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Full Length Research Paper

Design, synthesis and *in vitro* cytotoxic activity of Delavayin A: A cyclic hexapeptide

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Synthesis of new drug for anticancer activity has always been fascinating and interesting for researchers. Increase in the demand for new anticancer agents promotes the researchers for synthesizing new molecules with good activity on human cancer cell lines. Here design, synthesis and cytotoxic, antimicrobial activity of delavayin A is described. The structure of this synthesized compound was confirmed by (IR), 1H NMR, 13C NMR, Mass and elemental analysis. The synthesized compound was evaluated for *in vitro* cytotoxic activity by using Brine shrimp assay and on PC 3 and HL-60 cancer cell lines at Advanced Centre for Treatment Research and Education in Cancer (ACTREC), Mumbai. The compound was also evaluated for antimicrobial activity by using minimum inhibitory concentration (MIC) technique. The synthesized cyclopeptide possessed moderate cytotoxic activity against cell lines and found to show moderate activity against Gram positive bacteria.

Key words: Cyclopeptide, cytotoxic, antimicrobial, *p*-nitro phenyl ester method.

INTRODUCTION

Development of new anticancer drugs from natural sources is fascinating and interesting for researchers working in the field of medicinal chemistry and drug development, as increase of resistance by cancer cells towards current anticancer drugs is a major issue. In recent years, synthesis of naturally occurring cyclic peptides as anticancer agents has attracted much attention of the researchers owing to their wide range of pharmacological activities shown by peptide molecules, which includes antimicrobial, anthelmintic, insecticidal, cytotoxic, anti-inflammatory activities (Chaudhary et al., 2012; Dahiya et al., 2010; 2011; Fernandez et al., 1992; Shinde et al., 2008; 2010). Keeping in view the significant biological activities exhibited by various cyclic peptides,

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as a part of ongoing study, an attempt was made towards the synthesis of a phenyl alanine rich cyclic hexapeptide, delavayin-A, cyclo (Gly-Ser-γ-hydroxy Ile-Phe-Phe-Ala-), which was isolated from the roots of *Stellaria delavayi* and belongs the family Cariophyllacea (Morta et al., 1997).

The synthesis was carried out by using solution phase technique and the synthesized compound then subjected to cytotoxic screening against PC-3 and HL-60 human tumor cell lines. The synthesized molecule was also evaluated for antimicrobial activity. The synthesized cyclopeptide possessed moderate cytotoxic activity when tested against PC 3 and HL-60 cancer cell lines comparable with the standard drug adriamycin. The compound had also shown prominent activity against Gram positive bacteria's and moderate activity against pathogenic fungi in comparison with benzyl penicillin and fluconazole, as standard.

MATERIALS AND METHODS

General

All L-amino acids, di-*tert* butyldicarbonate (Boc_2O), diisopropylcarbodiimide (DIPC), trifluoroacetic acid (TFA), triethylamine (TEA), pyridine and *N*-methylmorpholine (NMM) were procured from Spectrochem Limited (Mumbai, India). Melting points of all intermediates and final compound were determined by using digital melting point apparatus, make SYSTRONIC. The IR spectra were recorded by using KBr pellets or utilizing chloroform and NaCl cells on JASCO 4100 FTIR spectrophotometer. 1H NMR and 13C NMR spectra were recorded on Bruker AC NMR spectrometer using dimethyl sulphoxide (DMSO) as a solvent. The mass spectrum of the cyclopeptide was recorded at 70 eV on JMS-DX 303 Mass spectrometer.

