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Synthesis, Characterization and Studies on Anticancer activity of certain Polymers containing Arylidene moiety in their main chain

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Abstract
A series of novel random copolyesters and copolyesteramides containing arylidene moiety in their main chain were synthesized from 4.4'- oxybis(benzoic acid)/ 2,5-pyridine dicarboxylic acid and a common monomer diol, namely 2, 7-bis (4-hydroxy3-methoxybenzylidene) cycloheptanone with varying diols and diamines such as 1,8-dihydroxy anthraquinone, 4,4'- dihydroxy diphenyl, 1,4-cyclohexanediol, 4,4'- diamino diphenyl methane and 1,6- diaminohexane using diphenyl chlorophosphate as the condensing agent. Their structural features were investigated by FTIR, ^{1}HNMR and ^{13}CNMR spectral data and thermal behaviour was established by differential scanning calorimetry (DSC) and hot stage optical polarized microscopy (HOPM). Some of the synthesized polymers were tested for anticancer activity against lung cancer cells using MTT assay. These polymers showed strong anticancer activity against lung cancer cells and therefore have potential applications in biotechnology and biomedical science.

Keywords: Anticancer activity, MTT assay, Differential Scanning Calorimetry, Hot Stage Optical Polarized Microscopy

Introduction
Cancer, the uncontrolled growth of cells is a leading cause of death worldwide.[1] Radiotherapy and surgery are the most commonly used methods for the treatment of local and non-metastatic cancers, but chemotherapy is the main treatment for metastatic cancers. Chemotherapy uses anticancer drugs which are intended to inhibit the fast proliferation of cancer cells but inevitably, the lack of selectivity will damage healthy tissues leading to adverse side effects. These facts originate the need for higher drug dose administration, without undesired side effects.[2- 4] Many researchers reported that bis(benzylidene) cycloalkanones (BBCA) can be used as cost effective drug against cancer. Dimmock *et al.*[5,6] synthesized a series of 2, 6-bis(arylidene) cycloalkanones and reported that they are used as potential cytotoxic analogue against human tumour cells.
This review aims to investigate the anticancer activity of some of the synthesized copolyesters and copolyesteramide against lung cancer cells using MTT assay.

Experimental
Materials and Methods
Materials
Lithium chloride (Merck, India), dry methanol, pyridine (Merck, India), N, N-Dimethyl formamide and DMSO were used after purification.
Aldrich samples of Vanillin (99%), 4, 4'-oxybis (benzoic acid) (OBBA), 2,5-pyridinedicarboxylic acid (PDCA), 1,8-dihydroxyanthraquinone (99%) (DHAQ), 4,4'-dihydroxydiphenyl (DHDP), 1,4-cyclohexanediol, 4,4'-diaminodiphenylmethane (DADPM), 1,6-diaminohexane (HMDA), diphenylchlorophosphate (99%) (DPCP) and cycloheptanone (99%) (CH) were used as supplied.

Cell line
Cell lines were obtained from National center for cell sciences, Pune. The cells were maintained in Minimal Essential Media (MEM) supplemented with 10% FBS, penicillin (100 /ml) and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37 °C.

Reagents

MEM, Fetal bovine serum (FBS), Trypsin, methylthiazolyl biphenyl tetrazolium bromide (MTT) and Dimethyl sulfoxide (DMSO) were purchased from Hi media & Sigma Aldrich Mumbai.

Method

Preparation of Monomer Arylidene diol (ADCH) -2, 7-bis (4-hydroxy 3-methoxy benzylidene) cycloheptanone[7- 9]

Arylidene diol [2, 7-bis (4-hydroxy3-methoxybenzylidene)cycloheptanone] used as one of the common monomer in the synthesis of random copolyesters and copolyesteramides was prepared by the condensation of cycloheptanone and vanillin in the mole ratio 1:2.

