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Original Article

The Effect of Synthesized Triazole and Ciprofloxacin Conjugated Peptide Compounds on Biological systems

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Abstract

Breast cancer is the most common cancer among women and only second in terms of cancer-related death in women. Finding new approaches to treat such cancers is critically important anti-cancer peptides (ACPs) offer the possibility of efficient-cancer drugs, and therefore, the development of drug delivery systems using ACPs as a synergism factor is an attractive strategy to address the current drawbacks of cancer therapeutics. This work investigated the cytotoxicity for a series of synthesized compounds based on triazole or ciprofloxacin conjugated peptides against T-47D breast cancerous cells and possible antibacterial effects. Wang resin was used for constructing peptide sequences on a solid support using the method of solid-phase peptide synthesis (spps) with the Fmoc strategy. Cytotoxicity of the synthesized peptide compounds was evaluated by MTT assay. The antimicrobial effect of synthesized peptide compounds was evaluated by agar well diffusion and Broth microdilution method. Most of the peptide compounds showed a cytotoxic effect toward T-47D cells. The antimicrobial effect of the peptide compounds was examined by agar well diffusion test and broth microdilution method. *E. coli* and S. aureus strains have shown the least amount of resistance. In the end, we suggest a new design based on these compounds and modifications to gain better anti-cancer agents.

Keywords: Breast cancer, T-47D, Conjugated peptide anti-cancer drug, Cell-penetrating peptide, Cytotoxicity.

1. Introduction

Breast cancer is the most frequently diagnosed cancer among women accounting for nearly 1 in 3 cancers and the second leading cause of cancer-related death among women after lung cancer. Breast cancer is one of the most common malignancies in Iranian women between ages 40-49. Most of the diagnosed patients were diagnosed at stage 2 with lymph node involvement [1]. In 2008 alone, the International Agency for Research on Cancer (IARC) estimated 12.7 million new cancer cases and 7.6 million cancer deaths occurred,

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and the most commonly diagnosed cancers are lung, breast, and colorectal [2].

Modern drugs are mostly categorized into two groups: chemical compounds and biological drugs. In 1900 German scientist Paul Ehrlich dreamt about a magic bullet that targets specific tumor cells or microbes. After over a century, his dream became a reality [3].

The majority of drugs in the market are chemical drugs. Until the 1970s and early 1980s, all the drugs in the market were chemical, but since then, biological drugs or biosimilar drugs have become more available [4]. Peptide conjugates are gradually finding their ways in pharmacotherapy.

Antimicrobial peptides are essential for an alternative pathway to cure an infection. However, there is a new way to use these peptides with or without the anti-cancer drugs as a strategy to fight cancer [5].

Cell-penetrating peptides (CPP) are considered an efficient strategy to deliver anti-cancer drugs or bioactive molecules to target cells. There are various ways to deliver anti-cancer therapeutics via CPPs [6-8]. Peptide conjugated drugs by enhancing tumor cells' sensitivity, increase the immune system's chance to respond, and eliminate the risk factor. Peptides are a good choice for this goal they have low molecular weight and they can target the tumor cells. Yet, they have a less toxic effect on healthy tissues [9].

Cell-penetrating peptides (CPPs) are short peptides comprised of between 5 to 30 amino acids, mostly basic amphipathic amino acid containing cationic and hydrophobic residues [10-13]. CPPs are efficient in delivering drugs into the cells by crossing over the lipid bilayer [14]. Their mechanism of action is yet to be fully determined [6, 11, 15]. These peptides are aimed to be cell-specific. They show a higher affinity to the specific receptors that are over-expressed in the cell membrane [15].

