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Research Article

NANOPARTICULATE DRUG DELIVERY SYSTEM OF POORLY WATER SOLUBLE ANTICANCER DRUG AND THEIR *IN VITRO* CYTOTOXICITY STUDIES

M. KrishnaPillai[1]* and N. Thirumoorthy[2]

[1]Department of Pharmaceutics, Karpagam University, Coimbatore, Tamilnadu, India.
[2]Department of Pharmaceutics, Cheran College of Pharmacy, Coimbatore, Tamilnadu, India.

ABSTRACT

The objective of this investigation was to develop a microsphere and also attempts were made to control the release of silymarin. Multiple-unit of silymarin microspheres was prepared from Ethyl cellulose and EudragitRS100 polymer by using emulsion solvent evaporation method and Ionic cross linking technique. These microspheres were evaluated for Drug entrapment, Drug loading, Percentage yield Swelling index, scanning electron microscopy and *in vitro* drug release. All formulation showed total drug release duration more than 12 hours. It was observed that the result of *in-vitro* dissolution study reveal that the formulation ED3 & ES3 gave controlled release pattern. Silymarin microspheres (1:3ratio) showed cytotoxicity against HT-29 cells and DU145 Prostate carcinoma cellines. Hence the formulations can be effectively tested for its anticancer activity. The silymarin microspheres showed maximum release at 8 to 12 hours in pH 1.2 Maximum percentage cell viability is reduced up to 25 percentages at 12 hour meaning that 75 percentage cell deaths occurred in HT-29 cells and DU145 Prostate carcinoma cells. Most of the drug release was observed at 12hour and good cytotoxicity.

Keywords: Silymarin, colorectal cancer, microspheres, entrapment efficiency, Cytotoxicity studies.

INTRODUCTION

Microcapsule technology is the latest trend in cancer therapy. It helps the pharmacist to formulate the product with maximum therapeutic value and minimum or negligible side effects. Cancer is a disease in which the abnormal cells are quite similar to the normal cells, with genetic or functional changes. A major disadvantage of anticancer drugs is their lack of selectivity for tumor tissue alone, which causes severe side effects and results in low cure rates. Thus, Silymarin is a unique flavonoid complex containing silybin, silydianin, and silychrisin that is derived from the milk thistle plant. These unique phytochemicals from the milk thistle have been the subject of decades of research into their beneficial properties. Milk thistle's common name comes from the white markings on the leaves, its milky white sap, and its traditional use by nursing mothers to increase milk. But it is best known for its use as a liver protectant and decongestant, which can be traced to the Greeks and Pliny the Elder (23-79AD), who wrote that it was excellent for "carrying off bile." The famous English herbalist Culpepper (1616-1654) used milk thistle to cleanse the liver and spleen, and to treat jaundice and gallstones. Among the most promising cancer fighting strategies that researchers are trying to develop are angiogenesis inhibitors (which stop the proliferation of blood vessels that feed tumors), cell cycle regulators, and selective promoters of cancer cell death. Amazingly, silymarin has been shown to possess all of these abilities. A review of research into silymarin's effects on prostate cancer concluded that silymarin has a huge potential to interfere with many molecular events involved in cancer cell growth, progression, and angiogenesis. One study done in August 2008 indicated that silymarin may inhibit metastasis in prostate

cancer. Another study done in September 2008 identified the strong efficacy of silymarin in prostate cancer prevention and intervention[1].

EXPERIMENTAL

Materials and Methods

Silymarin was received as gift samples from Micro labs Pvt. Ltd. Bangalore, India. Ethyl cellulose was received from SD Fine chemicals, Mumbai, India. Eudragit RS100 was received from Thermonik pvt ltd, Hyderabad, India. All other chemicals used were of analytical grade.

Preparation of microspheres

Silymarin microspheres polymer and drug content was a mixture of silymarin and ethyl cellulose in ratios of 1:1 (ES1), 1:2 (ES2), 1:3(ES3) and 1:4 (ES4). The drug and polymer (in a ratio of 1:1) were dissolved in a 20 ml mixture of dichloromethane and ethanol (1:1) at room temperature. The solution was poured slowly as a thin stream into 150 ml of 0.01 % Tween 80 maintained at 30 - 40 0C. The emulsion was continuously stirred at a rotation speed of 300 rpm for 1 h to allow the volatile solvents to evaporate. The microspheres were collected by decantation while impurities were discarded along with polymer residues. The collected microspheres were dried over night in an oven at 40 ± 2^0C and stored in a desiccator containing calcium chloride as desiccators.[2]