Synthesis of random copolyesters and copolyesteramides

Direct polycondensation of one dicarboxylic acid and two different diols/ one diol and one diamine in the mole ratio 2:1:1 using diphenylchlorophosphate in pyridine was used for the synthesis of all the six novel random copolymers.[10, 11]

In vitro assay for Cytotoxicity activity (MTT assay)

The anticancer activity of samples on Lung Cancer cells (A549) was determined by the MTT assay.[12] Cells (1 × 10^5/well) were plated in 0.2 ml of medium/well in 96-well plates. Incubate at 5 % CO_2 incubator for 72 h. Then, add various concentrations of the samples in 0.1% DMSO for 24 h at 5 % CO_2 incubator. After the removal of the sample solution and 20µl/well (5mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) in phosphate- buffered saline solution was added. After 4 h incubation, 1 ml of DMSO was added. Viable cells were determined by the absorbance at 540 nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC50) was determined graphically. The effect of the samples on the proliferation of A549 cells was expressed as the % cell viability, using the following formula:

Calculation

% cell viability = A540 of treated cells / A540 of control cells × 100%

Results and Discussion

All the polymers were synthesized by the direct polycondensation of diacid and diol/diamine monomers using DPCP as the condensing agent. This method avoided the tedious preparation of acid derivatives. The time required for the polymerization is only 3 h under mild conditions. The shorter time duration and low temperature required for polymerization reduces the risk of degradation of monomers, thereby enhances the molecular weight of polymers. The inherent viscosities (ηinh) of the polymers listed in Table 2 indicated that the synthesized polymers have high molecular weight.

Solubility of copolymers

The polymers reported here are found to be soluble in highly polar solvents, less soluble in moderately polar solvents and sparingly soluble in non-polar solvents. The results of the solubility of polymers are presented in Table 1. The present investigation showed appreciable solubility in polar solvents. The incorporation of arylidenediol with 4, 4'-oxybis (benzoic acid) and 2, 5-pyridine dicarboxylic acid along with other monomer diol and diamine provide structural variants which enhances solubility of these polymers in common polar organic solvents.

Table 1: Solubility of copolymers

S.No	Polymer code	DMSO	DMF	DMAc	NMP	THF	$CHCl_3$	Pyridine	C_6H_6	C_6H_{14}	CH_3OH
1	OADA	++	++	++	++	++	+-	++	--	--	--
2	OADP	++	++	++	++	++	--	++	--	--	--
3	PADP	++	++	++	++	++	--	++	--	--	--
4	PACH	++	++	++	++	++	--	++	--	--	--
5	PADM	++	++	++	++	++	--	++	--	--	--
6	PAHA	+-	++	++	++	++	--	++	--	--	--

++ = highly soluble +- = less soluble -- = sparin gly insoluble

Spectral characterization

The structural characterization of polymers was done with the help of FT-IR spectra. The representative FT-IR spectra of the polymers OADA and PADM are shown in Fig.1 and FTIR data are indicated in Table 2.

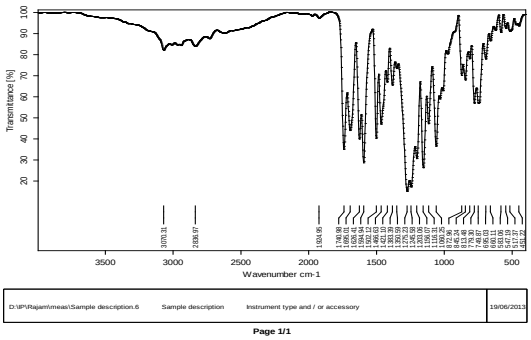
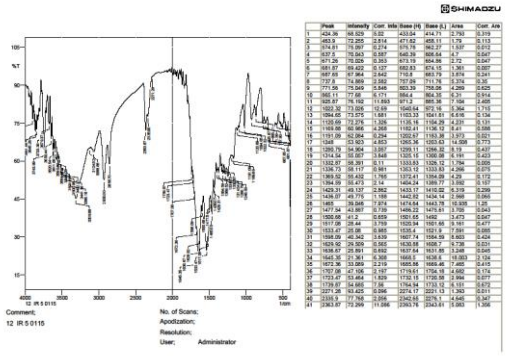