Langer et al. used Neuropeptide Y (NPY) as a model peptide and modified it by replacing the Glu-15 with a Cys to be able to attach daunorubicin. Cytotoxicity tests showed NPY-Dauno-HYD was as potent as free daunorubicin [16]. Nagy et al. attached doxorubicin and 2-pyrrolino-DOX to peptide fragments, where they tested conjugates on CFPAC-1 human pancreatic cancer, DMS-53 human SCLC, PC-3 human prostate cancer, and MKN-45 human gastric cancer cell lines. The IC50 of analog-DOX conjugates were lower than pure doxorubicin on these cell lines [17]. Dharap et al. used PEG as a linker to connect the luteinizing hormone-releasing hormone (LHRH) and camptothecin (CPT). The MTT test was performed on A2780 ovarian carcinoma cells. The results showed CPT conjugated PEG increased toxicity by more than 12 times the original [18]. Veronese et al. synthesized PEG-Peptide-DOX conjugates and tested them against B16F10 cells; the results showed 10-100 fold less toxicity than free DOX. The peptidyl linker (GFLG linker) displayed the greatest cytotoxicity [19]. Fu-Qiang et al. synthesized doxorubicin conjugated stearic acid-g-chitosan oligosaccharide polymeric micelles (DOX–CSO–SA) and, studied the antitumor activity of DOX–CSO–SA by in vitro release profile of doxorubicin from micelles in PBSAnti-cancer tests of MCF-7 (human breast carcinoma cell

line) and MCF-7/Adr (multi-drug resistant variant) showed that the MCF-7/Adr were doxorubicin-resistant and DOX–CSO–SA could reverse the drug resistance of MCF-7/Adr cells [20]. Kaha´ n et al. synthesized new LHRH analogs by consisting of one molecule of 2-pyrrolino-DOX-14-O-hemiglutarate coupled to the e-amino group of LH-RH. The results showed that these analogs were powerful antitumor agents, and receptor targeting chemotherapy may increase the concentration of cytotoxic agents in the tumor area and improve the treatment outcome [21]. Mazel et al. linked doxorubicin to peptide vectors such as pegelin and penetrating peptides. These peptides can cross the cell membranes. In their study, they used multidrug-resistance K562 cells. There was a similar result in both sensitive and resistant cells for conjugates, but the cytotoxic effects of doxorubicin conjugates in sensitive k562 cells were less than that of free doxorubicin. This result shows a different pathway for vector conjugates' uptake in these cells [22]. Liang et al. linked doxorubicin to a well-known cell-penetrating peptide, TAT, then the conjugate and free doxorubicin were tested on sensitive and drug-resistant MCF-7 cells. Doxorubicin-TAT showed a greater cytotoxic effect in drug-resistant cells [23, 24]. The work of DeFeo-Jones et al. introduces PSA (Prostate-specific Antigen) conjugated vinblastine as a new antitumor agent for the treatment of hormone-refractory prostate cancer in men [25].

Most of the antifungal drugs have azol rings in their structure imidazole compounds contain 2 nitrogen atoms in their azol ring and Triazole compounds contain 3 nitrogen atoms in their azol ring. Triazole antifungal drugs show less adverse effects in the body and in-vitro studies and have a broader spectrum of applications compare to imidazole drugs and amphotericin B. Triazole antifungals contain two generations, first generation triazole antifungal drugs are itraconazole and fluconazole. The second generation came to market about a decade later and contain voriconazole, posaconazole, albaconazole, ravuconazole, isavuconazole, and efinaconazole [26].

The 1,2,3-triazoles specifically are important heterocyclic compounds and showed anti- HIV [27], antimicrobial [28, 29], antiprotozoal, anticancer activities and more [30, 31].

Ciprofloxacin is structurally related to nalidixic acid and the second generation of fluoroquinolone. Ciprofloxacin inhibits bacterial DNA gyrase. It is a broad-spectrum antibacterial drug especially in Gram-negative bacteria [32].

Ciprofloxacin derivatives showed broad range of activities such as anti-HIV [33], anti-tumor [34, 35], anti-malarial [36] and etc [37].