In order to carry out the total synthesis of cyclopeptide, cyclo(Gly-Ser-γ-hydroxy Ile-Phe-Phe-Ala-) 12, it was disconnected into three dipeptide units, Boc-Gly-Ser-OMe 7, Boc- γ-hydroxy Ile-Phe -OMe 8, Boc-Phe-Ala-OMe 9 (Scheme 1). The required dipeptides were synthesized by coupling Boc amino acids 1,3 and 5 with respective amino acid methyl ester hydrochlorides 2,4 and 6 using DIPC, $CHCl_3$ and N-methyl morpholine according to Bondanszky procedure. The Boc-group of the dipeptide 8 was removed by using trifluoroacetic acid (TFA), 10% $NaHCO_3$ and the ester group of dipeptide 7 was removed by using LiOH. The deprotected units were then coupled to get a tetrapeptide Boc- Gly-Ser-γ-hydroxy Ile-Phe OMe 10. The hydroxyl group was introduced into isoleucine by using standard procedure (Pleissner and Wimmer, 2011) (Appendix 1). Similarly, the dipeptide 9 was coupled with tetrapeptide 10 after appropriate deprotection to get a linear hexapeptide 11 by using DIPC, N-methyl morpholine and chloroform. Finally by using p-nitrophenyl ester method (Bodanszky and Bodanszky, 1984) cyclisation of linear hexapeptide was carried out to get the titled compound 12. All intermediates and final product were recrystallized from $CHCl_3$.

The IR spectrum showed presence of -CO-NH- moiety in the cyclized product. The NMR spectrum of cyclized product clearly indicates the presence of all respective amino acid moieties. Furthermore, the mass spectrum of this cyclic hexapeptide showed a molecular ion peak at m/z 640.3. The synthesized cyclic hexapeptide was screened for cytotoxic activity by using Brine shrimp assay and also against human tumor cell lines by comparing with standard adriamycin at ACTREC Mumbai. The synthesized compound was also evaluated for antimicrobial activity by using tube dilution technique against four bacterial species and two fungal strains. Benzyl penicillin and fluconazole were used as standards for antibacterial and antifungal activity, respectively.

General method for preparation of Di/Tetra/linear hexapeptide

L-Amino acid methyl ester hydrochloride/dipeptide methyl ester/tetra peptide methyl ester (10 mmol) was added to chloroform (CCl_3, 20 ml). To the resulting solution, at 0°C, TEA (2.8 ml, 20 mmol) was added and the reaction mixture was allow to stir for 15 min. Boc-L-amino acid/Boc dipeptide/Boc tetrapeptide (10 mmol) in chloroform (20 ml) and DIPC (10 mmol) were added while stirring. The reaction mixture was allowed to stir for 24 h, filtered and the residue was washed with chloroform (30 ml) and added to the filtrate. Washing of the filtrate was done by using 5% $NaHCO_3$ and saturated NaCl solutions and the resultant organic layer was dried out by anhydrous Na_2SO_4, filtered and evaporated in vacuum. Recrystallization of the crude product was done from a mixture of chloroform and petroleum ether (b. p. 40 to 60°C). By using aforementioned procedure, compounds 1 to 7 were synthesized.

