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Synthesis and characterization of Linear PNC-28 Peptide, a p53-derived peptide

*[1]Deepshikha & [2]Pillai V.N. Rajasekharan

*[1]Research Scholar ,Department of Chemistry, Mewar University, Gangrar, Chittorgarh, Rajasthan, India, 312901 (India)
[2]Professor, Department of Chemistry, Mewar University, Gangrar, Chittorgarh, Rajasthan, India, 312901 (India)

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***Corresponding Author**
Email: *deepshikhaverma0341[at]gmail.com*

ABSTRACT

Linear PNC-28 Peptide (ETFSDLWKLL) was synthesized by using the solid Phase Peptide Synthesis method. The resin used in this method, as solid support was Wang resin, and it's loading capacity was 1.5 mmol/g. The chemical formula of PNC-28 Peptide is $C_{60}H_{90}N_{12}O_{17}$. The molecular weight of PNC-28 is 1251.5 g/mol and its sequence composition in percentage is: acidic=20%, basic=10%, neutral=20%, hydrophobic=50%. Nature of PNC-28 peptide is anticancer which treats pancreatic cancer and its origin is Antennapedia. The successful synthesis of PNC-28 Peptide is confirmed by LC-MS FTIR, 1HNMR and CD.

1. Introduction

Peptides are comprised of short chains of amino acids, all of which are linked together by peptide bonds. Structurally speaking, peptides are quite similar to proteins, as they are both made up of amino acid chains which are held together via peptide bonds. The main distinguishing factor between peptides and proteins, however, is their size. Whereas proteins are generally made up of 50 or more amino acids, peptides are often on the smaller side, consisting of only two to 50 amino acids. Naturally occurring synthesis of peptides can be found in all living organisms whereas the synthetic peptide production is often used by researchers to produce specific peptides. In particular, researchers synthesize those peptides that may be difficult to express in bacteria or to experiment with the incorporation of amino acids that are not typically found in peptides. The most common method for the synthetic peptide production is called solid-phase peptide synthesis. In our work we used this solid-phase peptide synthesis method for synthesizing Linear PNC-28 peptide. This method first pioneered by American biochemist Robert Bruce Merrifield; solid-phase peptide synthesis has since become the leading method for the synthetic production of peptides. Solid-phase peptide synthesis, often abbreviated as SPPS, streamlines the process of synthetic peptide production by creating multiple successive amino acid reactions on a singular porous apparatus. Peptide synthesis is often used in conjunction with epitope mapping and typically sees applications in medical sciences and biotechnology. Synthetic peptides are used to research potential cancer diagnoses and treatments and in the development of antibiotic drugs.[1]

Linear PNC-28 peptide contains p53 protein residues 17-28 (ETFSDLWKLL).[2] p53 is well known for its key role as a tumor suppressor protein. It is 393 amino acids (aa) in length with a predicted molecular weight of 44 kDa.[3,4] Tumor protein p53, also known as p53, cellular tumor antigen p53, phosphoprotein p53, tumor suppressor p53, antigen NY-CO-13, or transformation-related protein 53 (TRP53), is any isoform of a protein encoded by homologous genes in various organisms, such as *TP53* (humans) and *Trp53* (mice). p53 helps in regulation or progression of the cell cycle, apoptosis, and genomic stability and also act as an important factor in aging.[5] There are various studies where we found that PNC-28 induced cancer cell death in a variety of human cancer cells by inducing tumor cell necrosis rather than apoptosis.[6,7] The anticancer activity and mechanism of PNC-28 (p53 aa17–26-penetratin) was specifically studied against human pancreatic cancer.[8]

Peptide properties	
Sequence:	ETFSDLWKLL
Length:	10
Mass:	1250.6525
Isoelectric point (pI):	4.00
Net charge:	-1
Hydrophobicity:	+11.13 Kcal * mol^{-1}
Extinction coefficient[1]:	5500 M^{-1} * cm^{-1}
Extinction coefficient[2]:	5500 M^{-1} * cm^{-1}

1.1. Experimental:

Materials: In the synthesis of PNC-28 Peptide the reagents we used are obtained from Sigma-Aldrich Company and these are Wang Resin, F-moc protected amino acids (ETFSDLWKLL), additives (HOBT and HBTU, HATU and HOAT), Diisopropylcarbodiimide, DMF, DCM, IPA, IPE, TFA, DI water, KCN, Pyridine, n-Butanol, Ninhydrin, Phenol, trifluoroacetic acid (82.5% v/v), phenol (5% v/v), water (5% v/v), thioanisole (5% v/v), 1,2-ethanedithiol (2.5% v/v), Diethyl ether. The equipment we used are also obtained from Sigma-Aldrich company and they are frit glass vessel, caframo, stopper test-tube, weighing machine, round bottom flask, desiccator, lyophilizer.