In our previous works, Baharloui et al. and Esfandiari et al. have reported the synthesis of triazole-based and ciprofloxacin-based peptides that were designed and predicted by E-Kobon et al. [38] evaluated in colon and skin cancer cells [39, 40] respectively. In this article, we discuss their cytotoxicity toward the breast cancer cells as well as their antimicrobial effects.

2. Materials and Methods

2.1. Peptide synthesis

The peptides were synthesized on a solid-phase method using Wang resin. The resin (0.5 g, 1.0-2.5mmol/g substitution) was swollen in a reactor (fitted at the bottom with a fritted glass filter) by the solvent mixture dimethylformamide (DMF) /dichloromethane (DCM) (1: 9, 10 mL) for one h and then the solvent was drained. The first amino acid (2.0 eq), Hydroxybenzotriazole (HOBt) (2.0 eq), and 4- dimethyl amino pyridine (DMAP) (0.1 eq) in 5 mL DMF were added to the reactor. Diisopropylcarbodiimide (DIC, 1.0 eq) was then added to the reaction vessel, and the reactor was shaken for three hours at room temperature. After three hours, the mixture was added piperidine/acetic anhydride (2.0 eq: 2.0 eq) and the reaction was stirred for 30 min at room temperature. Following the removal of the solvent by filtration, the resin was washed with DMF (3 × 5 mL), DCM (3 × 5 mL), and methanol (3 × 5 mL). Removing the Fmoc protecting group of the amino acid attached to resin was performed by treating resin with a solution of piperazine/ DMF (10%) for 20 min. The solution was then drained, and the resin was washed with DMF (2× 5 mL). The second amino acid was used with HOBt and DIC (without DMAP) for attaching to the first amino acid bound to the resin. This was followed by washing the resin with DMF and DCM. Deprotection was also performed by the N-terminal Fmoc removal of the newly formed peptide bound to the resin. Other amino acids were used for bonding to the above peptide linked with resin followed by deprotection, accordingly [39, 40].

2.2. Cell culture

2.2.1. Cell line preparation

T-47D (ATCC HTB-133) cells were grown in RPMI 1640 medium supplemented with 10% (v/v) FBS (Fetal Bovine Serum, Gibco, USA) and 1% penicillin/streptomycin (10,000 units of penicillin, 10,000µg of streptomycin 100x, Gibco, USA) at 37º c in humidified incubator containing 5% CO_2. Cells were sub-cultured every two days using trypsin.

2.2.2. The cytotoxic assay

The MTT assay was used for the in-vitro cytotoxicity evaluation study. Cells (briefly 6×10^4 cells/well) were transferred into 96 well tissue culture plates and incubated for 24 hours. Cells were treated with pure peptides (0.1, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 250, and 1000 nM) and triazole or ciprofloxacin conjugated peptides (0.1, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 250, and 1000 nM) and Cisplatin (100, 50, 25, 12/5, 6/25 and 3/125 µM) as a control then incubated for 24 hours. After 24 hours of incubation, 10 µlit MTT reagent with the final concentration of 0.5 mg/mL was added into each well. The plates were incubated again for 3-4 hours at $37^{\circ C}$ to form formazan crystal (covered with aluminum foil). Each well was washed, and then 200 µlit of Dimethyl Sulfoxide (DMSO) was added to each well to dissolve the MTT formazan crystals. The plates were shaken for 30 minutes. ELISA reader measured the absorbance from each well at 570

and 630 nm wavelengths. All the experiments were performed in triplicate [41].

2.3. Antimicrobial tests

2.3.1. Microorganisms

The microorganisms used included: *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 53338), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella typhimurium* (ATCC 14028), *Micrococcus luteus* (ATCC 9341), and *Bacillus cereus* (PTCC 1247).

