Available online at www.derpharmachemica.com

Scholars Research Library

Der Pharma Chemica, 2015, 7(11):251-257
(http://derpharmachemica.com/archive.html)

ISSN 0975-413X
CODEN (USA): PCHHAX

First total synthesis and biological screening of cyclic tetrapeptides from *onychocola sclerotica*

Nirmala V. Shinde[1,2*], Avinash S. Dhake[2] and Kishan P. Haval[3]

[1]*Department of Pharmaceutical Sciences, Jawaharlal Nehru Technological University, Kakinada, Andhra Pradesh, India*
[2]*Department of Pharmaceutical Chemistry, S.M.B.T. College of Pharmacy, Nandi Hills, Dhamangaon, Tah: Igatpuri, Dist: Nashik (MH) India*
[3]*Department of Chemistry, Dr. Babasaheb Ambedkar Marathwada University, Subcentre Osmanabad.(MH) India*

ABSTRACT

Synthesis and cytotoxic, antimicrobial screening of cyclic tetratapeptides from onychocolascleroticais described. Structureof synthesized compounds were confirmed by detailed spectral analysis including FTIR, ^{1}H NMR, ^{13}C NMR, MSand elemental analysis. The spectral data showed that the synthesized compounds areidentical to the naturally occurring compound.The results of screeningindicated that synthesized compounds possessvery good antimicrobial activity against Gram positive bacteria, moderate antifungal activity against pathogenic fungi and poor cytotoxic activity.

Keywords: cyclic peptide, anticancer, p-nitrophenyl ester method, solution phase synthesis.

INTRODUCTION

In recent years, synthesis of naturally occurring cyclic peptides as therapeutic agents has attracted much attention of the researchers, owing to their captivating chemical structures and potent biological activity. The therapeutic potential shown by cyclic peptides is a result of greater resistance towards in *vivo* enzymatic degradation as well as greater bioavailability than non-cyclic analogs. Cyclooligopeptides, especially medium ring-sized peptides have been reported from diverse sources, including fungi, marine sources and shown to possess wide range of pharmacological activities like antimicrobial, anthelmintic, insecticidal, cytotoxic, anti-inflammatory activities[1-7]. As only minute quantities are obtained by isolation from natural sources, synthesis of such naturally active cyclopeptidesis of major interest for the development of new therapeutic agents. Keeping in view ofthe significant biological activities exhibited bycyclic peptides, as a part of ongoing study,an attempt was made towards the synthesis of a cyclic tetrapeptides, cyclo(L-N-MePhe–L-Val-L-N-MePhe–L-Val) and cyclo(L-N-MePhe–L-Ile-L-N-MePhe–L-Ile), which were isolated from a fermentation broth offungus *Onychocolasclerotic*[8]. Structure of synthesized compounds were confirmed by detail spectral analysis. The synthesis was carried out by using solution phase technique via coupling of dipeptide segments.In addition, the synthesized products were further subjected to cytotoxic activityagainst PC 3 and HL-60 human tumor cell lines. The compounds were also evaluated for antimicrobial activity studies against bacterial strains and pathogenic fungi. The results of biological activity showed that, the compoundsareprominently active against Gram positive bacteria's and moderately active against pathogenic fungi in comparison with benzyl penicillin andfluconazole, as standard.