Silymarin microspheres prepared by another method Ionic cross linking technique polymer used for this method EudragitRS100 using TPP as cross linking agent. EudragitRS100 solutions of varying concentrations were prepared by dissolving the dilute acetic acid (1% v/v). Tween 80 was added into the solution as a surfactant. The core material, Silymarin, dissolved in CH_2Cl_2 (2:10), was mixed with the aqueous phase (Eudragit solution) in a homogenizer at 5000 rpm for 20min. The volume ratio of CH_2Cl_2: aqueous phase was 1:10. The emulsion was cross linked by dropping through a spray gun into the TPP solution (10%). After cross linking was allowed for varying time, microspheres were washed with distilled water repeatedly and vacuum dried for 12h. Four different formulations with drug: polymer ratios (1:1, 1:2, 1:3 &1:4) were prepared and coded as ED1, ED2, ED3 and ED4.

EVALUATION OF MICROSPHERES

Identification of silymarin[2]

Identification of silymarin was by comparison with that of an authentic sample and verification of the presence of functional groups in its infra-red (IR) spectra. Also, various concentrations of the drug in 0.1M HCl were evaluated by ultraviolet (UV) spectroscopy (Shimadzu –1700) to determine if it would obey Beer's law.

Percent Yield of Microspheres[2]

The prepared microspheres were calculated and weighed from different formulations. The measured weight was divided by the total amount of all nonvolatile components which were used for the preparation of microspheres.(Table:2)

$$\text{Percentage yield} = \frac{\text{Actual weight of the product}}{\text{Total weight of drug and polymer}} \times 100$$

Drug Entrapment Efficiency[2]

The various formulations of the silymarin microspheres were subjected for drug content.50mg of silymarin microspheres from all batches were accurately weighed and powdered. The powdered samples were dissolved with 10ml ethanol in 100ml volumetric flask and made up the volume with 0.1NHCl.The resulting solution is then filtered through whatman filter paper No: 44, after filtration from this solution 10ml was taken out and diluted up to 100ml with 0.1NHCl.again from this solution 2ml was taken out and diluted up to 10ml with 0.1NHCl and absorbance was measured at 287 nm against 0.1NHCl as a blank. The percentage drug entrapment was calculated as follows.(Table:1)

$$\text{Percentage drug content} = \frac{\text{Calculated drug concentration}}{\text{Theoretical drug concentration}} \times 100$$

Angle of Repose[4]

Flow property of silymarin microspheres is usually assessed by determining angle of repose of the silymarin microspheres. It is maximum angle that can be obtained between the free flowing surfaces of silymarin microspheres. Heap and the horizontal plane. The angle of repose of microspheres was determined by fixed funnel method. The silymarin microspheres were allowed to fall freely through a funnel until apex of conical pile just touched the tip of the funnel.(Table:2)

The angle of repose

$$\text{ø} = \tan^{-1} h/r$$

Where = height of pile

r = radius of the pile formed by silymarin microspheres

Degree of Swelling[2]

Swelling properties of the beads were studied by soaking the beads at pH 1.2 in a glass beaker. Beads were removed at different time intervals and weighed after drying. The ratio of water uptake was calculated as:

$$\text{Ratio of water uptake} = \frac{[Wet\ \ Weight\ - Dry\ Weight]}{(Dry\ Weight)} \times 100$$

All mass measurements of the swollen beads were taken on single pan balance.(Table:2)

Scanning electron microscopy study

SEM photographs were taken with JSM 5600 scanning Microscope (Japan) to examine the morphology and surface structure of the beads at the required magnification at room temperature. The beads were deposited on brass hold on sputtered with a thin coat of gold under vacuum. Acceleration voltage used was 20kV with the secondary electron as a detector. (Fig: 1a, 1b)

***In vitro* Dissolution studies[2]**

The dissolution studies were carried out using USP XII dissolution rate test apparatus type I at 100 rpm and 37±0.5°C. The formulated microspheres equivalent to 200 mg of silymarin were filled in to colorless hard gelatin capsules and placed in basket separately. The dissolution medium was 0.1 N HCl pH 1.2 as simulated gastric fluid (SGF) for the 12 hour, 5 ml samples were withdrawn at specified time intervals and was replaced immediately with an equal volume of fresh medium. Samples were suitably diluted and analyzed at 287 nm (Shimadzu 1700). All the tests were carried out in triplicate. (Fig:2,3)

***In vitro* cytotoxicity studies**

Materials and Method (*In vitro* Studies)

From the *In vitro* release data for silymarin microspheres, formulation code (ED3, ES3) showed better results, hence ratio 1:3 of Ethyl cellulose and Eudragit RS100 silymarin microspheres was taken further for cytotoxicity studies against human colorectal adenocarcinoma HT-29 cell line. and DU145 Prostate carcinoma cell line.

