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Anti-bacterial activity, anti-cancer activity and nanofiber formation of certain poly (ester amides) from 2,5-pyridine dicarboxylic acid

V. Chitra[1*], D. Roop Singh[2]

[1]Research Scholar, PG &Research Department of Chemistry, Presidency College, University of Madras,Chennai-600 005, India

[2]Head & Associate Professor, PG & Research Department of Chemistry, Presidency College, University of Madras, Chennai-600 005, India

ABSTRACT

A new series of four poly(ester amides) were synthesized by direct polycondensation of 2,5-pyridine dicarboxylic acid with two different varying diols and diamines in pyridine medium using diphenylchlorophosphate as a condensation agent. The two diamines employed in the synthesis were 4,4'-diamino diphenyl methane and 1,4-diamino benzene. The arylidenediols 2,5-bis(4-hydroxy-3-methoxybenzylidene)cyclopentanone and 2,6-bis(4-hydroxy-3-methoxybenzylidene) cyclohexanone were also used. The synthesized poly(ester amides) were characterized by qualitative solubility test, FT-IR, ^{1}H and ^{13}C-NMR spectra. The monomeric moieties were found by spectroscopic analysis to be well incorporated in the polymer back bone. The thermal phase transition behavior of the poly(ester amides) were investigated by differential thermo gravimetry (DTG). The nanofibers of synthesized poly(ester amides) with PVC and composite fibers with PVC/nanoclay were formed by electrospinning. The morphology of these composite fibers was studied by scanning electron microscopy (SEM). These poly(ester amides) were screened for their anti-bacterial potential *in vitro* against *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus* and *Bacillus subtilis. In vitro* anti-cancer activities of synthesized polymers were also evaluated against MCF7 human breast carcinoma cells. The results show that the polymers exhibited significant anti-bacterial and anti-cancer activity.

Keywords: Poly (ester amides), Anti-bacterial activity, Nano fiber, Anticancer activity.

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***Address for Correspondence:**

V. Chitra, Research Scholar, PG &Research Department of Chemistry, Presidency College, University of Madras,Chennai-600 005, India

INTRODUCTION

Poly(ester amides) (PEAs) are considered as promising polymeric materials because they possess the properties of both polyesters and polyamides[1,2]. Recently much emphasis has been given to biomedical applications of PEAs which focused on their applications in drug delivery systems[3]. PEAs have attracted great interest since they can be modified based on the great variety of monomers which can be used. This leads to the development of PEAs with different composition and microstructure which make them suitable for wide range of applications[4,5]. Polymeric drug delivery systems are an important developing technology. The new and improved durg delivery methodology in the several polymer-drug systems were discussed by Suhas Thatte and co workers[6]. Laxshmi S. Nair *et al*[7]. reviewed the synthesis, biodegradability and bio medical applications of both natural and synthetic polymeric biomaterials. Synthetic biomaterials are generally biologically inert having tailored property profiles for specific applications. Jonathan *et al*[8]. reported the preparation of 2,6 –bis (arylidene cyclohexanones) and related analogues. They found that these compounds were cytotoxic to a number of human tumours *in vitro*, particularly towards colon cancer and leukemic cells. Electrospinning method is an efficient technique to produce polymeric nanofiber layers. The polymeric nanofibers exhibit unique characteristics for several applications have been addressed and focusing the influence of electrospinning on recent developments in the biomedical field[9]. The preparation of nanofiber layers of poly(ester amides) and the effect of the system parameters on morphology of fiber layers being formed during the electrostatic wet spinning was mentioned[10]. Electrospun poly(ester amides) with appropriate modification provide a versatile material for scaffold fabrication in tissue

engineering[11]. Functionalized PEAs can be subjected to subsequent chemical attachment of bioactive agents and thus making these polymers effective for drug delivery system[12]. The present investigation is to focus on the study of anti-bacterial, anti-cancer activities and nanofiber formation of the synthesized PEAs.

MATERIALS AND METHODS

Materials

Aldrich samples of 2,5-pyridine dicarboxylic acid, 4,4′-diamino diphenyl methane, 1,4-diamino benzene, diphenylchlorophosphate (99%) and anhydrous lithium chloride (99%) were used as received. cyclopentanone and cyclohexanone were purchased from SRL. They were freshly distilled at their boiling point. Merck sample of pyridine was used after purification. Merck samples of other solvents such as tetrahydrofuran and methanol were distilled at their boiling point before use. Spectral grade DMSO-d_6 (Aldrich) containing TMS as internal standard was used as such for recording NMR spectrum.

