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Research Article

Synthesis, Characterization and *In Vitro* Anticancer Evaluation of Itaconic Acid Based Random Copolyester

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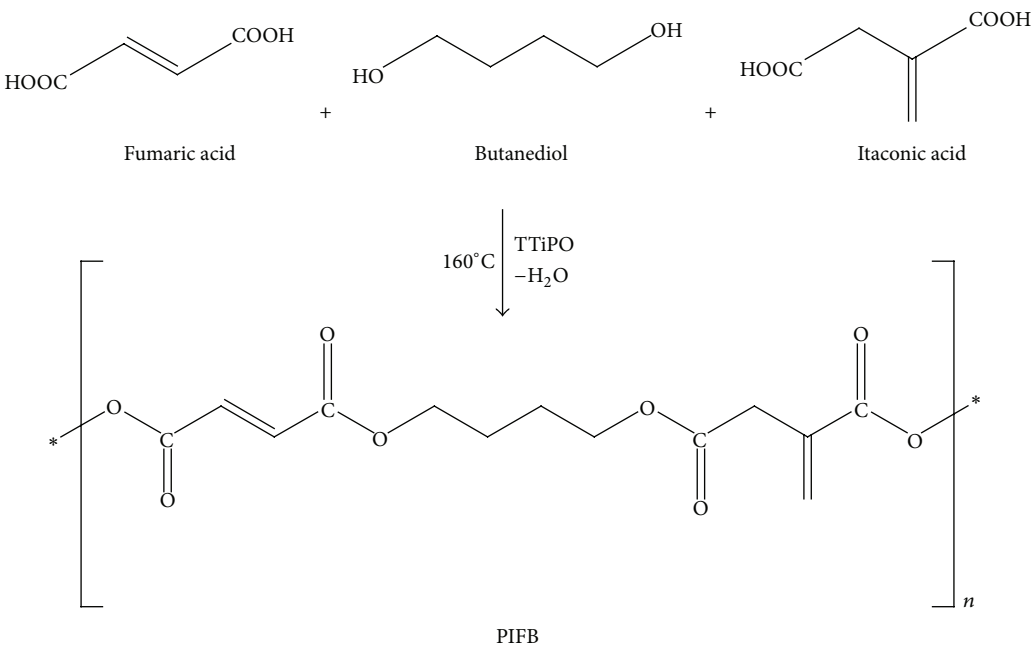
The present study deals with the synthesis and characterization of an aliphatic copolyester, poly [butylene fumarate-co-butylene itaconate] (PIFB) copolymer was obtained from itaconic acid, fumaric acid, and 1,4-butanediol using titanium tetraisopropoxide (TTiPO) through a two step process of transesterification and melt polycondensation. The synthesized aliphatic random copolyester was characterized with the help of FT-IR, ^{1}H-NMR, ^{13}C-NMR, viscosity measurements, Gel Permeation Chromatography (GPC) and X-ray diffraction (XRD) analysis. Thermal properties have been analyzed using thermogravimetric analysis (TGA) and Differential Scanning Calorimetry (DSC). Hydrolytic degradation studies were carried out in acid and alkaline regions of various pH values. The synthesized copolymer was subjected to *in vitro* anticancer activity studies against human breast cancer (MCF-7) cell line.