Method for cyclization of linear hexapeptide

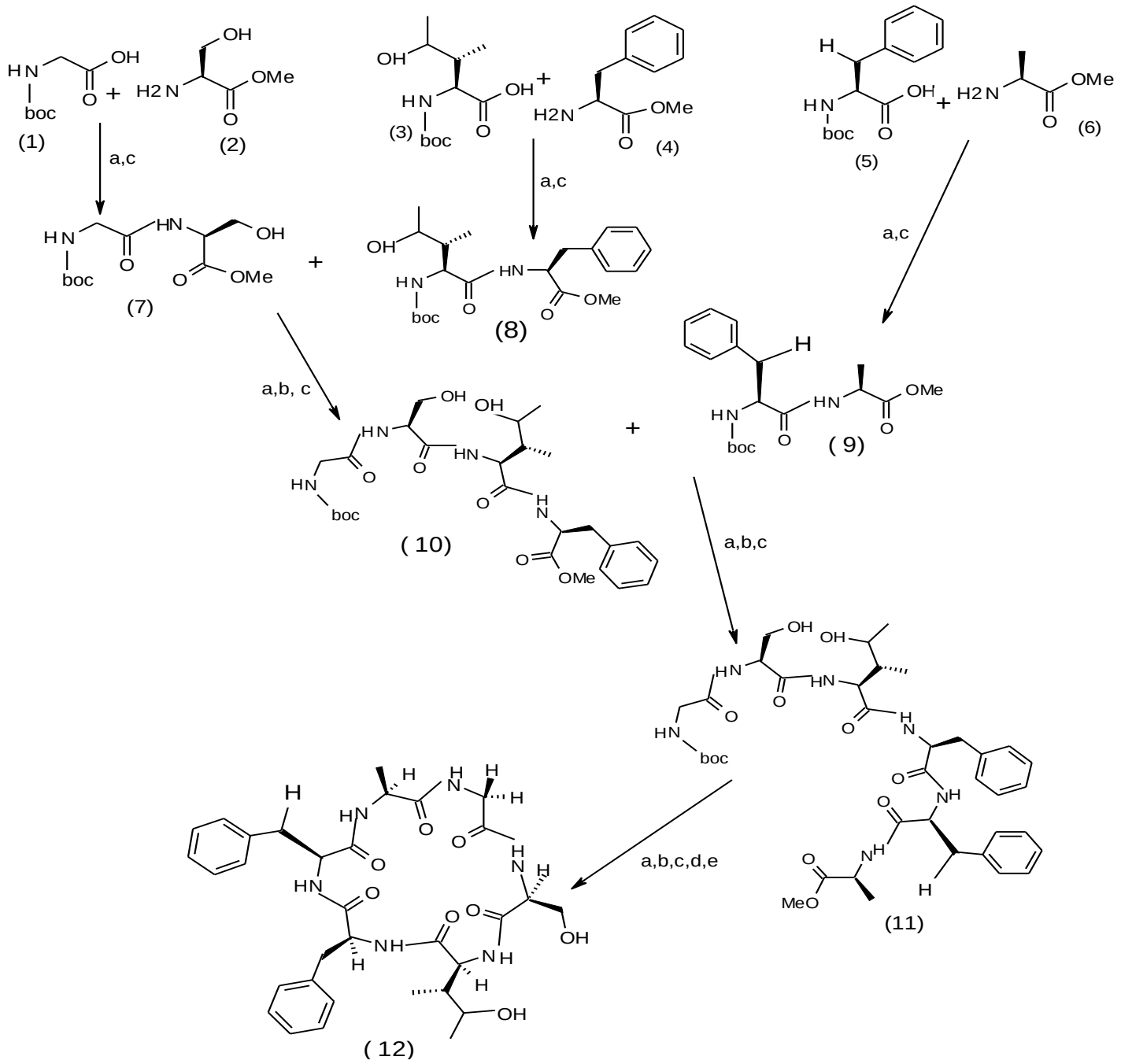
The cyclization of linear octapeptide was done by using p-nitrophenyl ester method (Bodanszky, 1984). The ester group of linear fragment was detached by using LiOH and the p-nitrophenyl ester group was introduced. In order to introduce p-nitrophenyl ester group, the Boc-peptide carboxylic acid (1.5 mmol) was added to chloroform (15 ml) at 0°C, to which p-nitrophenol (0.27 g, 2 mmol) was added, and allowed to stir for 12 h at RT. The reaction mixture was filtered and filtrate was washed by using $NaHCO_3$ solution (10%) until excess of p-nitrophenol was removed. The filtrate was finally washed with 5% HCl (5 ml) to obtain Boc-peptide-pnp ester. To the already mentioned Boc-peptide-pnp-ester (1.2 mmol) in $CHCl_3$ (15 ml), CF_3COOH (0.274 g, 2.4 mmol) was added and allowed to stir for 1 h at room temperature. The reaction mixture was washed with 10% $NaHCO_3$ and the organic layer was dried over anhydrous Na_2SO_4. To the Boc-deprotected peptide-pnp-ester in $CHCl_3$ (15 ml), N-methyl morpholine (1.4 ml, 2 mmol) was added and kept at 0°C for 7 days. The reaction mixture was washed with 10% $NaHCO_3$ till the byproduct p-nitrophenol was removed completely.