2. Method

For, the production of synthetic peptides in the lab, the established method is known as solid-phase peptide synthesis (SPPS).[9,10,11] 1963 Merrifield had developed the SPPS method; insoluble carrier: crosslinked polystyrene; Nα-protecting group:

Boc. Solid Phase Peptide Synthesis (SPPS) can be defined as a process in which a peptide anchored by its C-terminus to an insoluble polymer is assembled by the successive addition of the protected amino acids constituting its sequence. Each amino acid addition is referred to as a cycle consisting of: a) cleavage of the Nα-protecting group, b) washing steps, c) coupling of a protected amino acid, d) washing steps.[12,13,14] As the growing chain is bound to an insoluble support the excess of reagents and soluble by-products can be removed by simple filtration. Washing steps with appropriate solvents ensure the complete removal of cleavage agents after the deprotection step as well as the elimination of excesses of reagents and by-products resulting from the coupling step.[15,16,17] To synthesize PNC-28 Peptide, we proceed step by step to get the final synthesized PNC-28 peptide. First step is selection of resin. We choose Wang resin for synthesis and it's loading capacity is 1.5mmol. Second most important step for synthesis is a loading of first amino acid. We took 5-fold excess of Leucine, HOBT and 0.1-fold excess DMAP in a stoppered glass tube. To the tube added approximately 2ml DMF containing 4% DIPEA. Added this content to drain bed of swollen Wang resin. To the above vessel added 116.8 (0.1g X 1.5 X 5 X 126.2= 94.65, 94.65/0.81=116.8) microliters of DIC in a drop wise manner. Capped the vessel and started mixing or coupling on Caframo for overnight. Third important step was washing the material after overnight coupling, and washing steps are: 2 X DMF, 2 X DCM, 1 X IPA, 2 X DCM, 2 X DMF. Fourth step for synthesizes was Kaiser's Test: Took 10-15 beads of resin in a test tube. Added 2-3 drops of each reagent A, reagent B, reagent C. Heated the tube at 110ºC for five minutes. The result was colorless solution and beads were also colorless which showed the complete coupling. Fifth step was Deprotection of Fmoc group of amino acid Leucine with 20% Piperidine for half-an-hour and then follow the above-mentioned steps of washing. The deprotection result has been observed through Kaiser's test. Took 10-15 beads of resin in a test tube. Added 2-3 drops of each reagents; reagent A, reagent B, reagent C. Heated the tube at 110ºC for five minutes. The result was blue colored solution, and dark blue colored beads. It showed the complete deprotection. Repeated these steps until the final peptide sequence has been completed. Final washing had been done because the sequence of amino acids of PNC-28 peptide was finished and washing steps are: 3 X DMF (3ml) X 5 min, 3 X DCM (3ml) X 5min, 2 X IPA (3ml) X 5min, 2 X IPE (3ml) X 5min, To dry the material kept at vacuum for 2 hours. Cleavage and Precipitate the sequence from resin by using cocktail-K: 8.3ml TFA+ 0.25ml EDT+ 0.5g Phenol+ 0.5ml Thioanisole+ 0.5ml Water is used as cocktail K and chilled diethyl ether is used to precipitate. Cool the material at -80 degree C for an overnight. Decant the diethyl ether and lyophilize the final peptide sequence. Characterized the sequence by LC-MS. To synthesize the above-mentioned peptide, we used the manual Solid Phase Peptide Synthesis method. The general SPPS procedure is one of repeated cycles of alternate N-terminal deprotection and coupling reactions. The resin can be washed between each step. In the first step, the first amino acid is coupled to the resin. Subsequently, the amine is unprotected, and then coupled with the free acid of the second amino acid. This cycle repeats until the desired sequence has been synthesized. SPPS cycles may also include capping steps which block the ends of unreacted amino acids from reacting. At the end of the synthesis, the crude peptide is cleaved from the solid support while simultaneously removing all protecting groups using reagent strong acids like trifluoroacetic acid or a nucleophile. The crude peptide can be precipitated from a non-polar solvent like diethyl ether to remove organic soluble by products. The crude peptide can be purified using reversed-phase HPLC. Figure 1 shows the structure of PNC-28 peptide:

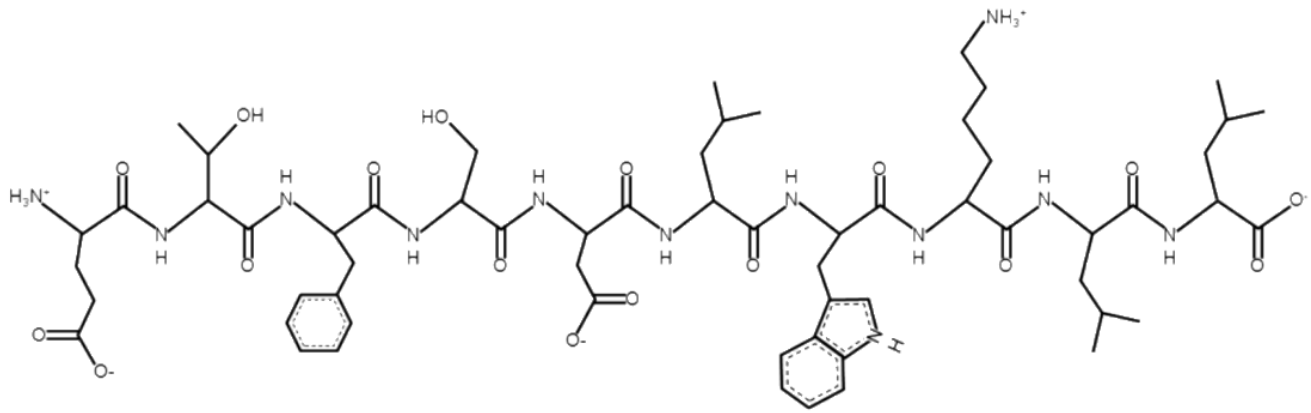

Fig1: PNC-28 Peptide (ETFSDLWKLL) structure

The basic concept in solid phase peptide synthesis is the step-wise construction of a peptide chain attached to an insoluble polymeric support shown in below figure 2:

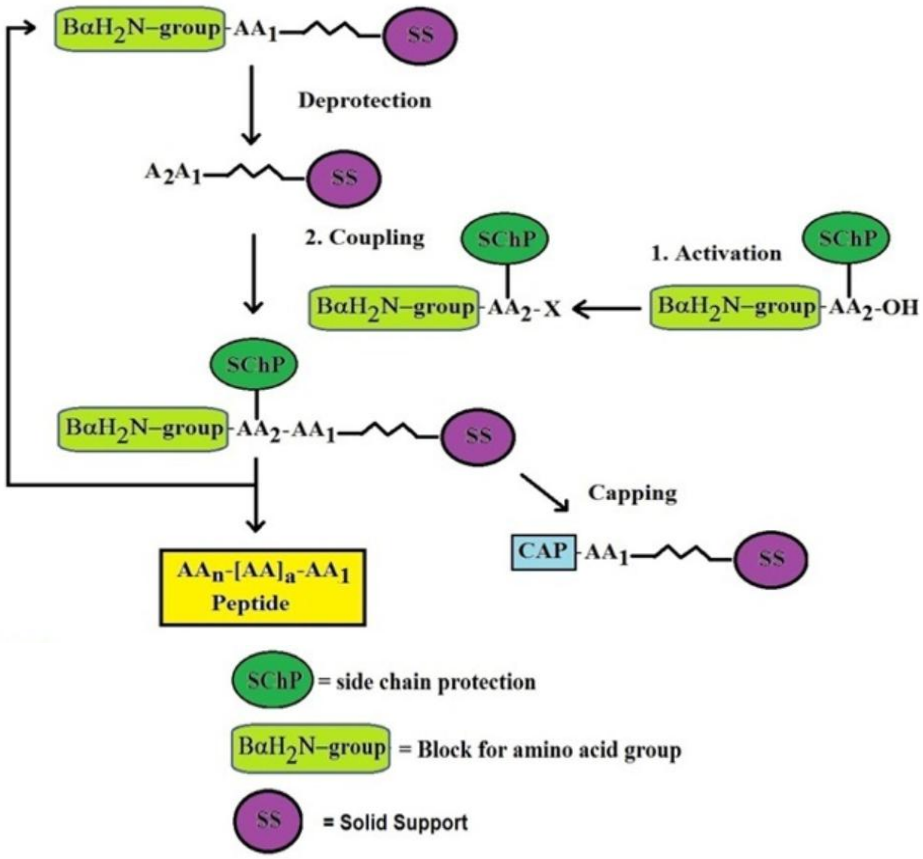

Figure 2: General Scheme for Solid Phase Peptide Synthesis.[12]

3. Result and Discussion:

FIGURE 3 represents the PNC-28 Peptide synthesis has been successfully done and was analysed through liquid chromatography mass spectroscopy which reveals the presence of PNC-28 Peptide (ETFSDLWKLL) with a molecular mass ≈1252g/mol.

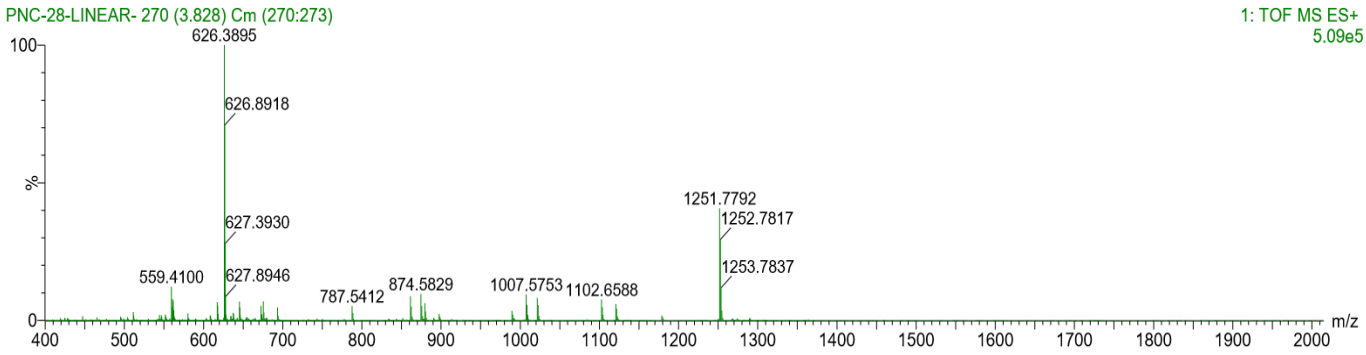

Figure3: LC-MS of PNC-28 Peptide

Figure 4 represents the ^{1}HNMR data of synthesised PNC-28 peptide in DMSO. The chemical shift values within the range 0.9 to 10.0 for ten amino acids (ETFSDLWKLL) were observed and these are: Glutamic acid (E) has chemical shift value HN= 8.0 HA=4.5 HB= 2.2,2.1β and other 2.3,2.2γ, Threonine (T) has chemical shift value HN= 8.0 , HA= 4.5, HB= 4.4β, other= 1.2γ, phenylalanine (F) has chemical shift value HN= 8.0, HA= 4.5, HB= 3.0,2.8β ring: H-C (3) 6.5 to 7.6, Serine (S) has chemical shift value HN=8.0, HA= 4.5, HB=3.7,3.6β, Aspartic acid (D) has chemical shift value HN= 8.0, HA= 4.6, HB=2.5,2.3β, Leucine (L) has chemical shift value HN= 8.0 , HA=4.4, HB=1.7,1.6β, other= 1.6γ and 1.0δ,0.9δ, Tryptophan (w) has chemical shift value HN= 8.0, HA= 4.5, HB= 3.1,2.9β ring: H-C (5) 6.5 to 7.9, NH:10.0, Lysine (K) has chemical shift value HN=8.0, HA=4.4, HB= 1.6,1.7β other= 1.5,1.4γ 1.7,1.6δ 3.0,3.1ε NH3:6.9.