Fig. 1: FTIR Spectrum of OADA



FTIR Spectrum of PAHA

Table 2: Physical properties of random copolymers and their FTIR data

S. No	Polymer code	Monomers	Yield %	Colour	η_{inh} dL/g	C=O	C=C	C=C Aromatic	CONH	N-H	C-OH	C=O Arylidene ketone
1	OADA	OBBA+AD +DHAQ	75	Orange	1.005	1740	1594	1502	-	-	-	1696
2	OADP	OBBA+AD +DHDP	70	Grey	1.041	1740	1594	1501	-	-	-	1694
3	PADP	PDCA+AD +DHDP	62	Grey	1.15	1715	1595	1479	-	-	-	1686
4	PACH	PDCA+AD +CHD	60	Grey	1.18	1718	1596	1483	-	-	-	1671
5	PADM	PDCA+AD +DADPM	70	Dark Brown	1.17	1715	1592	1509	2924	1651	3363	1697
6	PAHA	PDCA+AD +HMDA	54	Dark Brown	1.09	1706	1566	1512	2930	1646	3304	1691

Note: OBBA: 4, 4'-oxybis(benzoic acid), PDCA: 2, 5-pyridine dicarboxylic acid,
DHAQ: 1,8- dihydroxyanthraquinone, AD: 2, 7-bis (4-hydroxy 3-methoxy benzylidene) cycloheptanone,
DHDP: 4, 4'-dihydroxydiphenyl, CHD: 1, 4- cyclohexanediol,
DADPM: 4,4'-diaminodiphenyl methane, HMDA: 1,6- diamino hexane

Structural units of the polymers were identified by ^{1}HNMR and ^{13}CNMR spectra. The representative ^{1}HNMR spectra of polymers OADA and PADP recorded in DMSO-d_6 are exposed in Fig.2 and ^{13}CNMR spectrum in Fig.3.

The signal at 2.5 δ is due to CH_2 protons of cycloheptanone. The aromatic protons of dicarboxylic acid OBBA and PDCA appeared in the region of 7.8 to 8.5 δ. The benzylidene aromatic protons appeared in the region of 7.2 to 7.7 δ. The protons attached with exocyclic carbon- carbon double bond are indicted by the signal at 7.5 δ and –OCH_3 protons at 3.3 δ. The aromatic protons of monomer DHAQ appeared in the region of 7.2 to 7.4 δ and DHDP around 8 δ. This concludes that all the three monomeric units are present in the random polymeric backbone.

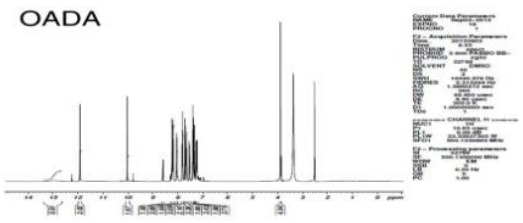
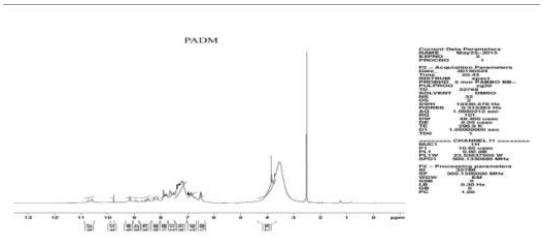


Fig.2: ^{1}HNMR spectrum of OADA



^{1}HNMR spectrum of PADM

The micro structure of repeat units in the polymer chain can be identified satisfactorily using ^{13}CNMR spectra. The signal at 54 ppm was assigned to the presence of –OCH_3 group. The signal in the region of 121 – 142 ppm was significantly aromatic carbons. The olefinic carbons of arylidene moiety appeared at 149- 151ppm. The ester carbonyl carbons were indicated by the signals from 156 – 166 ppm. The signals at 179 and 189 ppm were attributed to the carbonyl group of cycloheptanone and anthraquinone.