Cytotoxic activity

Preliminary cytotoxic activity by brine shrimp lethality assay (BSLA)

Brine shrimp eggs were obtained from the aquarium shop, Nashik. Artificial sea water was prepared from (1% NaCl) prepared by using nitrate, phosphate, and silicate-free sea-salt and distilled water (35 g/L) at 25°C under constant illumination. The salt water solution was aerated continuously during incubation with an aquarium air pump.

The seawater was placed in a small plastic container (hatching chamber) with a partition for dark (covered) and light areas. Shrimp eggs were added into the dark side of the chamber while the lamp above the other side (light) will attract the hatched shrimp. Two days were allowed for the shrimp to hatch and mature as nauplii



Scheme 1. Synthetic route for Delavayin A. Where: a = DIPC, NMM, CHCl3, RT, 24h, b = TFA, NMM, RT, 1 h, C = LiOH, THF:H_2O(1:1), reflux, 15 min, d = pnp-, CHCl3, RT, 12 h, e = NMM, $CHCl_3$, 0°C, 7 days.

(larva). After two days, when the shrimp larvae are ready, 4 ml of the artificial seawater was added to each test tube containing different concentration of drug and 10 brine shrimps were introduced into each tube.

Thus, there were a total of 30 shrimps per dilution. Then the volume was adjusted with artificial seawater up to 5 ml per test tube. The test tubes were left uncovered under the lamp. The number of surviving shrimps were counted and recorded after 24 h. Using probit analysis, the lethality concentration (LC_{50}) was assessed at 95% confidence intervals. The percentage mortality (%M) was also calculated by dividing the number of dead nauplii by the total number, and then multiplied by 100%. This is to ensure that the death (mortality) of the nauplii is attributed to the activity of the compound (Bussmann et al., 2011; Tagne et al., 2014). The

Table 1. Results for Cytotoxic activity by using brine shrimp assay.

Compound	Conc. (ppm or µg/ml)	Number of surviving Nauplii after 24 h			Total number of survivors	% Mortality
		T1	T2	T3		
Dela A	1000	4	4	5	13	56.66
	500	5	4	5	14	53.33
	250	6	6	7	19	36.66
	125	7	6	7	20	33.33
	62.5	7	8	8	23	23.33
	31.25	8	9	9	26	13.33
	0	10	10	10	30	0

*T=Trial, LC_{50}=460.9 µg/ml.

Table 2. Data of cytotoxic activity against human tumor cell lines.

Compound	% Mortality at various concentration						LC 50 (µg/ml)
Conc.(µg/ml)	25	50	100	150	200	250	
Dela A	16.66	30	43.33	63.33	80	90	117.5

results of activity are shown in Table 1.

In vitro cytotoxic activity against Human tumor cell lines

The cytotoxic activity against human tumor cell lines PC3 and HL 60 was carried out at ACTREC Mumbai, by following standard protocol (www.actrec.gov.in). The activity was carried out by sulphorhodamine B assay (Vichai, 2006; Skehn, 1990). The cell lines were grown in RPMI 1640 medium containing 2 mM L-glutamine and 10% fetal bovine serum. For screening, cells were inoculated into 96 well microtiter plates in 100 µl, followed by incubation at 37°C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to the addition of experimental drugs. After 24 h, one 96 well plate containing 5 × 103 cells/well was fixed *in situ* with TCA to represent a measurement of the cell population (Tz) at the time of addition of drug. Experimental drugs were initially added to DMSO at 100 mg/ml and diluted to 1 mg/ml using water and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed and diluted to 100, 200, 400 and 800 µg/ml with complete medium. Aliquots of 10 µl of these different drug dilutions were added to the appropriate microtiter wells already containing 90 µl of medium, resulting in the required final drug concentrations, that is 10, 20, 40 and 80 µg/ml. Plates were incubated at standard conditions for 48 h and assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 µl of cold 30% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4°C. The supernatant was discarded; plates were washed and air dried. SRB solution (50 µl) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 min at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing with 1% acetic acid and air dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on a plate reader at a wavelength of 540 nm with 690 nm reference wavelength. Percent growth has been calculated on a plate-by-plate basis for test wells relative to control wells. Percent growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells * 100. Six absorbance measurements [at time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels (Ti)] were used to calculate the percentage growth inhibition. Percentage growth inhibition at each of the drug concentration was calculated as:

[(Ti–Tz)/(C–Tz)] × 100 for concentrations for which Ti ≥ Tz (Ti–Tz) positive or zero
[(Ti–Tz)/Tz] × 100 for concentrations for which Ti < Tz. (Ti–Tz) negative

Adriamycin was used as a standard for carrying out the activity. The data of cytotoxic activity against different human tumor cell lines is shown in Table 2. The results of activity are plotted as graph of drug conc. vs % control growth and are shown in Figures 1 and 2. The activity of standard drug Adriamycin and synthesized compound against human tumor cell lines are shown in Figures 3 and 4.

Antimicrobial assay

Antimicrobial assay for synthesized cyclic heptapeptide was carried out by using tube dilution technique (Bauer, 1996; Shinde, 2008, 2010). The bacterial strains and fungal strains were obtained from the National collection of industrial micro-organisms (NCIM), branch of National chemical laboratory (NCL) Pune, India. The antibacterial activity was performed against four bacterial species *B. subtilis* ATCC NO 6051, *S. aureus* ATCC NO 25923, *E. coli* ATCC NO 25922 and *P. aeruginosa* ATCC NO 9721) and antifungal activity was performed against *Candida albicans* ATCC NO 2091 and *Aspargillus niger* ATCC NO 10594. For carrying out antibacterial and antifungal activity, Benzyl penicillin and Fluconazole were used as standards. A solution of the compound was prepared in DMF and a series of doubling dilutions prepared with sterile pipettes so

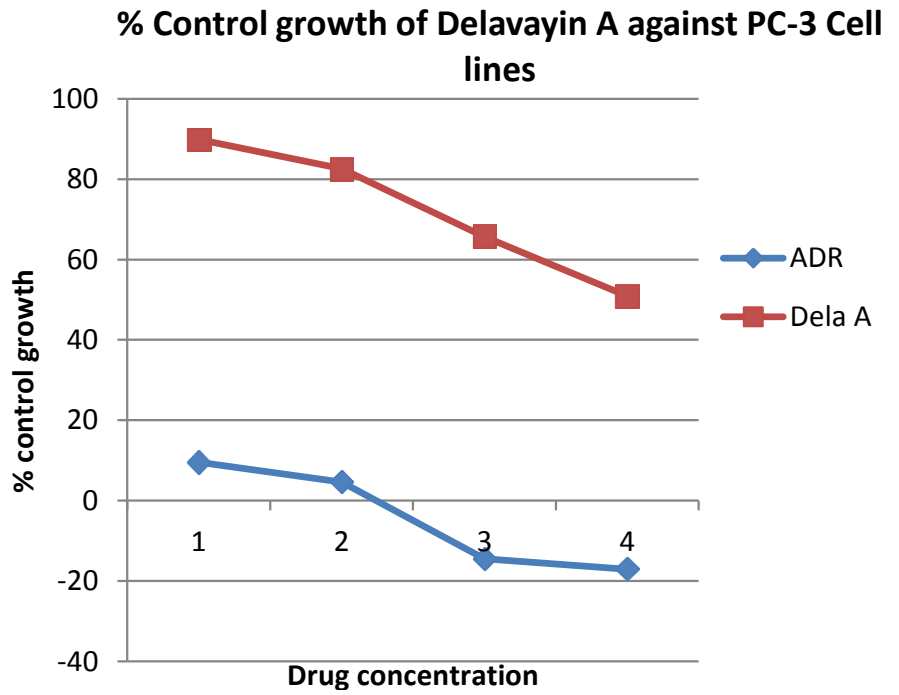


Figure 1. Cytotoxic activity of Delavayin A against PC-3 cell lines at different concentration in comparision with Adriamycin.