Preparation of Arylidene diol

The arylidene diol namely 2,6-bis(4-hydroxy-3-methoxy benzylidene) cyclohexanone was prepared by the procedure reported by Mayavathi and coworkers[13] (Figure 1).

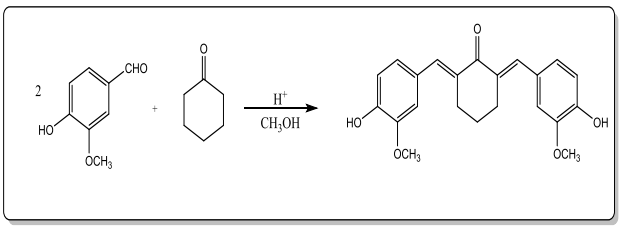


Figure 1: Synthesis of 2,6- bis(4-hydroxy-3-methoxy benzylidene) cyclohexanone

Synthesis of poly (ester amides)

The polymers (Table 1) were synthesized by direct polycondensation by the following procedure: In a three necked 250 ml round bottomed flask equipped with a condenser, thermometer and mechanical strirrer (400 rpm/min) in an oil bath, 0.835 g (5 mmol) of 2,5-pyridine dicarboxylic acid in 10 ml pyridine and 2.694 ml (13 mmol) DPCP were added. After stirring for 20 min, 0.4250 g (10 mmol) of LiCl in 10 ml pyridine was added and stirring was continued at room temperature for 30 min. The reaction mixture was slowly heated for 20 min. To this mixture, 0.92g (2.5 mmol) of diol 2,6-bis(4-hydroxy-3-methoxy benzylidene) cyclohexanone in 5 ml pyridine and 0.27 g (2.5 mmol) of diamine 1,4-diamino benzene in 5 ml pyridine were added and maintained at 120 ^{0}C for 3 hours. The solution then allowed to cool and poured into 500 ml water/methanol (1:1 v/v) mixture. The product was filtered, washed with hot methanol and dried in sunlight.

Table 1: Polymer code, Monomers used, Yield, Inherent Viscosity and Colour

S.No	Polymer Code	Monomers	Yield (%)	Inherent viscosity η_{inh}(dL/g)	Colour
1	PAPM	PD+AP+DM	84	1.16	Grey
2	PAPB	PD+AP+DB	65	1.20	Black
3	PAHM	PD+AH+DM	78	1.18	Black
4	PAHB	PD+AH+DB	80	1.23	Brown

PD: 2,5-pyridine dicarboxylic acid; AP: 2,5-bis(4-hydroxy-3-methoxy benzylidene) cyclopentanone;
AH: 2,6-bis(4-hydroxy-3-methoxybenzylidene) cyclohexanone; DM: 4,4′-diamino diphenyl methane;
DB: 1,4-Diamino Benzene.

Nanofiber formation

THF (5 ml) was taken in 10 ml closed container with pellet in which 0.3 g polyvinylchloride (PVC) was added and stirred for 30 min and then 0.15 g of PAPM was added and stirring continued for 30 min. The solution was removed and placed in an ultrasonicator. The ultrasonicator was run for 20 min to get homogeneous mixture of sample solution. The homogeneous solution was taken in a 2 ml syringe and fitted with the adjustable knob of the electrospinning instrument. A positive voltage was applied to the polymer blend solution through the needle attached to the syringe containing the solution. The solution jet was formed by electrostatic force, when the electrical potential inceased to 20 KV. The flow rate of the solution was set at 0.5 ml/h, which was adjusted by computer controlled syringe pump. The distance between the needle tip and collector was maintained at 12 cm and the drum collector rotation speed around 1800 rpm. The PAPM/PVC nanofibers in a nonwoven form were collected on an aluminium foil.

Antibacterial Study

The antibacterial activity of all the four poly (ester amides) was studied against *pseudomonas aeruginosa, escherichia coli, staphylococcus aureus and bacillus subtilis* pathogens by well diffusion method. The following concentrations of the PEAs were taken in DMSO 25, 50, 75 and 100µg/ml. The target microorganisms were cultured in Mueller–Hinton broth (MHB). After 24 hours the suspensions were adjusted to standard sub culture dilution. The Petri dishes containing Muller Hinton Agar (MHA) medium. The agar plates were seeded with freshly prepared different pathogens. The standard drug streptomycin (10µg) was used as a positive reference standard to determine the sensitivity of each polymer tested. Then the plates were incubated at 37 ºC for 24 h. The diameter of the clear zone around the disc was measured and expressed in millimeters as its anti-microbial activity.