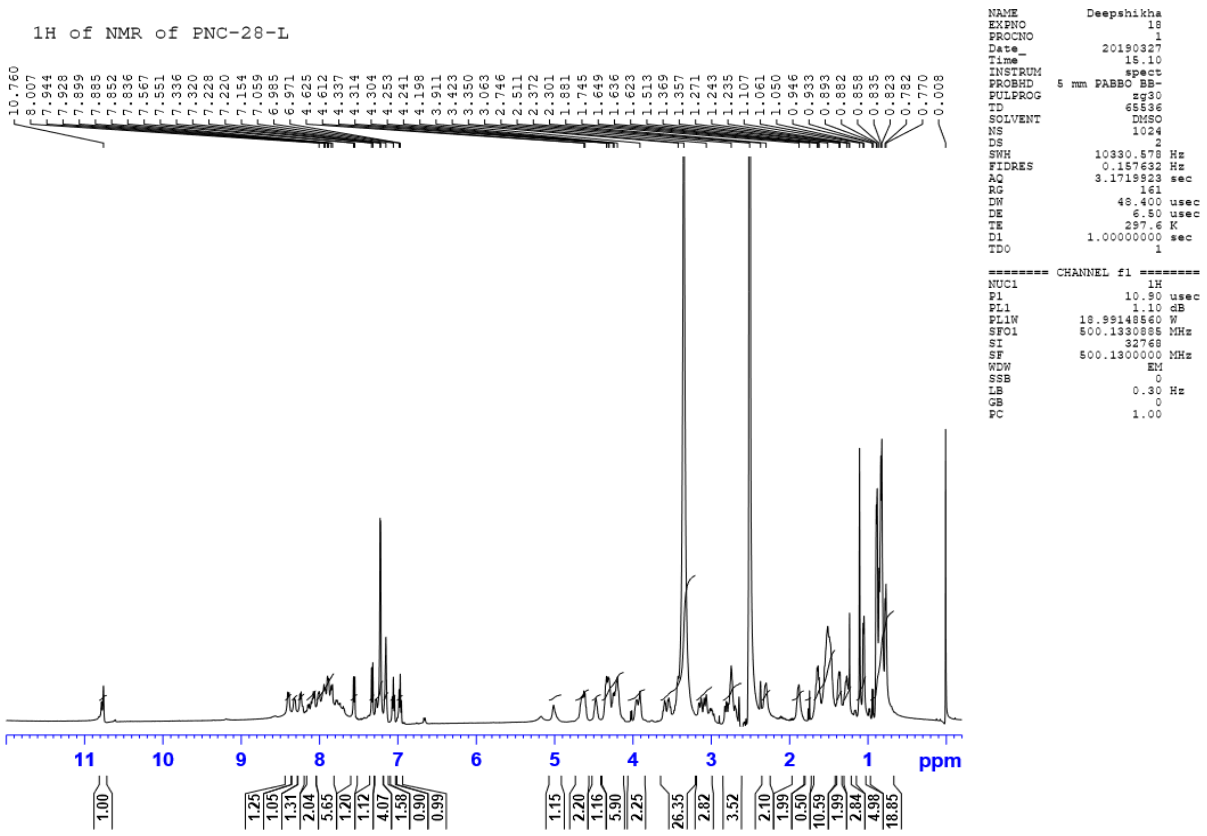

Fig 4: NMR Spectra of PNC-28 Peptide

Figure 5 represents the FTIR spectra of PNC-28 peptide. Infrared spectroscopy is an emerging technique for peptide analysis. Amide vibrations involve C=O, C-N and N-H groups of a peptide bond (amide bond), which result in characteristic spectral features of proteins. Three major spectral regions (amide I, amide II and amide III) have been identified based on theoretical and experimental studies.[18,19,20,21,22]

Three major spectral regions (amide I, amide II and amide III) have been identified for PNC-28 peptide and these are the amide I region 1651 corresponds to the C=O stretching, the amide II region 1529 represents C-N stretching, the amide III region 1199 is N-H in-plane bending coupled with C-N stretching. . Amide I, II and III are most commonly used IR spectral regions used for peptide structure-function analysis.