Cytotoxicity against cell lines *in vitro*

Silymarin microspheres (1:3 ratio) of both Ethyl cellulose and Eudragit RS100 along with the pure sample was checked for its cytotoxicity against human colorectal adenocarcinoma cell line HT-29 cell line and DU145 Prostate carcinoma cell line using MTT assay. All the above three samples were incubated at pH 1.5 for 12 hours, in a rotating agitator. Sampling was done at one hour interval for first 4 hours after that every 2 hours interval continued for 12 hours, starting from zero time. The samples collected at different time intervals were centrifuged and the drug concentration was measured using spectrophotometer. Human colorectal adenocarcinoma HT-29 cell line and DU145 Prostate carcinoma cell line expressing high levels of Cox-2 were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal calf serum, penicillin (100 U) and streptomycin (100 µg).The HT-29 cells and DU145 cells were plated and treated with samples collected at different time intervals from the rotating agitator. Cell viability was measured using MTT assay.[5]

Cytotoxicity Screening

Determination of Mitochondrial Synthesis by Micro culture Tetrazolium (MTT) Assay

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0×10^5 cells/ml using medium containing 10% new born calf

serum. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once and 100µl of different drug concentrations was added to the cells in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations recorded every 24 hours. After 72 hours, the drug solutions in the wells were discarded and 50µl of MTT in MEM was added to each well. The plates were gently shaken and incubated for 3 hours at 37°C in 5% CO_2 atmosphere. The supernatant was removed and 50µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540nm. The percentage growth inhibition was calculated using the formula below:

$$\% \text{ Growth inhibition} = 100 - \left(\frac{\text{Mean OD of Individual Test group}}{\text{Mean OD of control group}} \right) \times 100$$

RESULTS AND DISCUSIONS

Formulations ED3 & ES3 exhibited highest drug loading and % entrapment efficiency values of 72.34 ± 0.55 % ,73.12± 0.22 and 90.45 ± 1.45% & 91.12±1.25% respectively, whereas ED1 Silymarin microsphere showed the least value of drug loading and % entrapment efficiency as 62.06 ± 0.21 % and 76.42 ± 0.88% respectively. Formulations ED3 & ES3 exhibited highest percentage yield values of 91.12 ± 0.25 %, 90.80± 0.12 respectively, whereas Silymarin microsphere showed the least value of % yield76.14 ± 0.31 % respectively. Silymarin microspheres Angle of repose values 22 º.61' to 31º 60' respectively. Average degree of swelling silymarin microspheres values 0.7916 to 0.9227.The result of SEM (Fig: 1) SEM revealed that microspheres of silymarin using Eudragit RS100, were discrete and spherical in shape with cracks on rough outer surface which may be due to crosslinking of polymer, Microspheres of silymarin using ethyl cellulose were spherical and their surface was smooth and devoid of cracks giving them good appearance, the dissolution studies showed that the release of drug from silymarin microspheres (ED3 & ES3) was found to be 92.45±0.077%, 91.32±0.055% In the *in vitro* drug release studies, the highest cumulative drug released by the cellulose microspheres after 12 h was found to be92.45±0.077%, Eudragit microspheres was found to be 91.32±0.055%. The formulation with the lowest drug release was found to be Eudragit microspheres after 12 hour 76.16±0.44.Various release kinetic models were applied to determine the mechanism of drug release from microspheres and observed that the highest correlation coefficient (r^2) found for Higuchi square root of time profile indicated that the drug release from the microspheres formulations occurred via diffusion mechanism suggesting uniform dispersion of water soluble drug in swellable polymer matrix. Drug releases from the microspheres were retarded till 12 h. The release data appeared to fit the zero order model better for the silymarin microspheres as compared to Higuchi matrix model. This suggested that water insoluble drug from polymer matrices is released in a way which is proportional to the amount of drug remaining in its interior, in such a way that the amount of drug released by unit of time diminishes (Table:3). *Invitro* cytotoxicity study done all the three samples, pure sample (Silymarin), Ethyl cellulose microspheres (ES3) and EudragitRS100 (ED3) showed cytotoxicity against HT-29 cells. Hence the formulations can be effectively tested for its anticancer activity. Pure sample Silymarin showed drug release from the first hour itself at pH 1.5, followed at each hour. Maximum percentage cell viability it reduced up to 25 percentages. Meaning that maximum of 64 percentage cell death occurred in HT-29 cells and DU145 cells.Ethyl cellulose and Eudragit RS100 (ES3, ED3) Silymarin microspheres showed very significant results compared with the pure drug.(Table:4,5) The silymarin microspheres showed maximum release at 8 to 12 hours in pH 1.2 Maximum percentage cell viability is reduced up to 25 percentages at 12 hour meaning that 75 percentage cell death occurred in HT-29 cells(Fig:4) and DU145 Prostate carcinoma cells(Fig:5). Most of the drug release is observed at 12hour showed good cytotoxicity.