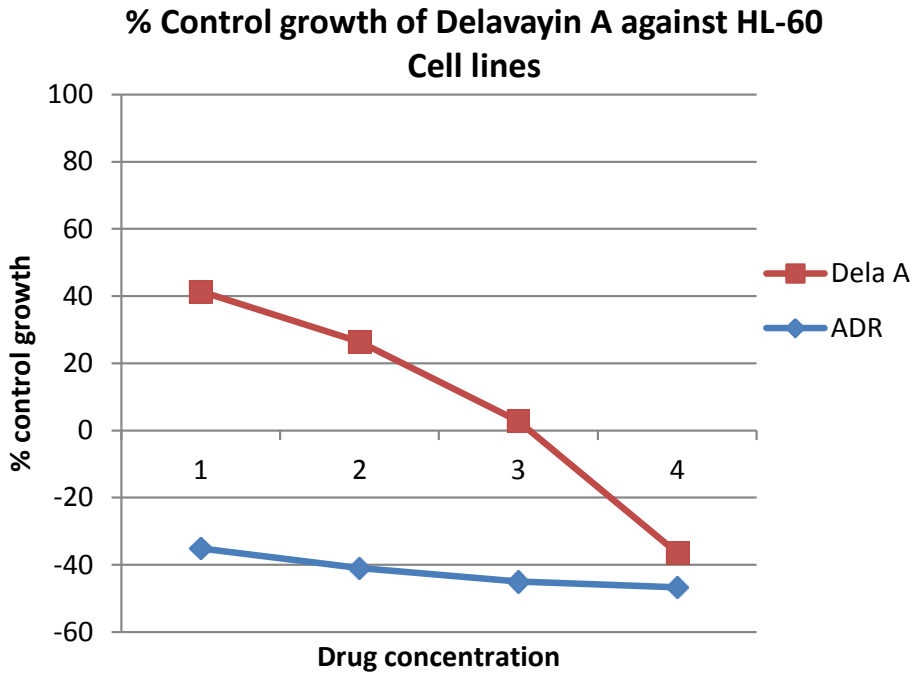


Figure 2. Cytotoxic activity of Delavayin A against HL-60 cell lines at different concentration in comparision with Adriamycin.

as to make concentrations as 100, 50, 25, 12.50, 6.25, 3.125, 1.562 and 0.781 µg/ml, respectively. A standard volume of nutrient broth medium was added to each of a series of sterile stoppered test tubes and a control tube containing no antimicrobial agent was also