1. Introduction

The polymer synthesis was found to be an effective development on human chemotherapy. Polyesters, polyanhydrides, and so forth are mainly used in pharmaceutical, biomedical, soft tissue engineering, and drug delivery. Drug device polyester is used for plasma expanders and also tablet coating. Whenever a new drug molecule is synthesized, it is given orally or injected into the affected tissues. Nevertheless, this system of intake has disadvantage like an undesirable effect, poor drug efficiency, duration, concentration, bioavailability, and the drug that might not be controlled. To overcome this drawback, a new controlled release technology was developed. In this technology, a drug remains inside the human body for a prolonged period of time by releasing in a controlled manner [1]. The first drug delivery application is reported using hydrogel in 1960 [2]. In the beginning, biodegradable poly (glycolic acid) and poly (lactic acid) were used for tissue engineering system [3–5]. Later, poly (lactide-co-glycolide) was synthesized for medical application like dental implant and scaffold for bone TE [6]. In recent years, biodegradable polyesters are widely used for drug delivery, especially for anticancer drugs [7, 8]. Biodegradable polyesters have also attracted much attention as green materials and biomaterials in biodegradable fibers, nonwovens, films, sheets, bottles, injection-molded products, pharmaceutical, medical, biomedical engineering applications including drug delivery systems, and functional materials in tissue engineering [9–11]. Polyesters have good biocompatibility and biodegradation property which are concluded by many researchers in the past decades. Aliphatic polyesters with alkane diol like ethylene glycol, propylene glycol, butane diol, and so forth have good biodegradation some of polymers have biocompatibility nature [12–15]. Biodegradable and biocompatible polyester poly (propylene fumarate) has been used for bone cement, bone tissue engineering, and drug delivery [16–18]. Nowadays biodegradable polyesters are studied for drug release application; here polyesters are used only for the purpose of controlled delivery at targeted position. While releasing a drug, polyester also undergoes some activity against targeted cells. It should happen when the polymer has cytotoxic and anti-cancer activity on it. It is possible when preparation of polyester is using bioactive monomers [19]. Bioactive monomers, on polymerization enhance their bioactive nature. In the present study, it is proposed to synthesize polyester using a bioactive



Scheme 1: Synthetic route of copolyester.

monomer which itself has good anti-cancer activity and drug release system. In order to enhance the biocompactibility and biodegradation, a polymer is designed from fumaric acid, 1,4-butanediol, and itaconic acid. Itaconic acid is a naturally occurring bioactive monomer and is also derived from citric acid. It can be obtained directly from fermentation of glucose [20]. Itaconic acid is used to enhance bioactivities when it undergoes polymerization and can be used in tissue engineering [21]. With all the above facts in mind, we have to synthesize an aliphatic copolyester using fumaric acid, 1,4-butanediol, and itaconic acid.

2. Experimental

2.1. Materials. Itaconic acid, fumaric acid and 1,4-butanediol were purchased from Sigma Aldrich. Titanium tetraisopropoxide, used as a catalyst, was purchased from Lancaster. All other chemicals and solvents (AR Grade) were used as such. MCF-7 cell line was utilized from King Institute, Guindy, Chennai. The cells were maintained in Minimal Essential Media (MEM) supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) in a humidified atmosphere of (50 μg/mL) CO_2 at 37°C. MEM was purchased from Hi Media Laboratories and Fetal bovine serum (FBS) was purchased from Cistron laboratories. Trypsin, methylthiazolyl diphenyl- tetrazolium bromide (MTT), and Dimethyl sulfoxide (DMSO) were purchased from Sisco Research Laboratory Chemicals, Mumbai. All other chemicals and reagents were purchased from Sigma Aldrich, Mumbai.

2.2. Instruments. FT-IR spectrum of copolyester was recorded using Perkin Elmer IR spectrometer in the range of 4000–400 cm^{-1} using KBr pellets. ^{1}H NMR and ^{13}C-NMR spectra were recorded on Bruker 300 MHz and 70 MHz instruments. Viscosity of polyester was performed in $CHCl_3$ at room temperature using Ubbelohde Viscometer. Gel Permeation Chromatography (GPC) analysis was used to determine number average molecular weight (M_n) and weight average molecular weight of the polymer (M_w) in THF. The flow rate was 1 mL/min which was performed at room temperature. Bruker B8 diffractometer with Cu K_α radiation was used for assessing the crystallinity of the polymer. The sample was scanned over the range angle (2θ), from 5° to 80°. Hydrolytic degradation study was done using phosphate buffer (PH-7.4) and gradual degradation of polymer was observed. DSC thermogram was recorded on a DSC Q200 V24.10 Build 122 differential scanning calorimeter. About 2–4 mg of the polymer sample was heated in an aluminium pan with pierced lid under nitrogen atmosphere at a scanning rate of 10°C/min between the temperature −80°C and 500°C.

2.3. Synthesis of Copolyester. The aliphatic copolyester was synthesized by a two-step melt polycondensation and transesterification method as follows. A mixture of fumaric acid (0.01 mol), itaconic acid (0.01 mol), and 1,4-butanediol (0.02 mol) with 0.1 mmol of TTiPO as catalyst taken in reaction bath was slowly heated to 160°C and stood for 2 h under dry nitrogen atmosphere and after 2 h the temperature was further increased to 190°C and kept under *vacuum* for 1 h to remove the traces of water. The white amorphous copolyester obtained was purified by dissolution in $CHCl_3$ and reprecipitated in 10 fold of ice cold methanol, then dried in a *vacuum* at 40°C (yield: 98%) (Scheme 1).