2.3.2. *Agar well diffusion test*

The activity of the synthesized peptides, triazole conjugated peptides, or ciprofloxacin conjugated peptides were tested against microorganisms that were mentioned above by agar well diffusion method. DMSO (10% NaCl) was used as a negative control, and ciprofloxacin was used as a positive control. McFarland turbidity standard 0.5 were prepared in NaCl and were plated thoroughly onto Mueller–Hinton agar in three directions with a sterile swap. 7 mm diameter wells were punched in plates (6 wells per plate). The wells were filled with 20 µl peptides or triazole conjugated peptides or ciprofloxacin conjugated peptides (containing 1, 0.5, 0.25, 0.125, or 0.062 mg/ml per well). The plates were incubated microaerophilically at 37ºC for 24 hours. The diameters of the inhibitory zones were measured in millimeters [42].

2.3.3. Broth microdilution method

10 µL of each bacterial suspension in Mueller–Hinton broth (1:19 diluted 0.5 McFarland standard) was added to the wells of a sterile 96-well microtitre plate already containing 100 µL of serially diluted peptides or triazole and ciprofloxacin conjugated peptides in DMSO (10% MHB). The final volume in each well was 110 µL. Positive control wells were prepared with bacterial suspension only, and negative control wells were prepared with the only peptide contains MHBs. The 96-well microtitre plates were incubated in 37ºC for 24h. The MIC was the lowest concentration, where no viability was observed after 24 hours. All the experiments were performed in triplicate [42].

3. Results and Discussion

3.1. The MTT assay

Antitumor activities of pure peptides, triazole conjugated peptides, or ciprofloxacin conjugated peptides were evaluated with MTT assay. All stock peptide solutions contain 10% DMSO and 90% RPMI as the solvent. Cisplatin was used as a control (IC_{50} =54.89 ± 2.47 µg/ml). The 50% cellular growth inhibitions (IC_{50}) of the peptide solutions with different peptide concentrations against T-47D were determined and presented in Figure 1 and Table 1.

The antitumor activity of 9 new peptide sequences, nine peptide-triazole conjugates, and nine peptide-ciprofloxacin conjugates has been evaluated in breast cancer cells (Table 1) in this research. Cellular uptake of cationic cell-penetrating peptides is described as a process that does not involve endocytosis [43]. The cationic anticancer peptides can bind to the negatively-charged membrane of the cancer cells and disrupt the membrane stability and