MTT assay

The anticancer activity of samples on Breast Cancer cell (MCF7) and VERO cells were determined by the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide) assay was used to assess the cytotoxicity[14,15]. 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 hours. Then, add various concentrations of the samples in 0.1% DMSO for 48 hrs at 5 % CO_2 incubator. After removal of the sample solution and 20µl/well (5mg/ml) of 0.5%

(MTT) in phosphate- buffered saline solution was added. After 4hrs incubation, 1ml of DMSO was added. Presence of viable cells was determined by the absorbance at 540nm. 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 MCF7 cells was expressed as the % cell viability, using the following formula:

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

RESULTS AND DISCUSSION

Solubility of poly (ester amides):

The solubility of all the poly(ester amides) synthesized are listed in Table 2. The poly(ester amides) derived from aromatic dicarboxylic acid were found to be easily soluble in highly polar solvents such as dimethylformamide(DMF), dimethylacetamide (DMAc) dimethyl sulphoxide(DMSO) and tetrahydrofuran (THF) partially soluble in acetone, chloroform and sparingly soluble in common organic solvents like methanol, benzene and hexane.

Table 2: Solubility of the poly(esteramides)

S.No	Polymer	Hexane	Benzene	$CHCl_3$	THF	Acetone	CH_3OH	DMF	DMAc	DMSO
1	PAPM	-	-	+-	+	+-	-	+	+	+
2	PAPB	-	-	+-	+	+-	-	+	+	+
3	PAHM	-	-	+-	+	+-	-	+	+	+
4	PAHB	-	-	+-	+	+-	-	+	+	+

+ =highly soluble; +- = partially soluble; - = sparingly soluble

Spectral analysis

The representative FT-IR spectrum of poly(ester amide) PAHM is shown in Figure 2. The presence of a sharp band at 1738 cm^{-1} is attributed to the C=O stretching of the ester group. The arylidene ketone showed their characteristic carbonyl stretching frequency at 1672cm^{-1}. The bands at 1595 and 1521cm^{-1} indicates C=C stretching in aromatic rings. The absorption due to NH bond of amide linkage is observed at 3337cm^{-1}. The absorption in the range of 1266cm^{-1} indicated C-O-C of ester group and other characteristic absorptions appeared in the IR spectra indicate, that the monomers are well incorporated into the polymer backbone of the poly(ester amides).

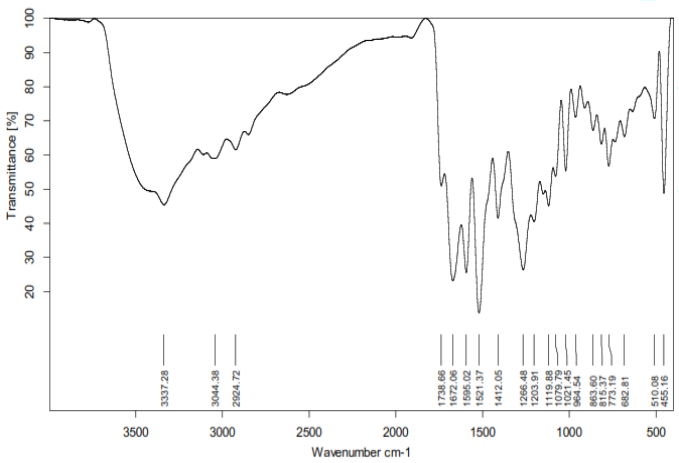


Figure 2: FT-IR Spectrum of PAHM

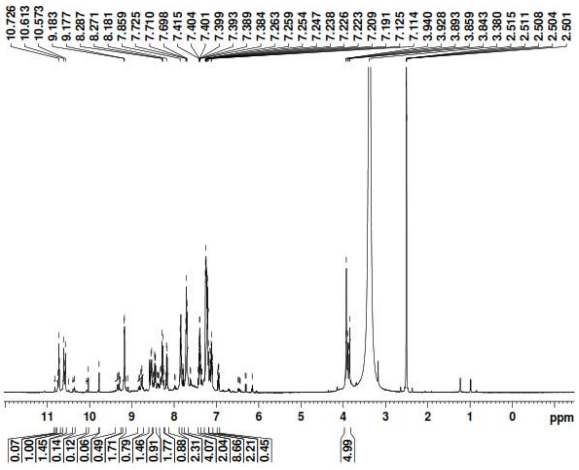


Figure 3: ^{1}H NMR Spectrum of PAHM

The structural units of the poly(ester amides) were identified by ^{1}H and ^{13}C NMR spectra. The representative ^{1}H NMR spectrum of poly(ester amide) PAHM recorded in DMSO-d6 is given in Figure 3. The peaks at 10.5-10.7δ is attributed to secondary amide proton. The aromatic protons of dicarboxylic acid appeared in the region of 8.1-8.2 δ. The benzylidene aromatic protons appeared in the range of 7.1-7.8 δ. The peak at 6.9 δ is due to vinyllic proton associated with the cyclohexyl ring. The peak corresponds to the methylenic proton attached to N- atom appeared in 2.5 δ. This data indicated that, all the monomers are present in the polymer backbone.