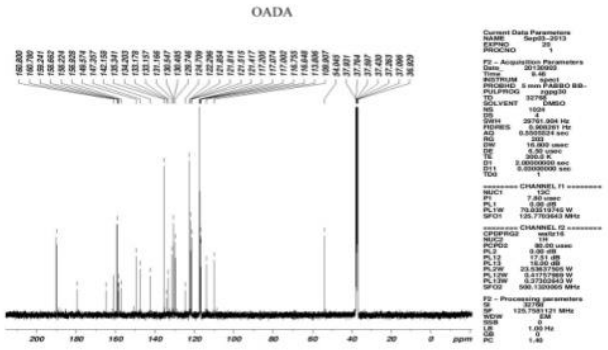
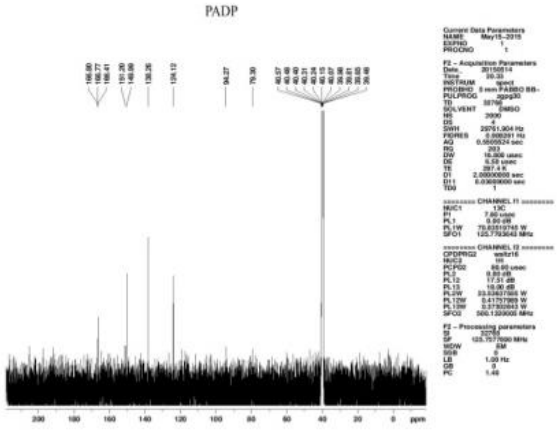


Fig.3: ^{13}CNMR spectrum of OADA



^{13}CNMR spectrum of PADM

Thermal properties

The thermal transition temperature of random copolymers was investigated from DSC thermogram and hot stage optical polarized microscopy. A representative DSC thermogram of OADA and its HOPM pictures are shown in Fig. 4 and Fig.5. The thermal properties deduced from DSC traces are presented in Table 3.

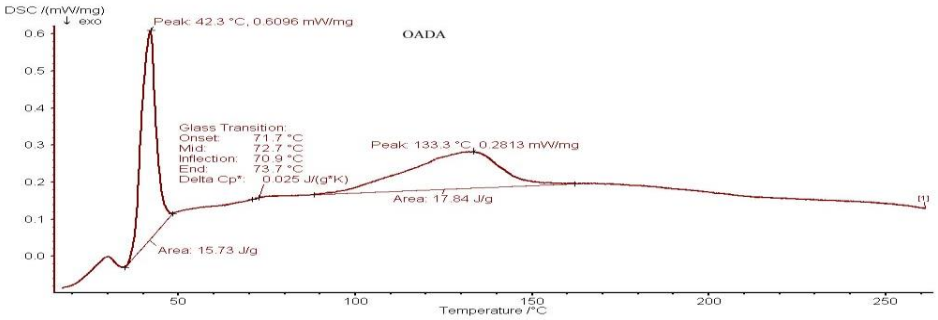


Fig.4: DSC thermo gram of OADA

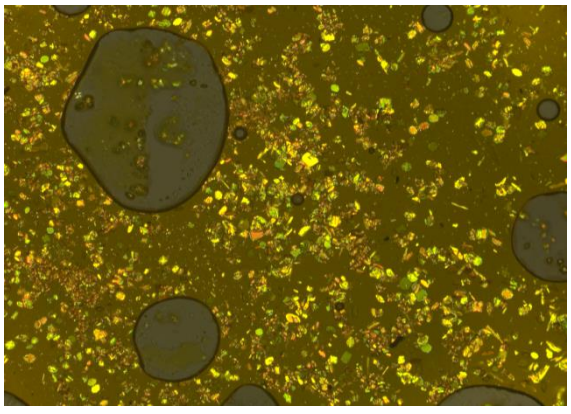


Fig.5: HOPM picture of OADA at 95 ^{0} C on cooling

Table 3: Thermal properties deduced from DSC traces

S. No	Polymer code	T_g ^{0}C	$T_{k\text{-}k}$ ^{0}C	T_m ^{0}C	ΔHJ/K	T_i ^{0}C
1	OADA	70.9	-	133.3	-	-
2	OADP	45.3	139.7	205.9	-	180.3
3	PADP	86.8	-	335.9	343	-
4	PACH	92.2	-	328.9	265	-
5	PADM	56.1	-	>340	-	
6	PAHA	84	146.7	>340	-	

The DSC thermo grams revealed that the synthesized polymers undergo more than one phase transition processes, when subjected to thermal treatment.