MATERIALS AND METHODS

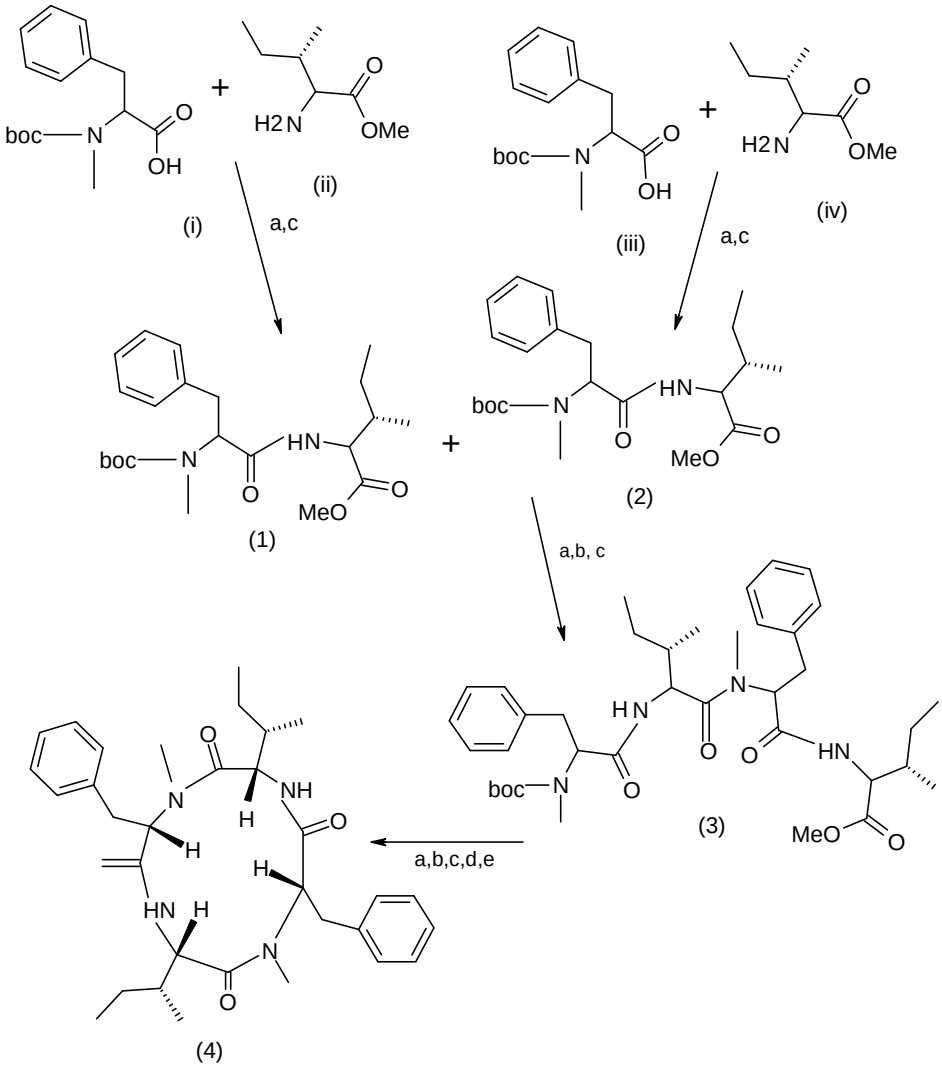
All L-amino acids, di-*tert*butyldicarbonate (Boc_2O), diisopropylcarbodiimide (DIPC) and other chemicalsrequired for synthesis were procured from Spectrochem Limited, Mumbai, India. Melting points of all intermediates and final

compound were determined by digital melting point apparatus, make SYSTRONIC. The IR spectra were recorded with the help of chloroform and NaClcellsor by using KBr pellets on JASCO 4100 FTIR spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AC NMR spectrometer using DMSO as a solvent. The Mass spectrum was recorded at 70 eV on JMS-DX 303 Mass spectrometer.