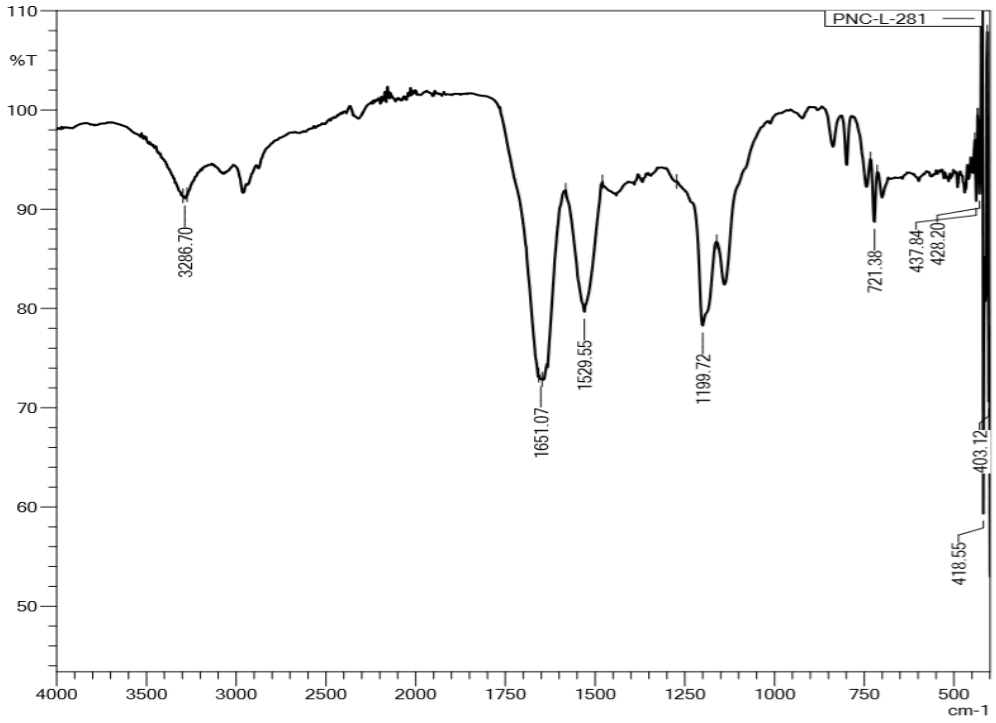

Fig5: IR Spectra of PNC-28 Peptide

Figure 6 and 7 represents the CD Spectra of PNC-28 Peptide.CD spectroscopy has been used in bioinorganic interface studies. Specifically, it has been used to analyse the differences in secondary structure of an engineered protein. The near-UV CD spectrum (>250 nm) of proteins provides information on the tertiary structure. The signals obtained in the 250–300 nm region are due to the absorption, dipole orientation and the nature of the surrounding environment of the phenylalanine, tyrosine, cysteine (or S-S disulfide bridges) and tryptophan amino acids. Unlike in far-UV CD, the near-UV CD spectrum cannot be assigned to any particular 3D structure. Rather, near-UV CD spectra provide structural information on the nature of the prosthetic groups in proteins, e.g., the heme groups in haemoglobin and cytochrome c.[23]

Integration of CD Data from zero:
i = 1 --> 61
x = 260 --> 200

Area		Peak at	Width	Height
59.09186	218	-16	-3.59216	

B represent the Phosphate Buffer solution CD absorption spectra. C, D, E, F represents the different concentration of synthesised peptide solution dissolved in water and make 400 microlitre solution. Sample C contained 15 microlitre PNC-28 Peptide in 385 microlitre water, Sample D contained 25 microlitre peptide in 375 microlitre water, Sample E contained 50 microlitre peptide in 350 microlitre water, and sample F contained 100 microlitre peptide in 300 microlitre water. The far-UV CD bands of proteins (between 200 to 260 nm) derive primarily from the peptide bonds and reflect the secondary structure of the protein (Fig. 7,8.) Circular dichroism (CD) spectra are recorded from proteins in phosphate buffer solution. The samples are filtered (0.2 µm) before use. The samples are placed in a quartz cuvette of 0.1 cm path length (Hellma) and measurements are performed using a JASCO-710 spectra polarizer. Spectra are recorded at room temperature from 260 to 200 nm at a scan speed of 50 nm/min and a resolution of 0.5 nm. 4 scans are accumulated and averaged. A spectrum of the buffer in absence of protein is recorded in the same manner.