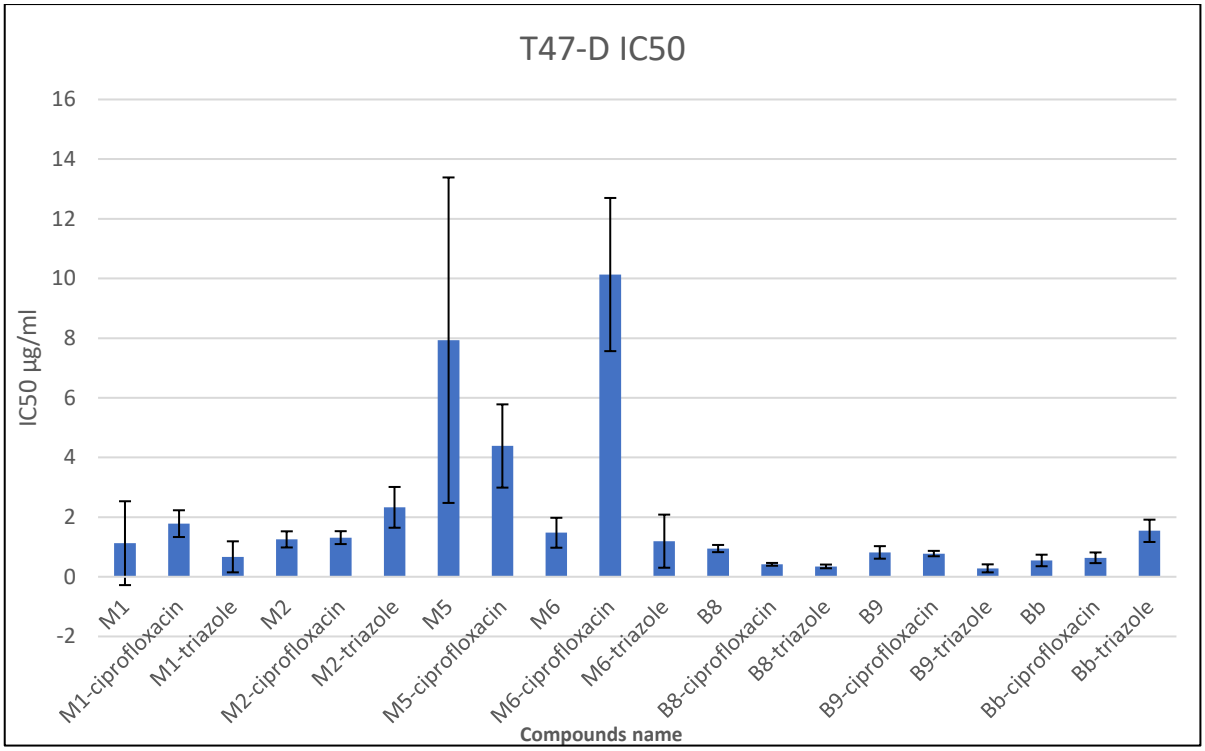

Figure 1. Synthetized peptides IC_{50} (µg/ml) comparison against T47-D cells (IC_{50}±SD).

fluidity, or barrel-stave model, or increase of calcium ion influx, leading to cell death. These peptides may inhibit cancer cell growth by one of the following paths: modification of lysosomal membrane, enhancement of proteasome activity, induction of mitochondrial pathway of apoptosis by the caspase cascade, activation of the immune-modulatory pathway, and inhibition of DNA replication-relating genes interfering the cell cycle [38].

Most of the peptides showed cytotoxic effects against T47-D. The results showed that the peptide-ciprofloxacin conjugates had lower IC50 compared to the triazole conjugated peptides. Ciprofloxacin alone showed no cytotoxic effect toward T47-D cells (data not shown) that can be justified using the logic that ciprofloxacin has higher lipophilicity compared with pure peptides. Peptides and conjugated peptides all showed lower cytotoxicity compare to cisplatin in T47-D cells [44]. According to E-Kobon et al. studies A. fulica mucus fractions significantly induced breast cancer cell death. They predict 16 different small cationic amphipathic peptides as potential anticancer peptides [38].

3.2. *Agar well diffusion*

Antimicrobial activities of pure peptides, triazole conjugated peptides, or ciprofloxacin conjugated peptides were evaluated by the agar well diffusion method. All stock peptide solutions contained 10% DMSO and 90% NaCl. The diameters of the inhibitory zones were measured in millimeters. All the experiments were performed in triplicate, as shown in Table 2.

Table 1: Cytotoxicity of synthetized peptides against T47-D cells.

Peptide name	Peptide sequence	Molecular weight(g/mol)	IC50 T47-D (µg/ml) n=3(Mean±SD)
M1	GGAMIMK	706	1.126776+2.431
M1- ciprofloxacin	GGAMIMK-C	1019.7	1.7814159+0.776
M1-triazole	GGAMIMK-T	953	0.6688154+0.9
M2	VVPVCTK	743	1.253441+0.468
M2- ciprofloxacin	VVPVCTK-C	1056	1.312608+0.374
M2- triazole	VVPVCTK-T	990	2.32749+1.183
M5	GYAAGNK	678	11.836524+26.344
M5- ciprofloxacin	GYAAGNK-C	992.8	4.38438+2.414
M6	GYAAGIK	677	1.362801+6.462
M6- ciprofloxacin	GYAAGIK-C	991	1.574699+13.59
M6- triazole	GYAAGIK-T	923	1.193439+1.54
B8	HANGGVLK	793.2	0.9462876+0.209
B8- ciprofloxacin	HANGGVLK-C	1106.2	0.41725864+0.081
B8- triazole	HANGGVLK-T	1040.2	0.34753082+0.107
B9	KLERAAGSK	957.3	0.8165769+0.363
B9- ciprofloxacin	KLERAAGSK-C	1270.3	0.77793172+0.156
B9- triazole	KLERAAGSK-T	1204.3	0.28264921+0.233
Bb	NAIATTTQK	945.3	0.5473287+0.335
Bb- ciprofloxacin	NAIATTTQK-C	1258.3	0.63695146+0.31
Bb- triazole	NAIATTTQK-T	1192.3	1.5404516+0.647