A typical C^{13} NMR spectra of poly(ester amide) PAHM is shown in Figure 4. The aromatic protons were indicated by the signals from 120-137 ppm. The signals from 148- 152 ppm was assigned to the olefinic carbon atoms of the arylidene moiety. The presence of ester and amide carbonyl groups in the polymer backbone was indicated by the signal at 162 and 166ppm respectively. The signals in

the range of 39-40 ppm assigned to the methylenic carbon of cyclohexanone.

Thermal Analysis:

DTG thermograms were recorded in nitrogen atmosphere at a heating rate of 10 °C/min from RT-600°C for the poly(ester amide)PAHM in order to detect the phase transitions (Figure 5). Similar DTG thermograms were obtained for all the PEAs. The thermal transition can be studied from the DTG thermograms. From the data, it can be observed that the sample melting from 263.9°C and the final clearance occurs at 453.8°C.

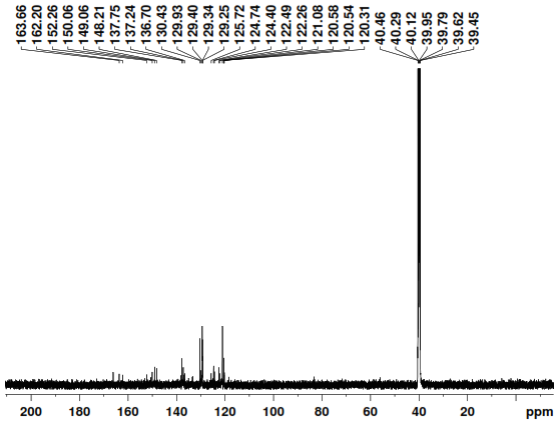


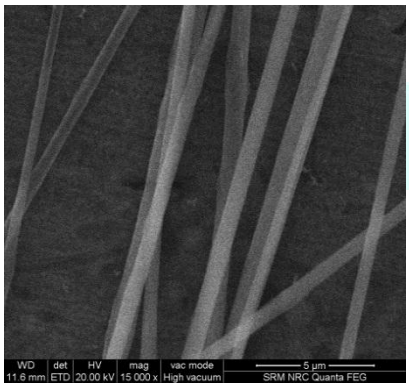
Figure 4: C^{13} spectrum of PAHM

Figure 5: DTG thermogram of PAHM

Morphological study by SEM

The electrospinning method was effectively utilized to embed poly(ester amide) in a polyvinylchloride(PVC) matrix, forming blend nanofibers. The morphology of polymer/PVC nano fibers and polymer/PVC/nano clay composite fibers has been studied with the help of SEM. The SEM images of all the four blend nanofibers of PEAs are shown in Fig.7. The fibers were found to be uniform, well dispersed and without beads on their surface. There is no significant disruption in the fiber structure due to the addition of PEA to PVC. These fibers are thus potential candidates for bio medical applications.

a



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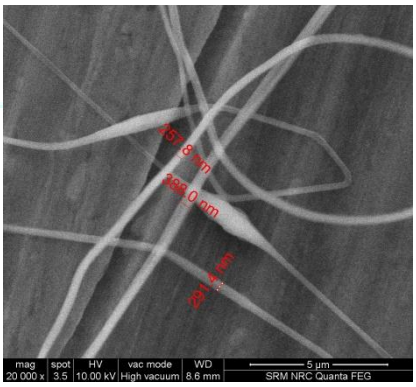

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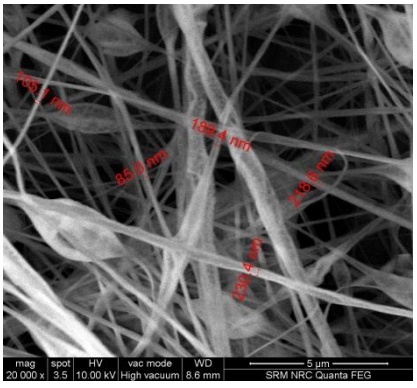

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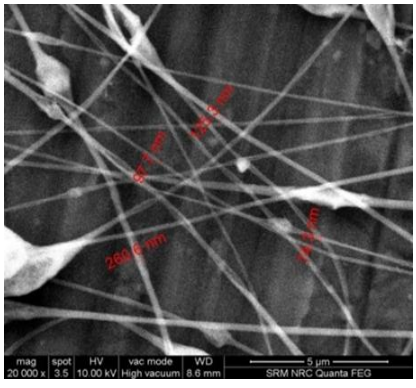