2.1 Synthesis of tetra peptides:

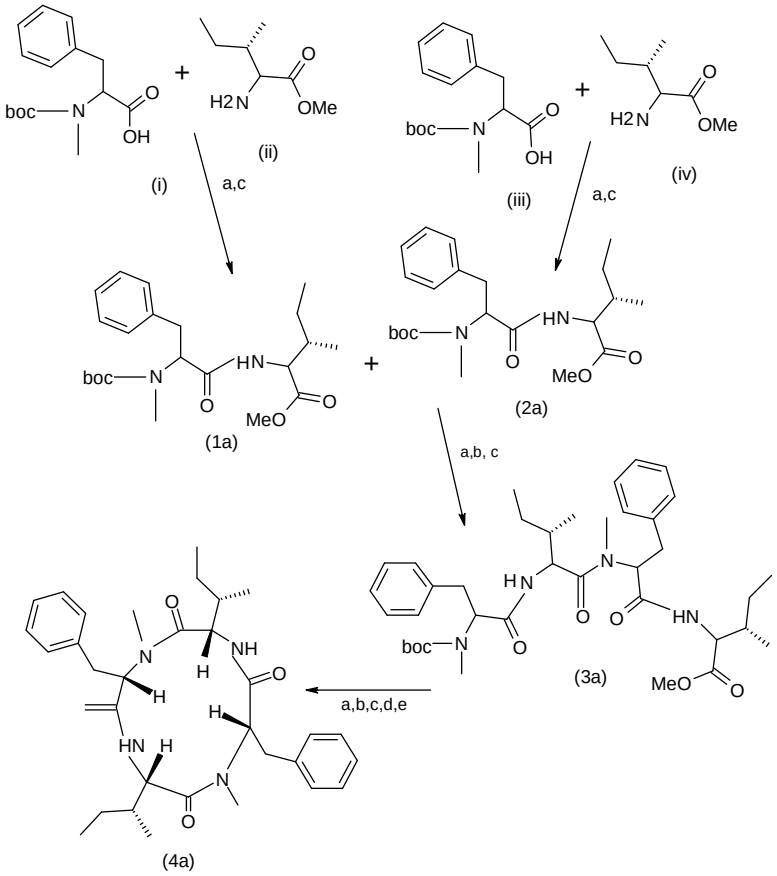
General method for preparation of Di/Tetrapeptide: Amino acid methyl ester hydrochloride/dipeptide methyl ester(10 mmol) was added to chloroform (CCl_3, 20 ml). To the resulting solution , at 0 $^{\circ}C$, TEA (2.8 ml, 20 mmol) was added and the reaction mixture was allow to stir for 15 min. Boc-N-Me-L-amino acid/ Boc dipeptide (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 (30ml) 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 over anhydrous Na_2SO_4, filtered and evaporated in vacuum. Recrystallization of the crude product was done from a mixture of chloroform and petroleum ether. By using above procedure, compounds **1-4**and **1a -4a**were synthesized.[9]

Scheme 1: Synthesis of tetrapeptide 4



Where: a= DIPC, NMM, CHCl3, RT, 24h,
b= TFA, NMM,RT,1h,
c= LiOH, THF:H_2O(1:1), reflux, 15 mins
d= pnp-, CHCl3, RT, 12h,
e= NMM, $CHCl_3$, 0°C, 7days

Scheme 2: Synthesis of tetrapeptide 4a



Where: a= DIPC, NMM, CHCl3, RT, 24h,
b= TFA, NMM,RT,1h,
c= LiOH, THF:H_2O(1:1), reflux, 15 mins
d= pnp-, CHCl3, RT, 12h,
e= NMM, $CHCl_3$, 0°C, 7days

Method for cyclization of lineartetrapeptide:

The cyclization of linear tetrapeptides**4** and **4a**was done by using p-nitrophenyl ester method. 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 $^{\circ}$C, to which p-nitrophenol (0.27 gm, 2 mmol) was added, and allowed to stir for 12h at RT. The reaction mixture was filtered and the filtrate was washed by using $NaHCO_3$ solution (10%) until excess of p-nitrophenol gets removed. The filtrate was finally washed with 5% HCl (5 mL) to get Boc-peptide-pnp ester.