2.4. Anticancer Evaluation. In vitro biocompatibility assessment was done using MCF-7 cell line which was maintained in Minimal Essential Media (MEM) sublimented with Fetal

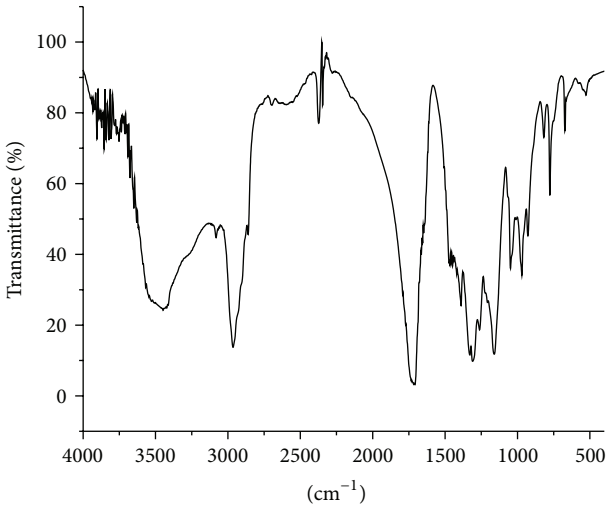


Figure 1: FT-IR spectrum of copolyester PIFB.

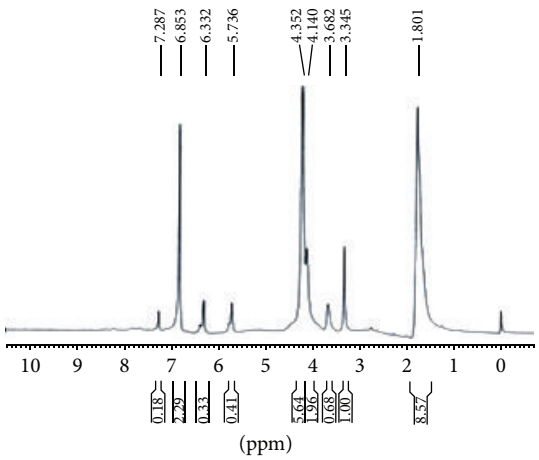


Figure 2: ^{1}H-NMR spectrum of copolyester PIFB.

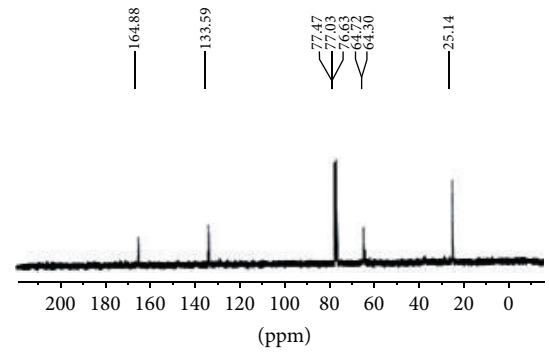


Figure 3: ^{13}C-NMR spectrum of copolyester PIFB.

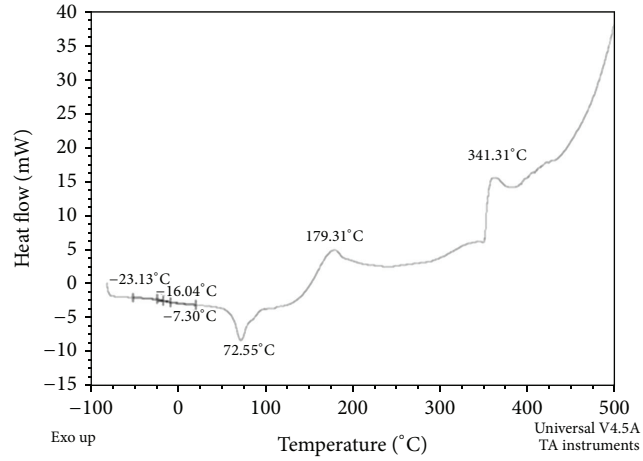


Figure 4: DSC thermogram of polymer PIFB.

bovine serum (FBS), penicillin (100 μg/mL) and streptomycin (100 μg/mL) in a humidified atmosphere of 50 μg/mL CO_2 at room temperature.