Figure 6: SEM images of (a) PAPM Polymer/PVC (b) PAPM Polymer/PVC/Nano clay

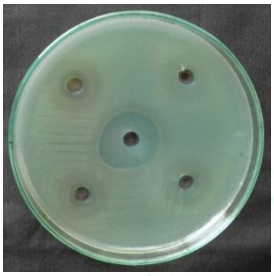
(c) PAHM Polymer/PVC/ (d) PAHM Polymer/PVC/nano clay

Antibacterial studies

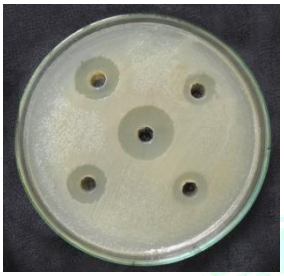
All of the polymers at different concentration were screened for their *in vitro* antibacterial activity against gram-positive bacteria namely *staphylococcus aureus and bacillus subtilis* and gram-negative bacteria namely *pseudomonas aeruginosa, escherichia coli* using Agar well-diffusion method by measuring the zone of inhibition in mm. Streptomycin (10µg/disk) was used as standard drug for antibacterial activity.The inhibition zones of the PEAs are listed in Table 3. The table shows that, each poly(ester amide) had high inhibition activity against specific bacteria. These results indicate that the antibacterial activity is mainly due to the presence of electron donating methoxy group in benzene ring[16].

Table 3: Inibition effects of the four poly(ester amides) on the growth of pathogenic bacteria.

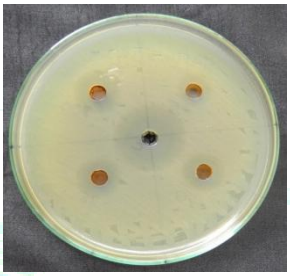
Micro Organism	PAPM				PABM				PAHM				PAHB			
	25	50	75	100	25	50	75	100	25	50	75	100	25	50	75	100
	Concentration µg/ml															
P.aeruginosa	-	4	6	9	-	-	-	4	-	4	7	10	-	5	8	10
E. coli	10	11	12	13	5	9	11	14	5	7	10	13	-	9	10	14
S. aureus	4	6	9	11	-	-	4	8	4	7	10	13	-	5	10	15
B. subtilis	9	11	12	15	2	3	5	12	4	5	12	16	-	-	-	4



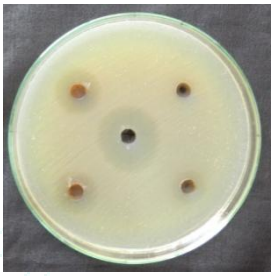
a **Pseudomonas aeruginosa**



b **Escherichia coli**



C **Staphylococcus aureus**



d **Bacillus subtilis**

Figure 7: Inhibition effects of poly(ester amide) PAHM

Anticancer Studies

To evaluate the cytotoxic activity of some of the synthesized poly(ester amides) namely PAPB and PAHM against human breast cancer cells (MCF7) and African Green Monkey kidney cell lines (VERO) were determined by MTT assay. The results of cytotoxicity assay are presented in Table 4, 5 and 6.

Table 4:% cell viability of PAHM

S.No	Concentration µg/ml	Absorbance 540nm	% cell Viability
1	100	0.03	2.5
2	50	0.09	7.7
3	25	0.21	18.1
4	12.5	0.43	37.0
5	6.25	0.86	74.1
6	3.12	0.98	84.4
7	Control cells	1.16	100

Table 5:% cell viability of PAPB

S.No	Concentration µg/ml	Absorbance 540nm	% cell Viability
1	100	0.01	0.9
2	50	0.04	3.9
3	25	0.09	8.9
4	12.5	0.21	20.7
5	6.25	0.45	44.5
6	3.12	0.79	78.2
7	Control cells	1.01	100

The effect of the polymers on the proliferation of MCF7 cells was expressed as the % cell viability. IC_{50} of the PEAs were determined and graphical representation is given in Figure 8, 9 and 10. Polymer PAHM and PAPB were able to inhibit the proliferation of the cancer cell (MCF7) (Figure 11 and 12) and the normal Vero cell viability of PAHM shown in Figure13. For polymer PAHM against human breast cancer cells (MCF7) & VERO cell lines, IC_{50} values were found to be 10.56µg and 18.75µg respectively. This value suggests that the polymer PAHM is more toxic to cancer cells than normal cells. IC_{50} values of the polymers PAHM and PAPB are found to be 10.56µg and 5.61µg respectively. This indicates that the polymer PAPB shows greater anticancer effect than polymer PAHM.