To the above Boc-peptide-pnp-ester (1.2 mmol) in $CHCl_3$ (15mL), CF_3COOH (0.274g, 2.4 mmol) wasadded and allow to stir for 1 h at room température. The reaction mixture waswashedwith 10% $NaHCO_3$ and the organiclayerwasdried over anhydrous Na_2SO_4.To the Boc-deprotected peptide-pnp-ester in $CHCl_3$ (15mL), N-methyl morpholine (1.4mL, 2mmol) was added and kept at 0°C for 7 days**.** The reaction mixture was washed with 10% $NaHCO_3$ till the byproduct p-nitrophenol gets removed completely, so as to obtain the titled compound **4**and**4a.[10]**

2.2 Biological activity:

i)*In vitro*anticancer activity against human tumor cell lines[11-13]:

For the synthesized compounds, cytotoxic activity against human tumor cell lines PC3 and HL 60 was carried out at ACTREC Mumbai, by following standard protocol. Adriamycin was used as a standard for carrying out the activity. From the result of activity, the synthesized tetra peptides are found to show poor cytotoxic activity against PC3 and HL60 human tumor cell lines in comparison with Adriamycin.

The data of cytotoxic activity against different human tumor cell lines is shown in Table 1 and figure 1 and 2 shows comparison of cytotoxic activity with Adriamycin as standard.

ii) Antimicrobial Screening[14-16]: The bacterial strains and fungal strains were obtainedfrom 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 25922and *P. aeruginosa*ATCC NO 9721) and antifungal activityagainst *Candida albicans*ATCC NO 2091 and*Aspargillus Niger*ATCC NO 10594by usingmodified Kirby-Bauer disc diffusion method and by tube dilution technique to obtain MIC values.

Benzylpenicillin was used as standard for comparision of antibacterial acitivity of the synthesized compounds and Fluconazole was used as standard drug for antifungal activity studies.

Disc diffusion method :
Modified Kirby Bauer Method is one of the official method among disk Diffusion Methods.Circular paper disk of 6mm diameter and 2mm thickness were sterilized by autoclaving at 121°C (151b PSIG) for 15 min and imprégnâted with specificamount of test sample.The discs were placed on a suitable Nutrient medium /Sabouraud's agar in a petri plate which was inoculated on its surface with one of the test organisms. After incubation, the plates were observed for the growth inhibition zones around the disks. The diameter of the zone of inhibition is proportional to the antimicrobial activity of the susbtance. The diamètres of zone of inhibition were compared with that produced by the standard antibiotics.

The results of antimicrobial activity by disc diffusion method are shown in Tables 2and figure 3 and 4 shows comparison of antibacterial, antifungal activity against standard.

Determination of MIC by Tube Dilution Technique:
Newly synthesized tetrapeptides were dissolved separately to prepare a stock solution of 1 mg/ml using DMFand a series of doubling dilutions prepared with sterile pipettes so 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 steriletest tubes and a control tube containing no antimicrobial agent was also included. All the tubes were inoculated with one loopful of one of the test bacteria/fungi. The process was repeated with different test bacteria/fungi and different samples. Tubes inoculated with bacterial/fungal cultures were incubated37º C for 24 h and examinedfor turbidity. The tube with highest dilution showingno turbidity was the one containing compound withMIC. From the screening data of antibacterial and antifungalactivity revealed that the synthetic peptide is foundto be active. The results of antimicrobial activity by tube dilution technique are shown in Tables 3.

RESULTS AND DISCUSSION

Spectral data:
Compound 4: cyclo (L-N-MePhe–L-Val-L-N-MePhe–L-Val-)
Physical state: White solid
IR(cm^{-1}): 3346, 1660 cm^{-1} are due to amino and amide carbonyl groups respectively ^{1}H NMR:δH4.47 dd, 3.60dd, 3.00 dd, 7.25 m, 7.33,7.24, for L-N-Me-Phe- and δ 4.22, 2.10 d, 0.71m, 0.86m for L-Val ^{13}C NMR:δC172.2(CO of L-N-Me-Phe), 64.6(α CH of L-N-Me-Phe), 35.4(β CH2 of L-N-Me-Phe), 138.5(γ C of L-N-Me-Phe), 128.5 (δ CH of L-N-Me-Phe), 173.4 (CO of Val), 57.3(α CH of Val), 30.2 (β CH of Val), 20.8 (CH of CH_3) MS: M^+at m/z 522, fragment ion peak at m/z 261 corresponding to (N-Me-Phe-l-Val) fragment