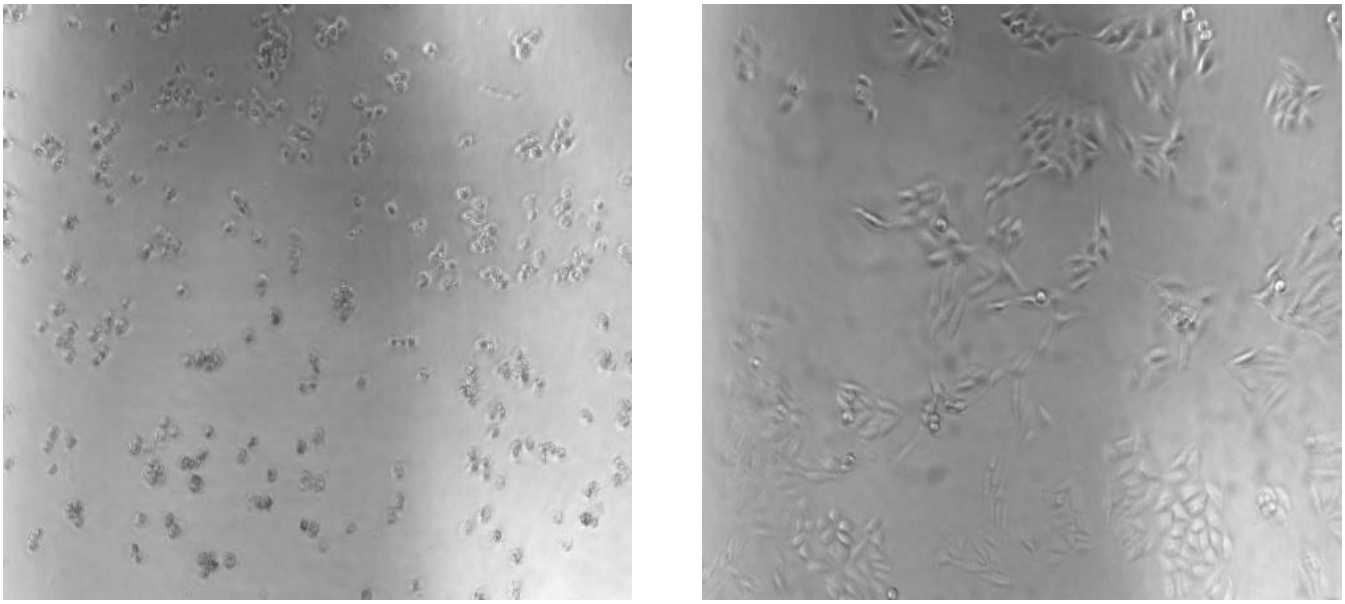


Figure 3. Cytotoxic activity of Adriamycin against PC3 cell lines (a) and HL-60 cell lines (b).

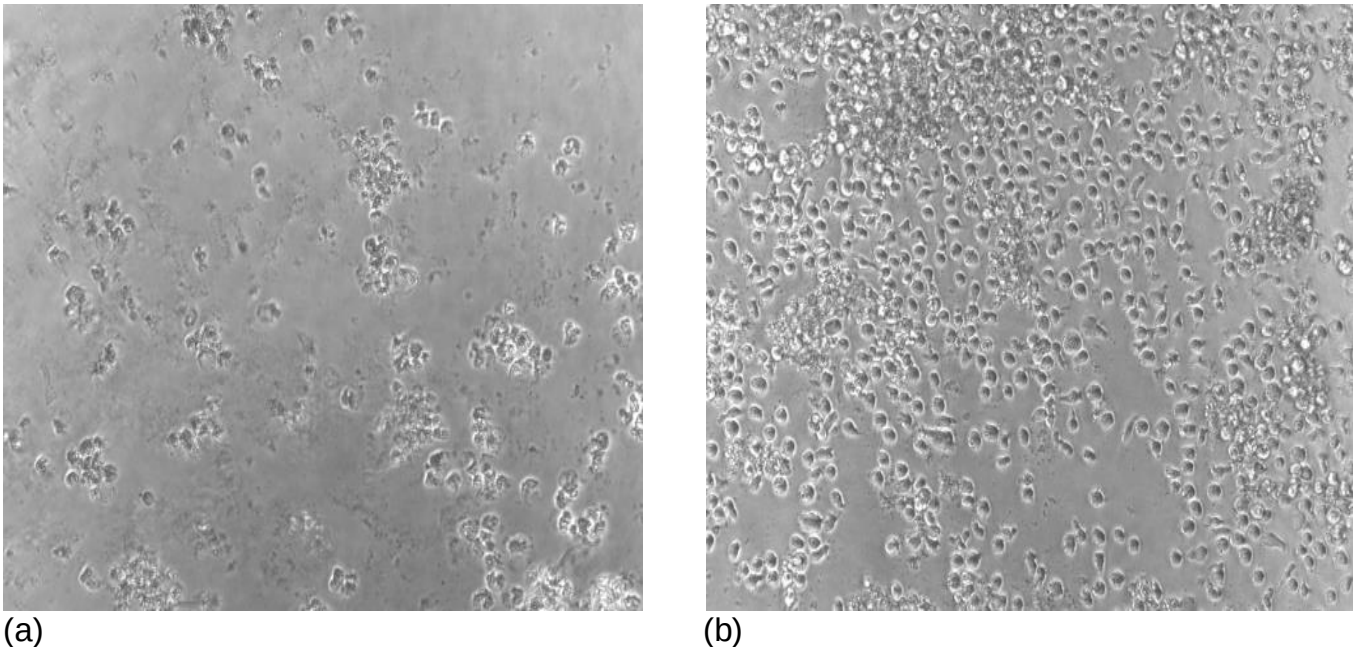


Figure 4. Cytotoxic activity of delavayin A against PC3 cell lines (a) and HL-60 cell lines (b).

included. The inoculum consisting of overnight broth culture of micro-organisms was added to separate tubes. The tubes were incubated at 37°C for 24 h and examined for turbidity. The tube with highest dilution showing no turbidity was the one containing compound with MIC. From the screening data of antibacterial and antifungal activity revealed that the synthetic peptide is found to be active. The results are shown in Table 3.