Studies on anticancer activity

In the present investigation anticancer activity of some of the synthesized polymers namely OADA, PADP and PADM against lung cancer cells (A549) was determined by MTT assay. Viable cells were determined by the absorbance at 540 nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC50) was determined graphically. The effect of the samples on the proliferation of A549 cells was expressed as the % cell viability. IC_{50} of the copolyesters OADA, PADP and copolyesteramide PADM were determined and shown in Table 4, Table 5 and Table 6. The assessment of cell viability by MTT assay through graphical representation is shown in Fig. 5 whereas the photographs are displayed in Fig. 6. Surprisingly, these observations indicate that all the polymers under study are showing excellent inhibition against lung cancer cell. The covalent conjugation of 4-arylidene moiety through ester and linkages increase its molecular size and steric hindrance which may improve its cellular permeability and restrict its cancer progression.[12, 13] Electron releasing methoxy group at the meta position of benzene ring also enhances the anticancer and antioxidant activity of copolyesters and

copolyesteramides.[14] In the present study IC50 values of the copolymers OADA, PADP and PADM are found approximately as 1.35 µg, 2.028 µg and 3.744 µg respectively.

Table 4: % Cell viability of OADA by MTT assay

S.No	Concentration µg/ml	Dilution	Absorbance 540nm	% cell Viability
1	200	Neat	0.00	0.0
2	100	1:1	0.00	0.0
3	50	1:2	0.00	0.0
4	25	1:4	0.02	2.3
5	12.5	1:8	0.07	8.2
6	6.25	1:16	0.19	22.3
7	3.12	1:32	0.21	24.7
8	1.56	1:64	0.37	43.5
9	Control	-	0.85	100

Table 5: % Cell viability of PADP by MTT assay

S.No	Concentration µg/ml	Dilution	Absorbance 540nm	% cell Viability
1	200	Neat	0.00	0.0
2	100	1:1	0.00	0.0
3	50	1:2	0.02	0.0
4	25	1:4	0.07	8.2
5	12.5	1:8	0.13	15.2
6	6.25	1:16	0.17	20.0
7	3.12	1:32	0.30	35.2
8	1.56	1:64	0.49	57.6
9	Control	-	0.85	100

Table 6: % Cell viability of PADM by MTT assay

S.No	Concentration µg/ml	Dilution	Absorbance 540nm	% cell Viability
1	200	Neat	0.00	0.0
2	100	1:1	0.00	0.0
3	50	1:2	0.03	3.5
4	25	1:4	0.09	10.5
5	12.5	1:8	0.15	17.6
6	6.25	1:16	0.27	31.7
7	3.12	1:32	0.45	52.9
8	1.56	1:64	0.53	62.3
9	Control	-	0.85	100