Table 1: Data of Cytotoxic activity against human tumor cell line

Cell line	**Percent control growth** (Average value, n=3)			
Conc.(µg/ml)	**10**	**20**	**40**	**80**
PC3 (against Compound 4)	90.6	87.6	63.7	58.9
PC3 (against Compound 4a)	90.2	84.8	65.4	60.7
PC3 (against Standard)	9.5	4.6	-14.2	-17.1
HL60 (against Compound 4)	76.5	67.4	47.8	10.3
HL60 (against Compound 4a)	72.1	64.7	54.1	8.9
HL 60 (against Standard)	-35.1	-41.0	-45.0	-46.7

Compound 4 a: cyclo(L-N-MePhe-L-Ile-N-MePhe-L-Ile)
Physical state:White solid
IR (cm^{-1}): 3326,1660 cm−1 are due to amino and amide carbonyl groups respectively ^{1}H NMR:4.48, d (for α H of L-N-Me-Phe), 3.65, d and 3.00, t for β H of L-N-MePhe, 7.28 m, 7.37 m for γ and δ of L-N-Me-Phe, 7.24 m, 2.88 S for N-CH_3 of L-N-Me-Phe, 4.28, d, for α H of isoleucine, 1.86, m for β H of isoleucine, 1.48, m and 1 m for γ H of isoleucine, 0.87, t for δ H of isoleucine, 0.87, t forδ H of isoleucine. ^{13}C NMR:δC 172.2(CO of L-N-Me-Phe), 64.6(α CH of L-N-Me-Phe), 35.1(β CH_2 of L-N-Me-Phe), 139 (γ C of L-N-Me-Phe), δ 129.7(δ CH of L-N-Me-Phe),

31.1(CH, N-CH_3), δ173.4 (CO of Ile), δ56.8(α CH of Ile), δ36.9 (β CH of Ile), δ 25.4(γ CH_2 of Ile), 11.9 (CH of CH_3) MS:M^+ at m/z550, fragment ion peak atm/z 275, corresponding to the N-MePhe-Ile fragment

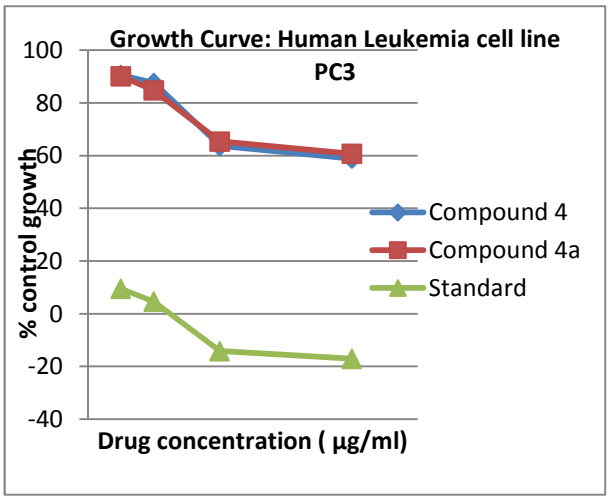


Figure 1: Cytotoxic activity of compound 4 and 4a against PC3 cell lines at different conc. in comparison with Adriamycin

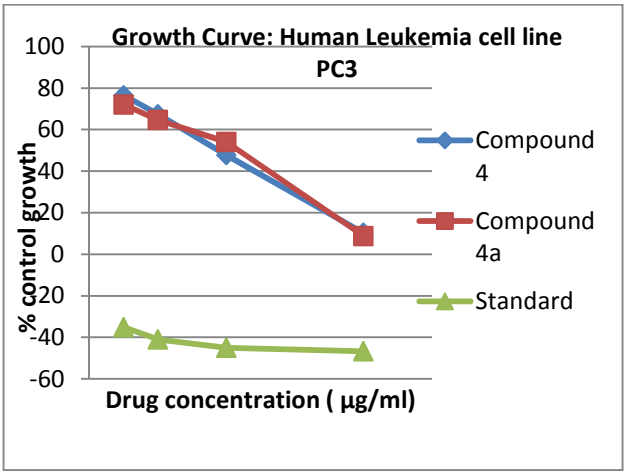


Figure 2: Cytotoxic activity of compound 4 and 4a against HL60 cell lines at different conc. in comparison with Adriamycin