RESULTS AND DISCUSSION

Spectral data

- Physical state: Semisolid mass
- IR data: 3316.3(NH-stretch.), 2932.2(aliph.-CH stretch.),

Table 3. Minimum inhibitory concentration for antimicrobial activity.

Compound	Percent control growth (Average value, n=3)			
Conc. (µg/ml)	**10**	**20**	**40**	**80**
PC3 (against sample)	89.9	82.6	65.7	50.9
PC3 (against standard)	9.5	4.6	-14.2	-17.1
HL 60 cell lines (against sample)	76.3	67.12	47.5	10.1
HL 60 cell lines (against Standard)	-35.1	-41.0	-45.0	-46.7

2854.9, 1648.5(C=O of amide), 1534.7(-NH bend), 1453.1 (-CH bend).
- FABMS: showed M^+ ion peak at *m/z* 640.3
- ^{13}C NMR: showed six amide carbonyl carbon (δ170.01, 171.00, 171.16, 171.56, 172.65, 173.60).
- δ 43.86 for α Gly, δ 170.08 for C=O of Gly, δ 55.45 for α position of Serine, δ 63.12 for β position of Serine, δ 60.39, 40.01, 15.11,21.09 for α,β,γ and δ positions respectively of α hydroxy isoleucine, δ 56.24 , 39.91, for α and β positions of Phe, δ 171.11 for C=O of Phe, 137.43 for γ position of Phe, δ 52.11 for α position of Ala, δ 16.57 for β position of Ala, δ 173.11 for C=O of Ala.
- ^1H NMR: showed six amide protons (δ 8.20, 8.70, 9.15, 9.25, 9.33, 9.46, 10.18).
- δ 4.90 (HN of Gly), δ 4.16(dd, Hα of Gly), δ 10.20 (dd, C=O of Gly), δ 5.25 (dd, Hα of ser), δ 4.40 (HN of Ser), δ 9.47 (d, C=O of Ser), δ 1.01 (3H,d,7.1)γ CH_3 of γ hydroxy Ile, δ 3.85(1H,dd,6.0,12.3) for γ hydroxy Ile, δ 5.32(1H,dd,3.9, 8.5, 11.1)α Phe, δ 7.39(2H,d,7.2) δ Phe, δ 4.69 (1H,dd,6.2,12.3) α Phe, δ 3.44 (2H,dd,6.3) β Phe, δ 4.48 (1H,d,6.6, 6.9) α Ala, δ 4.53(1H,d, 3.9, 6.9) for α Ala, δ 1.53(3H,d, 6.9) for β Ala, δ 9.31(1H,D, 3.6) N-H of Ala.
- Elemental analysis: C: 60.9 (60.17), H: 6.54 (6.63), N: 12.12 (13.16).

DISCUSSION

The compound was synthesized with good yield by using solution phase technique. Anti-cancer activity of synthesized compound was carried out by SRB assay and had shown to possess moderate activity against PC 3 and HL-60 cancer cell lines when compared against adriamycin standard. The synthetic peptide has shown prominent activity against *Bacillus subtilis* and *Staphylococcus aureus* (Gram positive bacteria) and less activity against *Escherichia coli* and *Pseudomonas aeruginosa* (Gram negative bacteria) in comparison with standard drug benzyl penicillin. The synthesized molecule has also shown moderate activity against fungal strains *Candida albicans* and *Aspargillus niger*. As N-methylated analogs of most of cyclic peptides are found to show increase in the activity, there is scope for synthesizing analogs of lead molecule for the development of potent cytotoxic and antimicrobial agent.

Conflict of Interests

The authors have not declared any conflict of interests.

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Appendix 1. Graphical abstract.