The cytotoxicity of polymers on MCF-7 was determined by the MTT assay [22]. Cells (1×10^5/well) were plated in 1 mL of medium/well in 24-well plates (Costar Corning, Rochester, NY). After 48 hours of incubation, the cell reaches the confluence. Then, cells were incubated in the presence of various concentrations of the samples in 0.1% DMSO for 48 h at 37°C. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4) 200 μL/well, 5 mg/mL of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) phosphate-buffered saline solution was added. After 4 h incubation, 0.04 M HCl/isopropanol was added. Viable cells were determined by the absorbance at 570 nm. Measurements were performed and the concentration requiredfor a 50% inhibition of viability (IC_{50}) was determined graphically. The absorbance at 570 nm was measured with a UV- Spectrophotometer using wells without sample containing cells as blanks. The effect of the polymers on the proliferation of MCF-7 was expressed as the % cell viability, using the following formula:

$$\%\ \text{cell viability} = \frac{\text{A570 of treated cells}}{\text{A570 of control cells}} \times 100\%. \quad (1)$$

3. Results and Discussion

3.1. Viscosity Measurement and GPC Analysis. Inherent viscosity of the polymer was reported using chloroform at the concentration of 1 mg/mL using Ubbelohde Viscometer by calculating the values of flow time of pure solvent and polymer. The inherent viscosity of the polymer PIFB is 0.86 dL/g. GPC is used to analyze the number average molecular weight ($M_n = 2.94 \times 10^3$) and weight average molecular weight ($M_w = 4.36 \times 10^3$). From these values, we can calculate poly dispersity ($M_w/M_n = 1.48$) of the synthesized polymer. In the GPC technique, a single peak which differs in magnitude and elution time was received. From the above polydispersity index and M_w value, we concluded that polymer formed in a random manner and lower molecular weight of the polymer is suitable for the biological application.

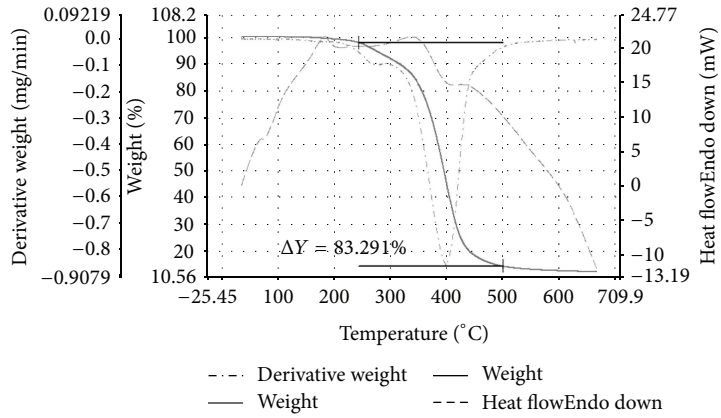


Figure 5: TGA thermogram of polymer.

Table 1: IR spectral data of copolyester PIFB in cm^{-1}.

S. no.	Absorption band (cm^{-1})	Assignment
1	1723	C=O stretching of ester group
2	1040	C–O stretching of ester group
3	2900	Aliphatic C–H stretching
4	1400	Aliphatic C–C stretching

3.2. IR Spectral Studies. FT-IR spectrum of synthesized copolyester is presented in Figure 1. The copolyester showed characteristic absorption band for ester carbonyl stretching at 1723 cm^{-1}. The polymer was observed peaks at 1040, 2900, and 1400 cm^{-1}. These peaks are assigned to C–O–C stretching, aliphatic C–H of methylene, and C–C stretching respectively. The IR data of the polymer is shown in Table 1. A new ester bond that was formed during polycondensation can be revealed from the report.