Table 2:Results of Antimicrobial activity by Disc diffusion method

Compound	Diameter of zone of inhibition (mm)					
	B. Subtilis	***S. Aureus***	***P.Aeruginosa***	***E.coli***	***C.Albicans***	***A.Niger***
Compound 4	15	14	9	8	18	12
Compound 4a	22	12	11	10	16	14
Benzyl Penicillin	25	15	17	16	-	-
Fluconazole	-	-	-	-	20	18
DMF	-	-	-	-	-	

(-) indicates no inhibition zone (no activity)

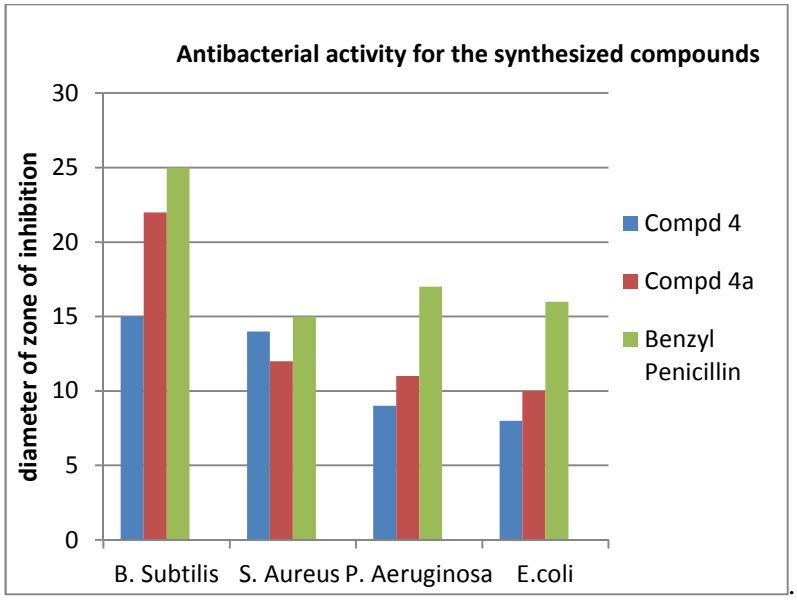


Figure 3: Antibacterial activity for compound 4 and 4a

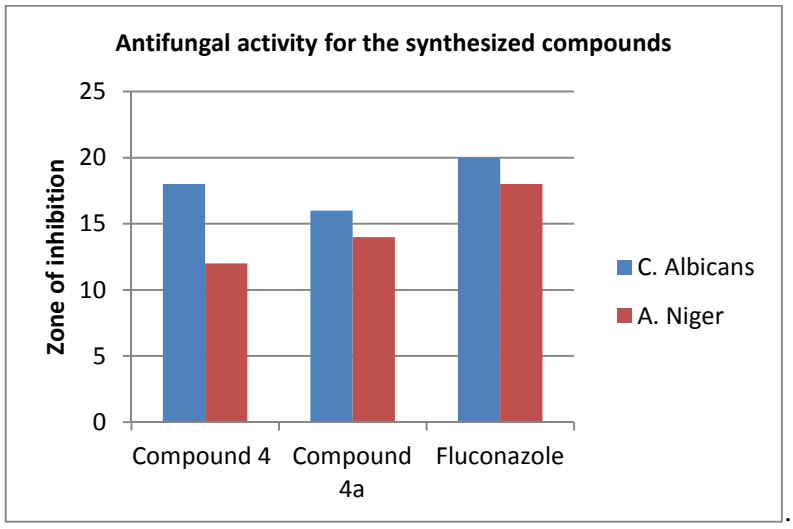


Figure 4: Antifungal activity for compound 4 and 4a

Table 3: Determination of MIC by Tube Dilution Technique

Compound	MIC (µg/ml)					
	B. Subtilis	***S. Aureus***	***E.Coli***	***P. aeruginosa***	***C.albicans***	***A.Niger***
Compd. 4	12.5	6.25	100	100	50	50
Compd. 4a	12.5	12.5	100	50	50	100
Benzyl Penicillin	1.562	0.781	50.0	25	-	-
Fluconazole	-	-	-	-	25	25

CONCLUSION

Thetitled compounds were synthesized with good yield by using solution phase technique. The compound had shown to possess poor activity against PC 3 and HL-60 cancer cell lines when compared against Adriamycin standard. The synthetic peptideshas shown prominent activity against *Bacillus subtilis* and *Staphylococcus aureus* (Gram +ve bacteria) in comparision with standard drug benzyl penicillin.The synthesized molecule has also shown goodactivity against fungal strains *Candida albicans*and*Aspargillus Niger*.

Acknowledgements
Authors are thankful to S.M.B.T. College of Pharmacy, Dhamangaon, Nashik for providing necessary facilities to do the research work. We also extend our thanks to Sophisticated Analytical Instrumentation Laboratory, Chandigarh for spectral analysis and ACTREC, Mumbai for carrying out cytotoxic activity.