Table 6: % cell viability of PAHM

S.No	Concentration µg/ml	Absorbance 540nm	% cell Viability
1	100	0.07	5.6
2	50	0.29	23.3
3	25	0.41	33.0
4	12.5	0.81	65.3
5	6.25	0.97	78.2
6	3.12	1.18	95.1
7	Control cells	1.24	100

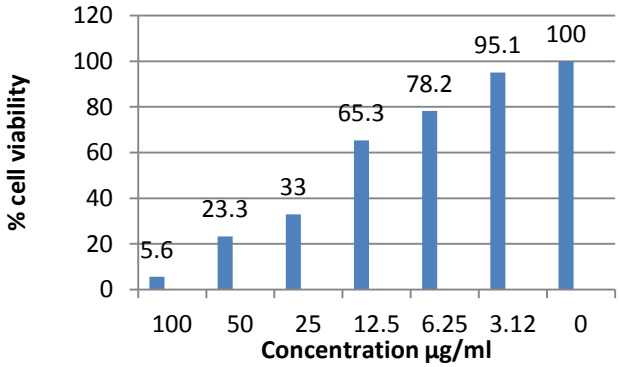


Figure 8: IC_{50} value of PAHM on VERO cell line

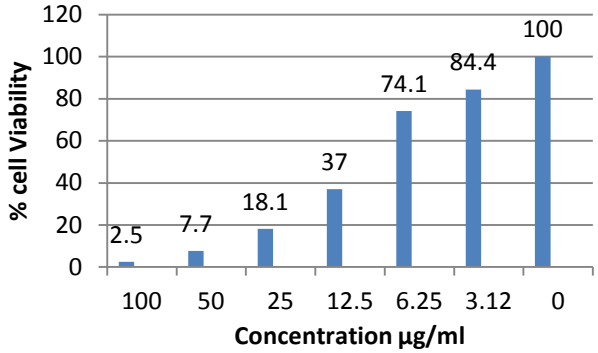


Figure 9: IC_{50} value of PAHM on MCF7 cell line

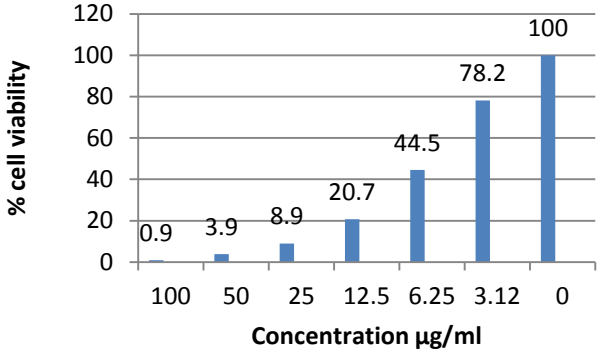


Figure 10: IC_{50} value of PAPB on MCF7 cell line