3.3. NMR Spectral Studies. ^{1}H-NMR spectrum of the copolyester (Figure 2) was recorded at RT in $CDCl_3$. The peak at 1.8 ppm was attributed to central methylene protons of 1,4-butanediol while peak at 4.25 ppm is due to terminal methylene groups of 1,4-butanediol moiety [23]. In addition, the peak at 3.3–3.6 ppm is due to methylene proton of itaconic acid 7.4 ppm for CH of fumaric acid. Copolyester prepared by molten state polycondensation has generally been considered to have a random distribution of the structural units because of the almost equal reactivities of the monomers and the random transesterification reaction during the polycondensation process [24]. ^{13}C-NMR spectrum of polymer (Figure 3), the peaks at 164, 133 & 25 ppm belongs to O–C=O, C=C of fumaric acid & methylene group of 1,4-butanediol respectively [25]. The signals at 64 and 77 ppm correspond to O–CH_2 group of 1,4-butadiol. Based on these spectral data, it may be concluded that the following structural units are randomly distributed in the copolyester.

3.4. DSC & TGA Thermal Studies. The DSC thermogram of the polymer PIFB shows glass transition temperature (T_g) at

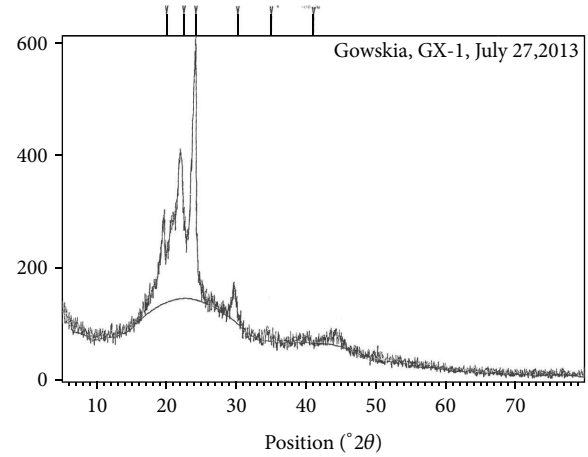


Figure 6: X-ray diffractogram of PIFB.

Table 2: DSC data of copolyester PIFB.

S. no.	Transition temperature	Obtained temperature (°C)
1	Tg	−23
2	Tm	72
3	Tc	179.31
4	Td	362

−23°C and melting temperature (T_m) at 72°C [14, 15] which are shown in Figure 4 and the values are in Table 2. These values show that these temperatures of the polymer lie in the range of temperature of the polymer used for drug delivery application, which is partially matched with the temperature of the human body and from the TGA, the decomposition temperature (T_d) of the polymer is observed to be 362°C which is shown in Figure 5.

3.5. X-Ray Diffraction Analysis. X-ray diffractogram of the synthesized polymer is shown in Figure 6. The crystalline nature of polyester was determined from X-ray diffractogram. Gaussian curves are used to describe the amorphous