REFERENCES

[1] R. Dahiya and H.Gautam, *Bulletin Pharm. Res.*,**2011**, 1(1), 1-10.
[2] S.Chaudhary, H. Kumar, H. Verma, A. Rajpoot, *Int J. Pharm Tech Res*., **2012**, 4(1), 194-200.
[3] N.V. Shinde, M. Himaja,S.K.Bhosale, M.V.Ramana and D.M.Sakarkar.,*Indian J Pharm Sci.*,**2008**, 70(6), 695-852.
[4] S. Chaudhary, H.Kumar, H.Verma, A. Rajpoot.,*Int Journal of PharmTech Research.*, 2012, 4(1), 194-200.
[5] D. Hernández et.al.,*Eur. J. Org. Chem.*, **2008**, 3389–3396.
[6] N.V.Shinde, M.Himaja, S.K.Bhosale, M.V. Ramana and D.M. Sakarkar., *Asian Journal of Chemistry*, **2010**, 22(2), 996-1000.
[7] H. Kawagishi, A. Somoto, J. Kuranari, A. Kimura and S. Chiba., *Tetrahedron*,**1993**, 34(21), 3439-3440.
[8] S. Onychocola, P. Ignacio, M. Jesús, G.M. Víctor,N.D.Pedro, E.A. Noureddineet.al. *J. Nat. Prod.*,**2012**, 75, 1210−1214.
[9] C. Saranya,S. Pavla and T. Istvan., *Molecules.*,**2013**, *18*, 4373-88.
[10] M. Bodanszky, A. Bodanszky.Practice of peptide synthesis, Springer-Verlog, New York,**1984**, 78.
[11] U. J.Joshi, A. S.Gadge, D. M. Priscilla, R. Sinha, S. Srivastava and G.Govil., *International J Research in Pharm and Biomedical Sciences*, **2011**, 2(4), 1756-1766.
[12] www. actrec.com
[13] P.Houghton, R. Fang, I. Techatanawat.,*Science Direct.*, **2007**, 42, 377–387.
[14] R.Dahiya, D. Pathak, M. Himaja, S. Bhatt**.,** *Acta Pharm.*,**2006**,*56*, 399–415.
[15] R. Dahiya and H. Gautam.,*African J Pharm and Pharmacology*, **2011**, 5(3), 447-453.
[16] M. Himaja, S.R. Ogale,M. M. Das, and A. Karigar.,*Unique J of pharm and Biosciences*, **2014**, 02 (03),1-4.