	-	-
	Found	1.9	2	2.1	-	-
10	Calculated	4	2	-	-	-
	Found	3.9	1.9	-	-	-
11	Calculated	-	2	2	2	-
	Found	-	1.8	2	1.79	-
12	Calculated	-	2	2	-	2
	Found	-	1.99	2.1	-	1.68
13	Calculated	2	2	2	2	-
	Found	1.9	1.8	1.9	2	-
14	Calculated	2	2	2	-	2
	Found	1.85	1.97	2.1	-	1.77
15	Calculated	6	2	-	-	-
	Found	5.8	1.89	-	-	-
16	Calculated	4	2	-	-	2
	Found	3.8	1.9	-	-	1.59
17	Calculated	2	2	2	2	-
	Found	1.8	1.9	2.1	1.7	-
18	Calculated	2	2	2	-	2
	Found	1.8	2	2.1	-	1.65
19	Calculated	6	2	-	-	-
	Found	5.69	1.8	-	-	-
20	Calculated	4	2	-	-	2
	Found	3.88	1.96	-	-	1.60

From the above table showed the results of most amino acid analysis equal of the theoretical and calculated, and all amino acids that were used in the synthesis of target peptides were existed in the chemical structure.

Spectroscopic studies on the synthesized peptide chains were carried out by IR measurements and the resulted data were summarized in **Table 2**.

Table 2. The absorption bands of the function groups and amino acids in the FT-IR spectra of the prepared peptide chains

Comp. no.	Characteristic absorption bands of the function groups/ (cm^{-1})				Characteristic absorption bands of amino acids/ (cm^{-1})			
	N-H stretching	C=O stretching	C-N, N-H bending	Alkyl C-H stretching	Glu		Ala	Tyr
					CO_2^- asymmetric stretching	CO_2^- symmetric stretching	CH_2 bending	Benzene ring vibration
3	3053	1654	1264	2985	1560	1420	1458	---
4	3052	1654	1265	2984	---	---	1458	---
7	3051	1655	1262	2984	---	---	1459	---
8	3053	1654	1264	2985	---	---	1459	1596
9	3052	1654	1263	2985	1560	1420	---	---
10	3051	1654	1262	2984	1560	1420	1458	---
11	3052	1655	1254	2984	---	---	1458	---
12	3052	1654	1263	2985	---	---	1459	1596
13	3052	1654	1264	2985	1560	1420	1460	---
14	3052	1654	1264	2985	1560	1420	1458	1596
15	3052	1654	1264	2985	1560	1420	1458	---
16	3053	1654	1264	2985	1560	1420	1459	1596
17	3052	1654	1264	2985	1560	1420	1458	---
18	3052	1654	1264	2985	1560	1420	1460	1596
19	3053	1654	1264	2985	1560	1420	1458	---
20	3052	1654	1264	2985	1560	1420	1458	1596

Fourier transform-infrared (FT-IR) spectroscopy is particularly useful for probing the structures of proteins [24, 25]. The characteristic absorption bands of the function groups; N-H (stretching), C=O (stretching), Alkyl C-H (stretching) and C-N, N-H (bending) that illustrated in the above table confirm the chemical structure of the synthesized peptides. Also, the appearance of characteristic absorption bands of Glutamic acid at (1560 cm^{-1} – COO^- asymmetric stretching and 1420 cm^{-1} – COO^- symmetric stretching), Alanine at (1458, 1459 and 1460 cm^{-1} – CH_2 bending) and Tyrosine at (1596 cm^{-1} – benzene ring vibration) prove the structure of the prepared peptides.

UV measurements are applied on all the free PEG-peptides. Ethanol was used as a solvent and all the results are recorded in **Table 3.**

Table 3. UV absorption bands of the PEG-Peptides

Comp. no.	W. L.	Abs.	Comp. no.	W. L.	Abs.
3	230	No beak	13	228	No beak
4	229	No beak	14	231	2.030
7	228	No beak	15	228	No beak
8	231	1.662	16	230	1.183
9	228	No beak	17	228	No beak
10	228	No beak	18	232	2.228
11	229	No beak	19	228	No beak
12	231	2.114	20	230	1.395

From the above table, it was observed that the compounds **3, 4, 7, 9, 10, 11, 13, 15, 17** and **19** showed no observed beaks. On the other hand, the compounds **8, 12, 14, 16, 18** and **20** showed obvious absorbance bands at λ_{max} 232, 231, 230, 230, 231, 231 nm respectively, which

may be attributed to the existence of chromophoric groups in the backbone structure of the prepared peptides and this elucidates the presence of Tyrosine in the peptide sequences.

Biological study

The results of antimicrobial activity for newly synthesized compounds were recorded in **Table 4**, and showed that most of the compounds have a week antibacterial activity effect against *Bacillus subtilits* except the compound no.**11** posses a moderate inhibition effect (I.Z 15.5 mm) in comparing with the antibacterial standard antibiotics used. A Gram-positive bacterium, infects the upper respiratory tract. So, these compounds can be use in the treatment of these pathoigens group. On contrary, there is no inhibition effect was noticed against *Escherichia coli* as example of Gram-negative group.