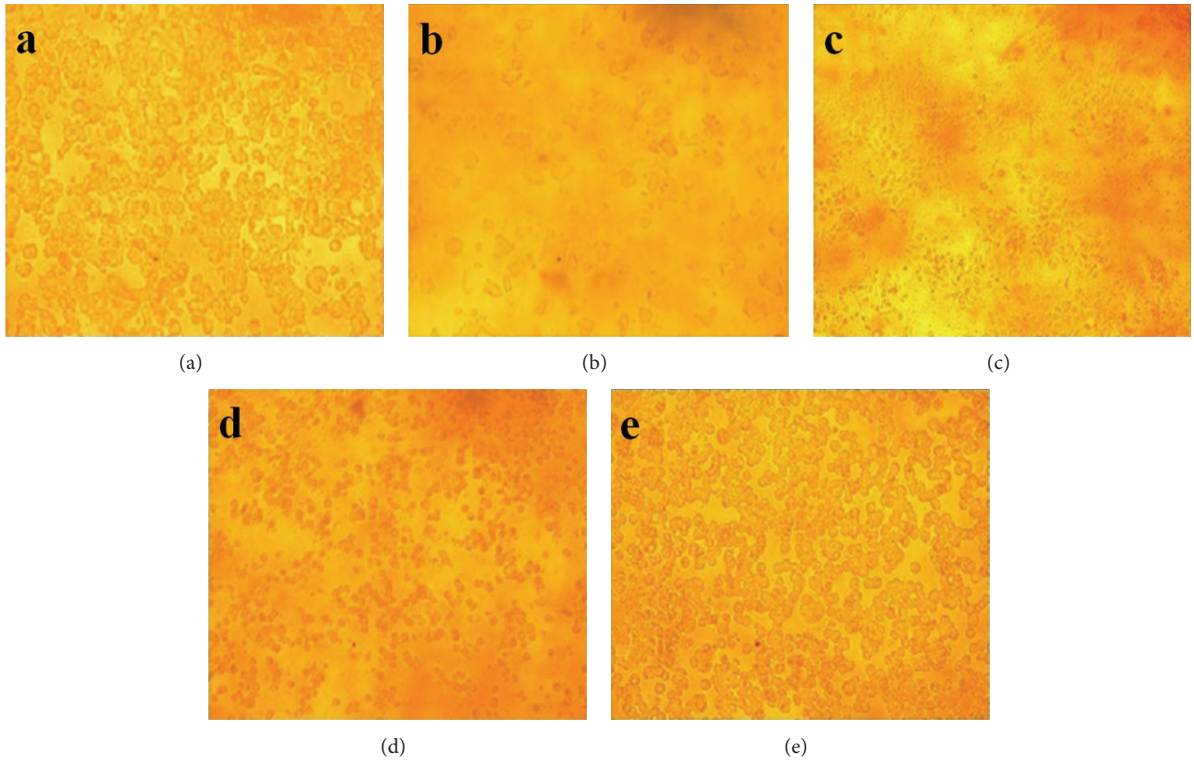


Figure 7: Cytotoxic anticancer evaluation of polymer on MCF-7 cell line at different concentrations: (a) normal MCF-7 cell line, (b) 1000 μg/mL (c) 125 μg/mL (d) 62.5 μg/mL (IC_{50}), and (e) 31.2 μg/mL.

phase and all crystal reflections of a diffractogram. In the X-ray diffractogram, the intensity of diffraction peaks increases with the increase in the length of the flexible spacer group. This is in accordance with the study of Chen et al. [26]. This indicates that the crystallinity of the polymer increases with the length of flexible segments. From the X-ray diffractogram, it is observed that PIFB is amorphous in nature.

3.6. Hydrolytic Degradation. The polymer is subjected to hydrolytic degradation in phosphate-buffered saline pH 7.4, alkaline, and acidic medium for 24 h. There is not any degradation that took place in an acidic medium. Very slight degradation takes place in alkaline medium but complete degradation takes place in phosphate buffer saline pH 7.4, which matches pH range of human body.

3.7. Cytotoxic Anticancer Evaluation of Synthesized Polymer. Viable cells were determined by the absorbance. Measurements were performed and the concentration required for a 50% inhibition of viability (IC_{50}) was determined graphically. The absorbance was measured with a UV- Spectrophotometer using wells without sample containing cells as blanks. The effect of the polymer on the proliferation of MCF-7 was expressed as the % cell viability. The affected MCF-7 cell line at different concentration was shown in Figure 7. IC_{50} of the polymer was determined and was shown in Table 3. In Figure 8, a graphical representation of the polymer effect on cancer cells by % cell viability is shown.

Table 3: Anticancer effect of copolyester on MCF7 cell line.

S. no.	Concentration (μg/mL)	Dilutions	Absorbance (O.D)	Cell viability (%)
1	1000	Neat	0.07	13.4
2	500	1 : 1	0.15	28.8
3	250	1 : 2	0.19	36.5
4	125	1 : 4	0.23	44.5
5	62.5	1 : 8	0.28	53.8
6	31.2	1 : 16	0.33	63.4
7	15.6	1 : 32	0.38	73.0
8	7.8	1 : 64	0.44	84.6
9	Cell control	—	0.52	100

4. Conclusion

The polyester PIFB was successfully synthesized by transesterification and melt polycondensation method and was characterized. The probable structure of the repeating units present in the polyester was assigned on the basis of NMR spectral data. The inherent viscosity value is found to be 0.86 dL/g which predicts that the degree of polymerization is high for the polymer. TGA analysis shows the decomposition temperature of the polymer at 362°C which is the usual decomposition temperature range for many polyester with drug delivery application and good biodegradation properties. The cytotoxic assay shows that the PIFB is toxic to