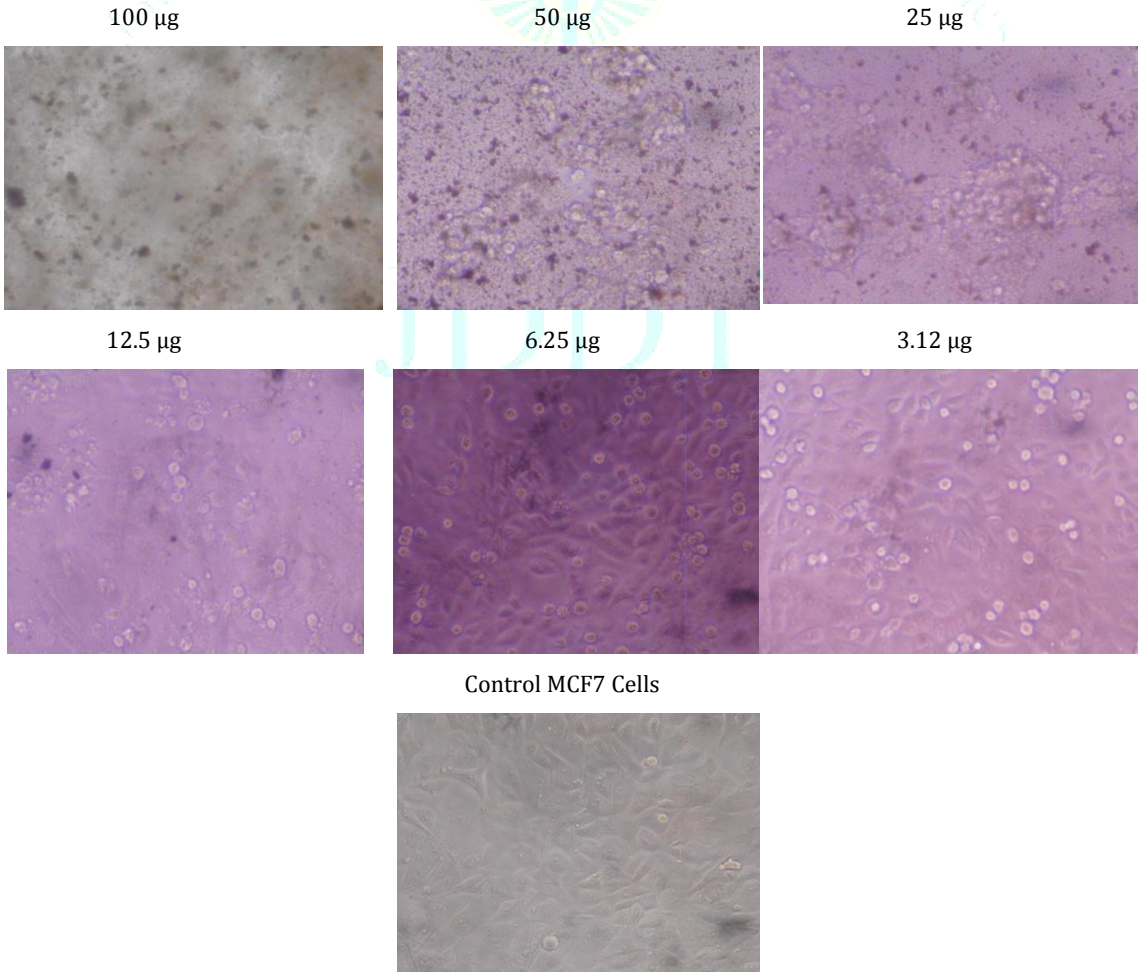


Figure 11: Cell viability of PAHM

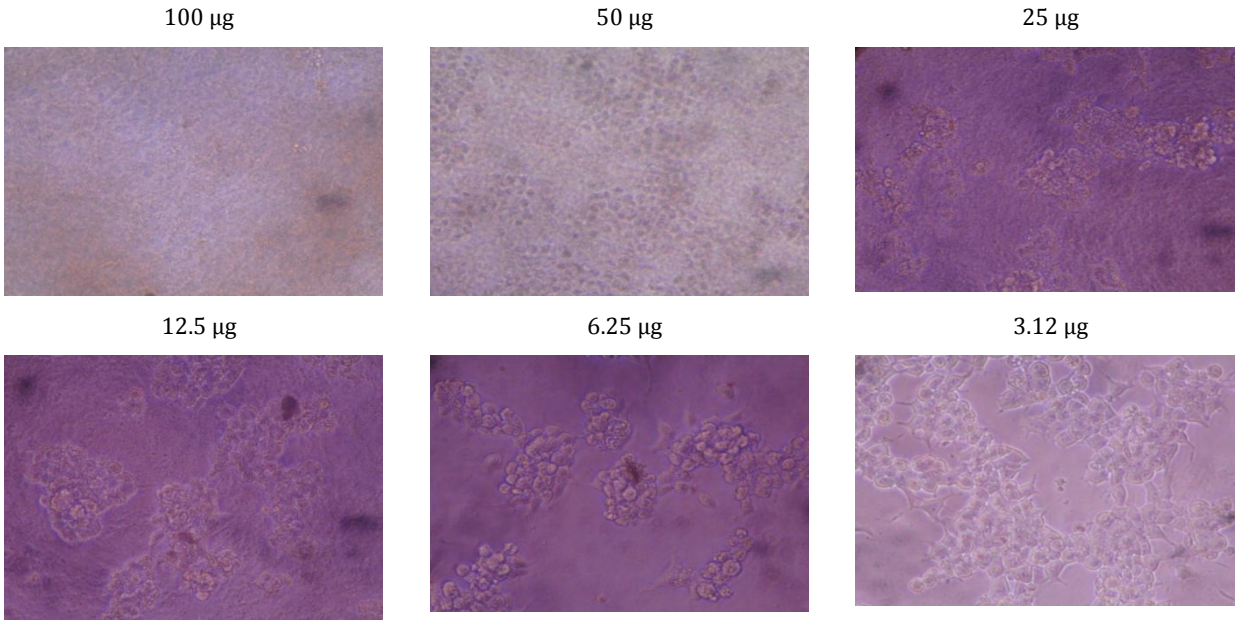


Figure 12: Cell viability of PAPB

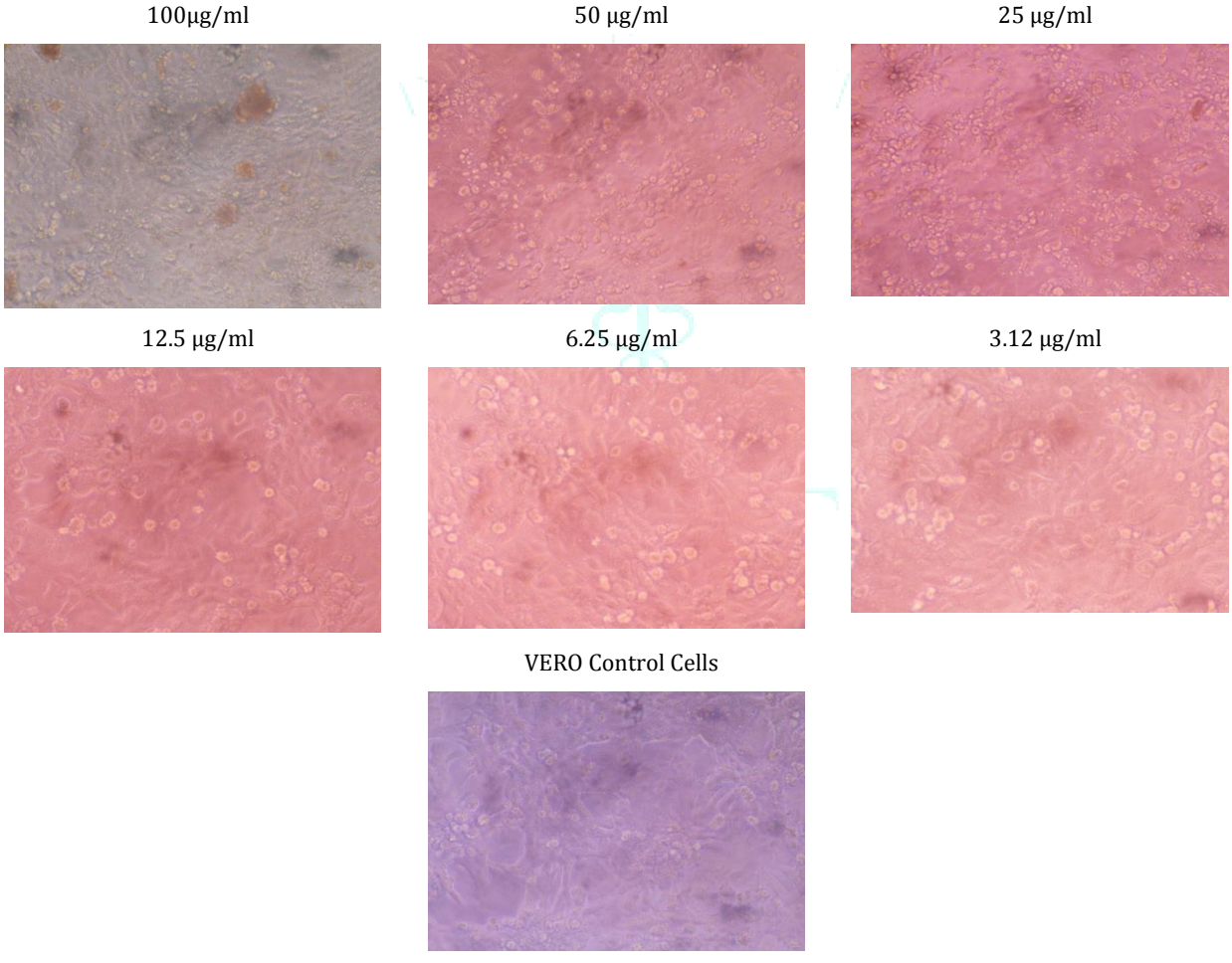


Figure 13:Cell viability of PAHM

CONCLUSION

A series of four new poly(ester amides) were synthesized and characterized. The spectral data supported the micro structure of polymer back bone. The resulting polymers showed good solubility in polar, aprotic solvents and the inherent viscosity data reveal that these polymers are of high molecular weight. Blending of the poly(ester amides) derived from 2,5-pyridine dicarboxylic acid with PVC produced smooth fibers. The polymers incorporated arylidene diol moieties are found to possess cytotoxic and anti-cancer properties. Polymers synthesized in this study were shown to demonstrate not only cytotoxicity but also possessed anti-bacterial properties. Reports indicate that these polymers could be useful in drug delivery systems

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