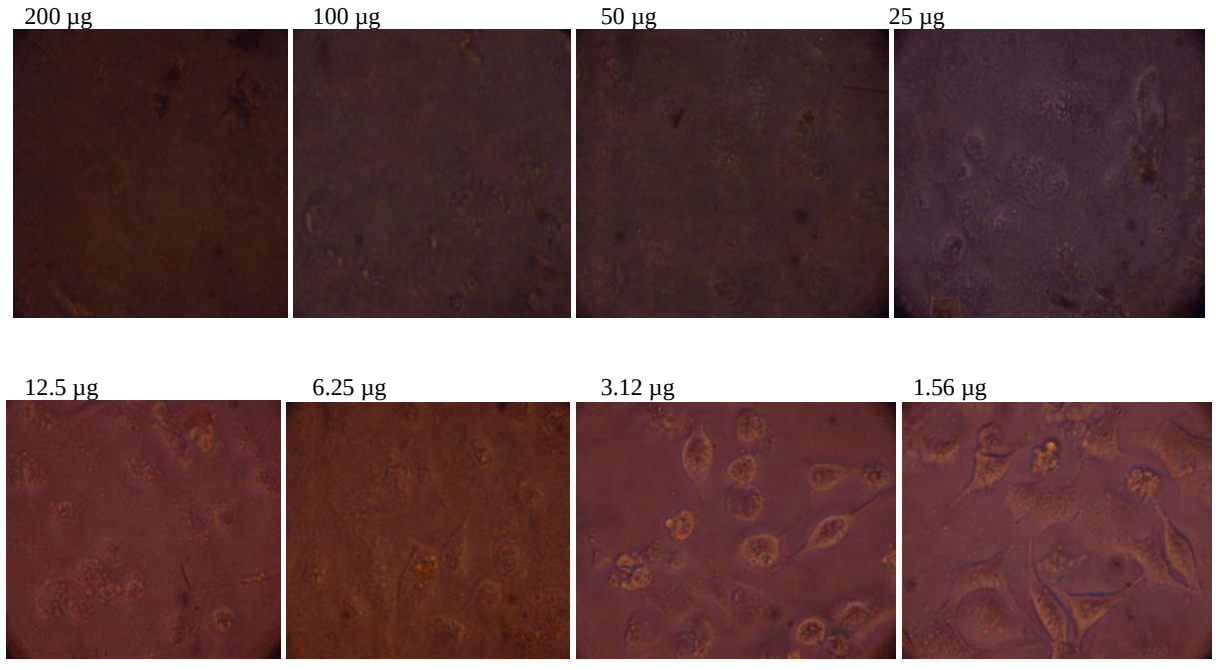


Fig. 6: Cell viability of OADA by MTT assay

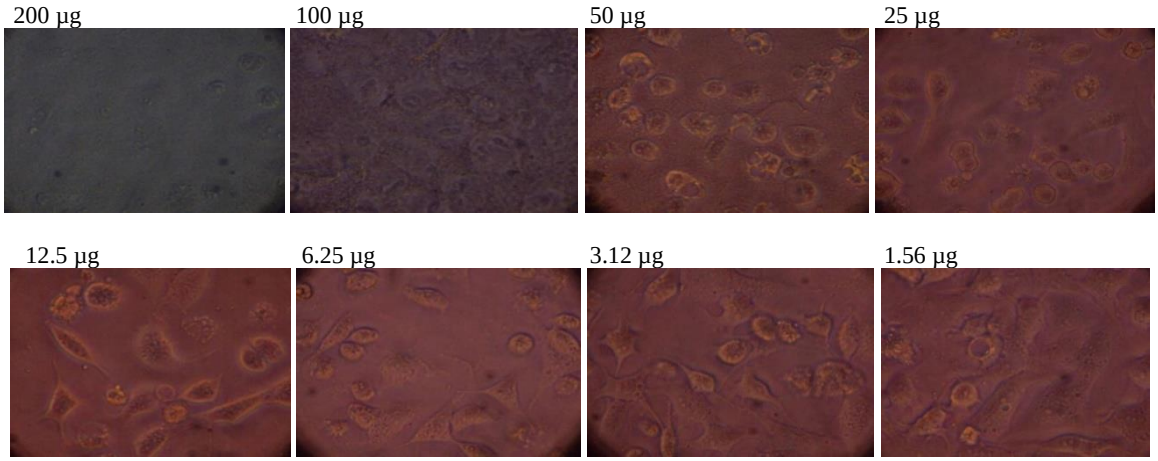


Fig. 7: Cell viability of PADP by MTT assay

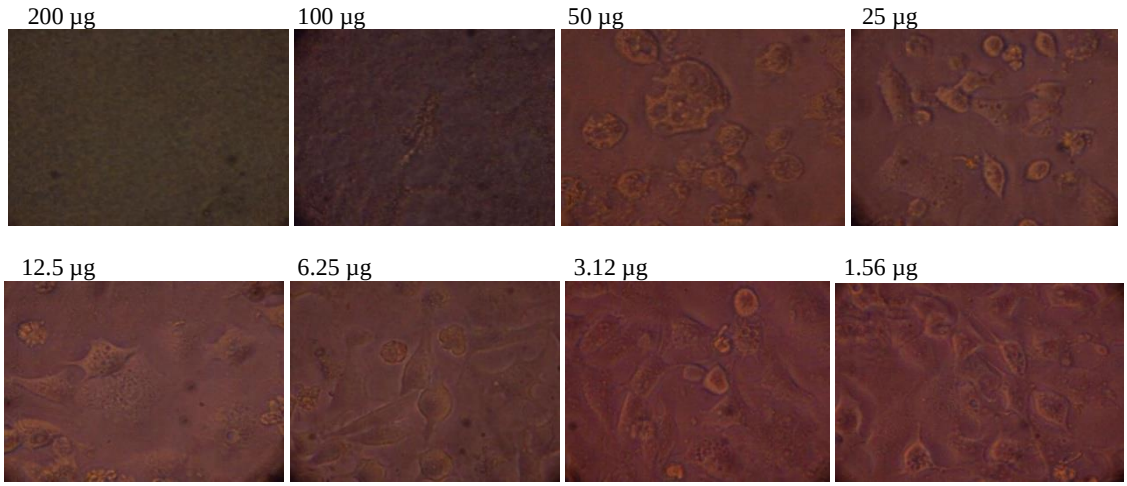
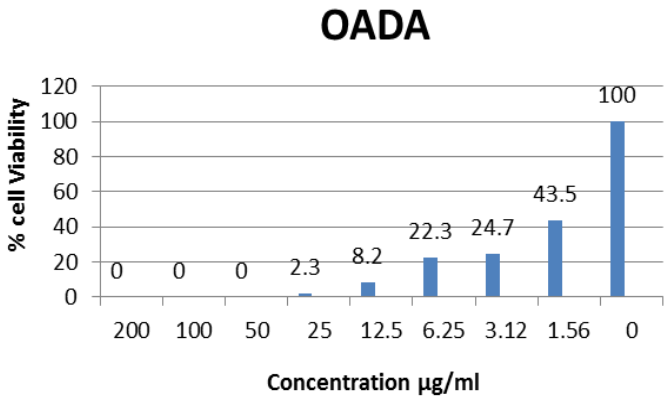


Fig. 8: Cell viability of PADM by MTT assay



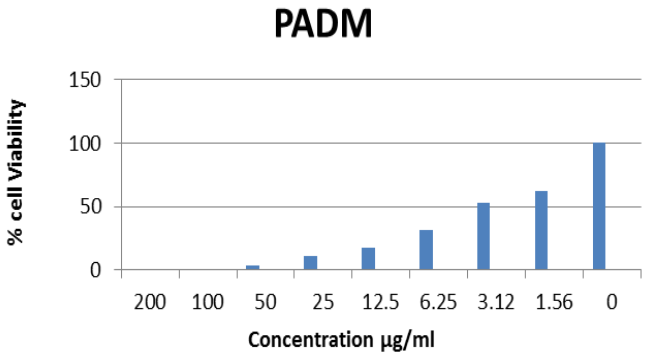
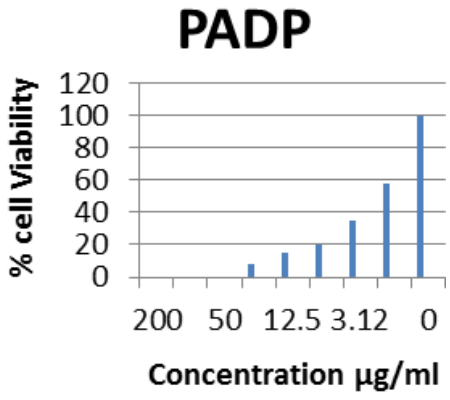


Fig. 6: %Cell viability by MTT assay



Conclusion

Six novel random copolyesters and copolyesteramides OADA, OADP, PADP, PACH, PADM and PAHA were synthesized successfully from 4,4'- oxybis(benzoic acid)/ 2,5-pyridine dicarboxylic acid and 2, 7-bis (4-hydroxy 3-methoxybenzylidene) cycloheptanone with varying diols and diamines such as 1,8-dihydroxy anthraquinone, 4,4'-dihydroxy diphenyl, 1,4-cyclohexanediol, 4,4'- diamino diphenyl methane and 1,6- diaminohexane by direct polycondensation with diphenylchlorophosphate and characterized spectroscopically. The spectral data supported the structural assignment of the polymers. The inherent viscosity data reveals that the polymers have high molecular weight. Presence of arylidene moiety has positive effect on the solubility of copolymers. DSC

thermograms and HOPM pictures revealed that the synthesized polymers undergo more than one phase transition, when subjected to thermal treatment. Some of the synthesized polymers tested for anticancer activity against lung cancer cells using MTT assay showed an excellent anticancer activity against lung cancer cells.
It can be concluded that the preparation of random copolyesters and copolyesteramides could find potential application in several areas of biotechnology and bio medical science.

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