3.3. *Broth microdilution method*

Using agar well diffusion, the antimicrobial effect of peptides was evaluated. Furthermore, the MIC values of peptides or conjugated peptides that had inhibition growth zones were determined in the previous tests, as shown in Table 3.

Peptides penetrate bacteria more easily than they do mammalian cells and cationic peptides such as prolin-rich antibacterial peptides have both intracellular and membrane

Table 3: Antimicrobial activity of synthesized peptide compounds by Broth microdilution method.

Peptides name	***S. aureus***	***S. enterica***	***M. luteus***	***P. aeruginosa***	***B. cereus***	***E. coli***
M1- ciprofloxacin	0.0625	0.03125	<0.0078	0.5	0.125	0.0156
M2- ciprofloxacin	0.125	0.03125	>4	0.25	0.125	0.007813
M5- ciprofloxacin	0.0625	0.003906	1	0.125	<0.03125	0.000977
M6- ciprofloxacin	0.5	0.0625	>4	1	0.5	0.03125
Bk- ciprofloxacin	0.25	0.0625	>4	1	0.25	0.03125
B9- ciprofloxacin	0.25	0.015625	>4	0.5	0.125	0.0156
B8- ciprofloxacin	0.5	0.125	<0.001953	4	0.5	0.125
Bb- ciprofloxacin	0.0625	0.125	1	0.125	0.0625	0.03125
M1-triazole	0.0625	0.001953	>4	0.125	0.125	0.001953
M2- triazole	>2	0.0625	>4	>4	>2	0.0156
M5- triazole	>2	>0.25	>2	>2	>2	>0.25
M5	1	0.125	>4	4	1	0.0625
M6	>4	1	2	2	2	0.5
Ciprofloxacin	<0.000122	<0.000122	0.000244	0.000122	0.000122	<0.000122

activities. Bower et al. present data to support it [45]. Sadler et al. tested synthetic fragments of Bac 7 for antimicrobial activity and cell permeability functions. Peptide fragments with high cationic amino acids like proline showed better cell-permeant ability. Even a fragment with 10 amino acids containing two arginine, was capable of delivering NeutrAvidin protein into cells [46]. Cho et al. synthesized a novel b-sheet peptide-resin conjugate as a novel antimicrobial agent. One of the conjugates (conjugate 1) showed a promising effect as potent synergism with vancomycin [47]. The antimicrobial effects of peptides and peptide conjugates were analyzed in vitro against six bacterial species. Most peptides studied in this work showed antimicrobial activity against some of the test microorganisms. *E. coli* as a gram-negative strains and *S. typhimurium* as a gram-positive strain have shown the least amount of resistance, respectively. All peptides showed less antimicrobial effect compared to ciprofloxacin alone. In total, gram-positive strains showed less resistance for these compounds compares to the gram-negative strains. Peptides M5 and M6 showed potential antimicrobial effects in *E. coli* and *S. typhimurium*. we suggest that these two agents be examined for the synergism with other current antibacterial.

In general, the peptides with histidine in their sequence showed a greater cytotoxic effect than other ones. In Rana et al. studies, they showed amphiphilic peptides favored helical-type secondary structures, and well defined cationic nanoparticle formulations were more potent toward lung cancer cells [48]. Karpinski et al. examined peptides from bacteria to discover new anti-cancer peptides. They showed that some peptides with low molecular weight and hydrophobicity have cytotoxic effects on cancer cells, and such a characterization is important for other bacterial peptides discovery [49].