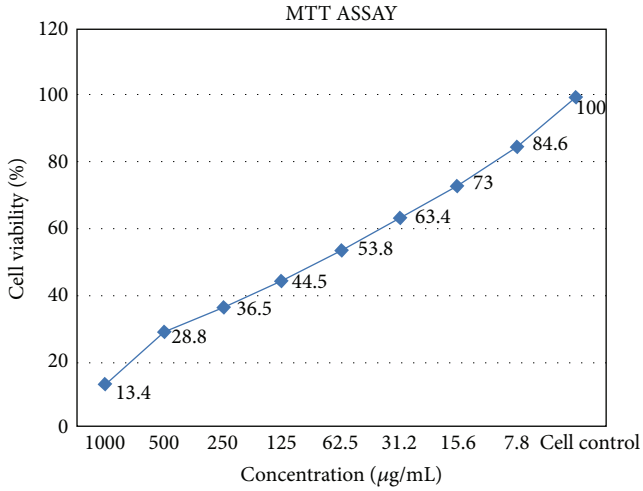


Figure 8: Graphical representation of polymer on MCF-7 cancer cell line.

the MCF-7 cell and 40 to 60% of these cells were killed after incubation for two days with the extract. These lines of evidence show that the polyester may further be studied for drug delivery application.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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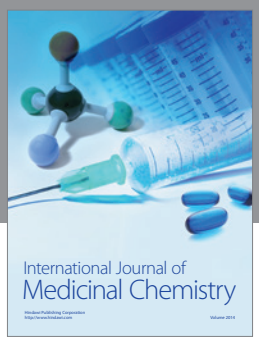

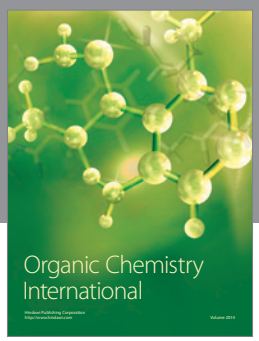

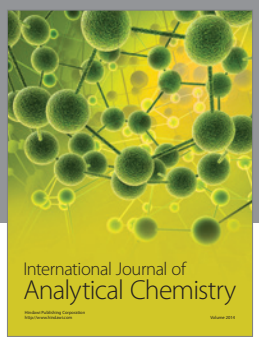

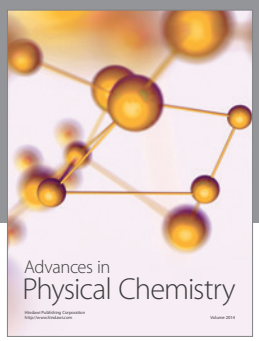

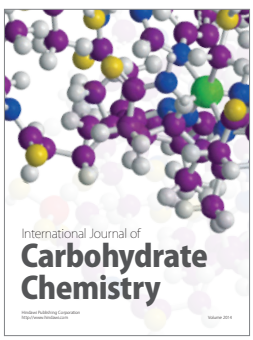

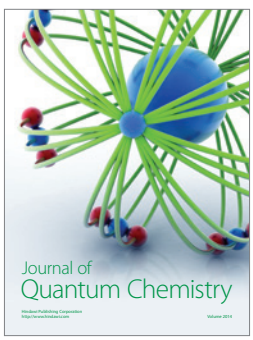

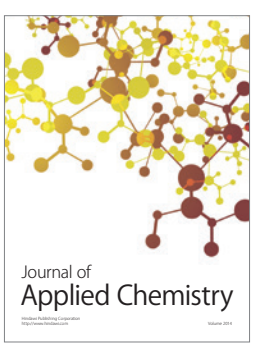

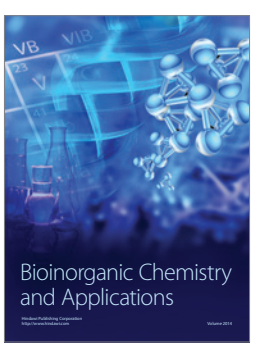

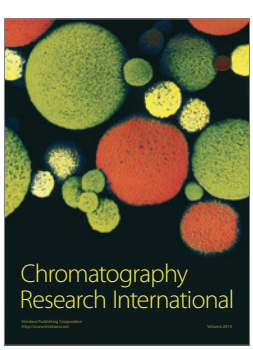

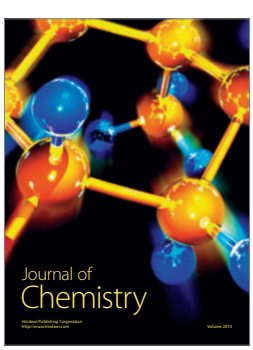

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