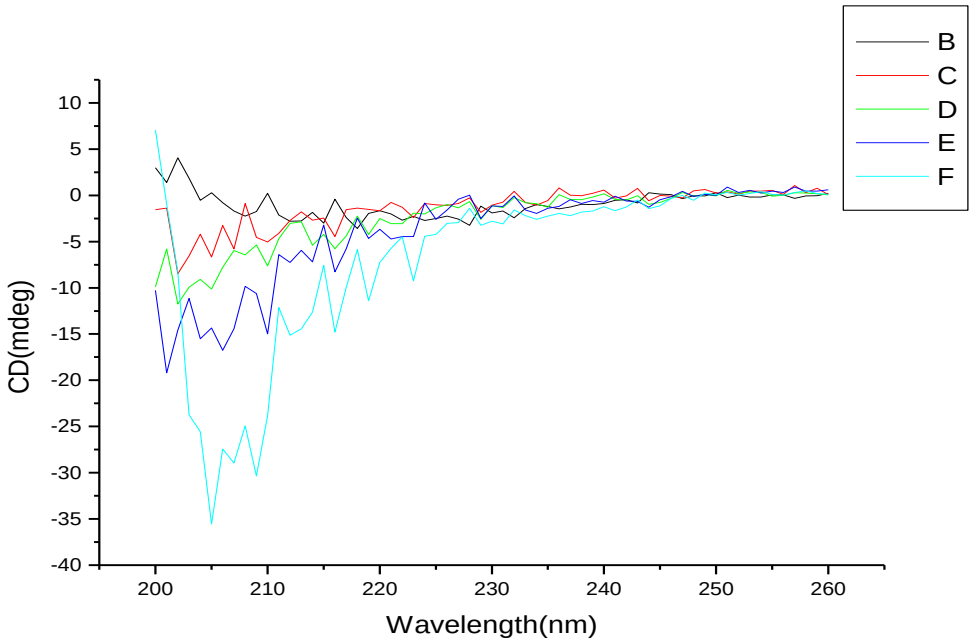

FIG 6: CD SPECTRA OF PNC-28 PEPTIDE

Integration of CD Data from zero:
i = 1 --> 61
x = 260 --> 200

Area	Peak at	Width	Height
-14.74447	200	-14	0.71023

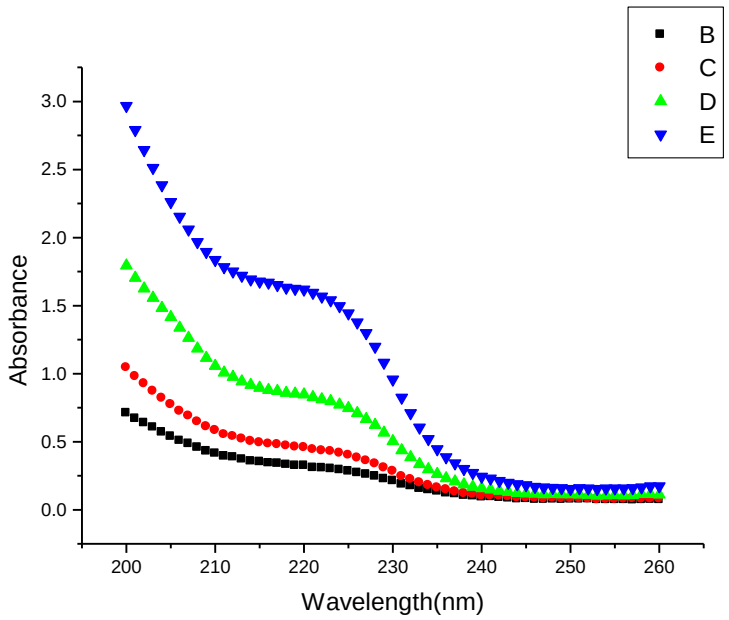

FIG 7: CD SPECTRA OF PNC-28 PEPTIDE

4. Conclusion

Our study presents the solid-phase peptide synthesis method for the synthesis of PNC-28 peptide manually without any difficulty and its characterization with the help of 1HNMR, CD, IR, LC-MS. PNC-28 induced cancer cell death in a variety of human cancer cells by inducing tumor cell necrosis rather than apoptosis. The anticancer activity and mechanism of PNC-28 (p53 aa17–26-penetratin) was specifically studied against human pancreatic cancer.

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