4. Conclusion

The present study is aimed to use this approach by synthesizing peptides containing a triazole ring moiety to further explore the anticancer activity of such combination. The peptides previously detected in different fractions as inhibitors of human breast cancer cell [38], were synthesized on solid phase method using Wang resin. The present study is aimed to use this approach by synthesizing peptides containing a triazole ring or Ciprofloxacin moiety to further explore the anticancer activity of such combinations.

In conclusion, the new design peptides presented potentially promising antimicrobial and anti-tumor effects at the cellular level. Preparation of the triazole and ciprofloxacin derivatives for each of them has provided good means of comparison for each peptide. We have synthesized and tested 9 of 16 predicted peptides. Based on this study, our result has shown that a single peptide may not solely be effective enough to inhibit cancerous cell growth, and the combinatorial effects of anticancer peptides should be more effective and worth to be further examined. Although some investigators might be in favor of using a combination of normal and cancerous cells to not only evaluate the anticancer effects, but also adverse effects of any under-examined new compound, but we did not feel to perform these experiments at this preliminary stage. Using of normal cells as control is

a beneficial critical step in introducing new anticancer drugs, but is remained for future studies once we have discovered the best agents and their mechanism of actions on the desired cell lines, to be selected from the cells of the same tissue that are sensitive to those cellular cascades. Although this is a basic preliminary work, enough hints are extracted to improve more peptide complex structures for better antibacterial or antitumor activities to be considered for potential clinical.

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Conflict of interest

The authors declare to have no conflict of interest.

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Table 2: Cytotoxicity of synthetized peptides against T47-D cells.

Peptide name	microorganism	Concentration					
		1(mg/ml)	0.5(mg/ml)	0.25(mg/ml)	0.125(mg/ml)	0.062(mg/ml)	0.031(mg/ml)
Bb- ciprofloxacin	*E. coli*	40	30	28	25	20	18
	P. aeruginosa	20	15	12	–	–	–
	S. enterica	34	30	27	25	20	15
	B. cereus	16	13	–	–	–	–
B9- ciprofloxacin	*S. enterica*	28	24	21	16	11	-
	E. coli	30	25	21	16	10	-
Bk- ciprofloxacin	*S. enterica*	22	19	16	-	-	-
B8- ciprofloxacin	*E. coli*	15	13	-	-	-	-
	S. enterica	16	11	-	-	-	-
M1- ciprofloxacin	*E. coli*	27	22	18	-	-	-
	S. enterica	23	21	17	11	-	-
M2- ciprofloxacin	*E. coli*	30	27	24	11	-	-
	S. enterica	28	23	20	13	-	-
M5- ciprofloxacin	*P. aeruginosa*	19	14	-	-	-	-
	S. aureus	22	20	-	-	-	-
	S. enterica	32	28	25	21	16	15
	E. coli	38	32	26	24	19	15
	B.cereus	14	13.5	-	-	-	-
M6- ciprofloxacin	*E. coli*	30	24	20	18	-	-
	S. enterica	25	21	18	14	-	-
M1-triazole	*P. aeruginosa*	17	9	-	-	-	-
	S. enterica	30	27	23	22	15	11
	B. cereus	11	-	-	-	-	-
	E. coli	34	30	27	26	21	12
M2- triazole	*E. coli*	27	24	16	-	-	-
	S. enterica	25	19	16	-	-	-
M5- triazole	*E. coli*	28	25	18	12	-	-
	S. enterica	25	22	18	13	-	-
M5	*E. coli*	25	18	14	-	-	-
	S. enterica	22	18	15	11	-	-
M6	*E. coli*	27	24	21	14	-	-
	S. enterica	26	23	19	14	-	-
Ciprofloxacin	*B. cereus*	68	68	60	58	52	48
	M. luteus	-	88	80	64	56	44