Table 4. Evaluation of antimicrobial activity for some compounds

Comp. no.	Wt. by mg / mL	Types of micro-organisms							
		Bacteria				Yeast		Fungi	
		G+ve *Bacillus subtilits*		G-ve *Escherichia coli*		Unicellular *Candida albicans*		Filamentous *Asperagillusniger*	
		Inhibition Zone; mm (Compounds concentration is 50 mg/mL)							
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
3	50	13.5	0.70711	--	--	13.5	0.70711	16.5	0.70711
4	50	12	0	--	--	14	1.41421	17.5	0.70711
9	50	13	0	--	--	15.5	0.70711	21.5	0.70711
10	50	13	0	--	--	15.5	0.70711	21	1.41421
11	50	15.5	0.70711	--	--	13.5	0.70711	18.5	0.70711
17	50	15	0	--	--	14	1.41421	22.5	0.70711
18	50	12	0	--	--	14	1.41421	16.5	0.70711
20	50	12	0	--	--	14.5	0.70711	20.5	0.70711
Inhibition Zone; mm (Compounds concentration is ranged from 2 to 10 mg/mL)									
7	5	--	--	--	--	14.5	0.70711	11.5	0.70711
8	5	--	--	--	--	11.5	0.70711	11.5	0.70711
12	7.5	--	--	--	--	12.5	0.70711	11.5	0.70711
13	5	11.5	0.70711	--	--	11.5	0.70711	11	0
14	2.5	11.5	0.70711	--	--	11.5	0.70711	11.5	0.70711
15	5	--	--	--	--	11.5	0.70711	11.5	0.70711
16	10	11.5	0.70711	--	--	11.5	0.70711	11.5	0.70711
19	2	--	--	--	--	11.5	0.70711	11.5	0.70711
Inhibition Zone; mm (Standard bacterial and antifungal antibiotics)									
St.1	S* =10 ug	14	0	12	0	--	--	--	--
St.2	TE* =30 ug	18	0	23.5	2.12132	--	--	--	--
St.3	N*=30 ug	--	--	--	--	16	1.41421	15	0
St.4	NS*=100 ug	--	--	--	--	--	--	15	0

S = Streptomycin; TE = Tetracyciline; N = Neomycin and NS = Nystatin

In case of unicellular fungi most of the compounds showed a moderate antifungal effect against *Candida albicans*. The compounds **3, 4, 7, 9, 10, 11, 17, 18** and **20** have been lowest activity against fungal organisms. In case of filamentous fungi thecompounds **3, 4, 9, 10, 11, 17, 18** and **20** have the strongest antifungal effect against *Aspergillusniger* while the others gave a weak inhibition effect in compared the antifungal standard antibiotic that used in this study. *Aspergillusniger* is one of the fungal pathogens that can affect the respiratory tract. A. niger is a causative agent of pulmonary diseases including aspergillosis, bronchial asthma and acute allergic alveolitis. The fungus

colonizes old tuberculosis or bronchiostatic acavities, in which it forms a large colony (aspergilloma); or it may actually invades the lung tissue to produce haemorrhagic and necrotizing pneumonia [26].

Finally, most of samples have a strongest antifungal activity and a moderate antibacterial activity. The demonstration of activity against both gram-positive bacteria and fungi is an indication that the compounds can be used in the treatment of gram-positive and fungus pathogens, as well as, peptides showing the best antifungal activity suggesting that these peptides can be useful in the treatment of infections caused by strains. This improves the ability of the peptides to kill gram-positive and fungus pathogens, slightly decreasing the activity against Gram-negative bacteria, Therefore, these peptides show promise as the basis for development of novel, broad-spectrum antimicrobial agents.

4. Conclusion

In an attempt to investigate the polymer bounded peptides which possess antimicrobial activity. The peptides are covalently bounded to polyethylene glycol of molecular weight 6000 Daltons. The physicochemical properties of PEG permits its penetration through the cell membrane, so antimicrobial measurements could be carried out on PEG-bounded peptides. The acid labile *tert*. butyloxycarbonyl group (*tert*. Boc) was employed as N-terminal protecting group, the first amino acid was covalently bounded to PEG. The N-protecting group (*tert*. Boc) was cleaved by dissolving the PEG-peptides in the mixture of TFA/CH_2Cl_2 (1:1). The crude PEG-peptides were obtained by dissolving in distilled water, lyophilized, subjected to HPLC and identified by amino acid analysis. Spectroscopic studies on the synthesized peptide chains were carried out by IR and UV measurements. The *in vitro* antimicrobial activity of the synthesized peptides was screened against Gram-positive (*Bacillus subtilits*) and Gram-negative (*Escherichia coli*) bacteria, yeast (*Candida albicans*) and filamentous fungi (*Asperagillusniger*). Most of the samples have a strongest antifungal activity and a moderate antibacterial activity. The demonstration of activity against both gram-positive bacteria and fungi is an indication that the compounds can be used in the treatment of gram-positive and fungal pathogens.

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