CONCLUSION

From the above experimental results it can be concluded that Oral controlled release of

silymarin microspheres achieved by Emulsion solvent evaporation technique using EudragitRS100, Ethyl cellulose as polymer. From the study it is evident that a promising controlled release microparticulative drug delivery of silymarin can be developed, further *in vivo* investigation is required to establish efficacy of these formulations. The study also indicated that the amount of drug release decreases with an increase in the EudragitRS100, Ethyl cellulose concentration Drug release from the formulation followed Zero order kinetics and the mechanism of drug release was diffusion controlled. *In vitro* cytotoxicity results showed that all the above formulations showed anti cancer activity. Most important, the formulations were able to release the drug slowly and hence the effect of silymarin on cancer cells was observed till 12hrs. In case of pure drug, maximum activity was observed the cell viability near to 45 to 50 % only, then after the activity reduced significantly. Thus, we are able to make a formulation, which will have sustained release of the drug and hence this will be very much useful in treatment to reduce the number of daily doses. Although all four formulations were found to be good, we observe formulation code (ES3, ED3) deserved that they showed better anti cancer activity.

Table 1: Drug loading and Drug Entrapment of silymarin microspheres

Formulation code	Drug Loading	Drug entrapment Efficiency
ED1	62.06	76.42
ED2	68.42	82.34
ED3	72.34	90.45
ED4	66.54	78.24
ES1	72.24	90.25
ES2	71.4	88.14
ES3	73.12	91.12
ES4	68.52	82.62

Table 2: Angle of Repose, Percentage Yield, and Average degree of swelling silymarin microspheres

Formulation code	Angle of repose	% yield	Average degree of swelling
ED1	31º 60'	89.12	0.7916
ED2	29 º 24'	85.15	0.8612
ED3	30 º 96'	91.12	0.8983
ED4	22 º.61'	84.16	0.9227
ES1	25 º 20'	76.14	0.8946
ES2	27 º 10'	82.83	0.8714
ES3	27 º 40'	90.80	0.9118
ES4	28 º 10'	78.15	0.9238

Table 3: Correlation coefficient (r^2) values for the fit of different kinetic models

Formulation code	Zero order plots	Higuchi's plots	Peppa's plots	n
	r^2	r^2	r^2	
ED1	0.9686	0.8411	0.9988	1.429
ED2	0.9916	0.8526	0.9618	1.316
ED3	0.9847	0.8865	0.9864	1.234
ED4	0.9846	0.8515	0.9991	1.216
ES1	0.9714	0.8845	0.9926	1.064
ES2	0.9912	0.8534	0.9996	1.364
ES3	0.9824	0.8816	0.9912	1.286
ES4	0.9816	0.8564	0.8814	1.344

Table 4: In vitro cytotoxicity studies HT29 Colon carcinoma cell line

TIME (hour)	Pure silymarin		ED3(1:3)		ES3(1:3)	
	Concentration (µg)	% cell Viability	Concentration (µg)	%cell Viability	Concentration (µg)	% cell Viability
0	0	100	0	100	0	100
1	108	84	98	74	110	88
2	132	80	120	72	125	74
3	155	76	160	73	140	65
4	220	72	180	68	160	54
6	240	66	230	55	220	42
8	259	60	260	40	240	40
10	286	53	300	34	290	28
12	290	48	340	23	320	20

Table 5: *In vitro* cytotoxicity studies DU145 Prostate carcinoma cell line

TIME (hour)	Pure silymarin		ED3(1:3)		ES3(1:3)	
	Concentration (µg)	% cell Viability	Concentration (µg)	%cell Viability	Concentration (µg)	% cell Viability
0	0	100	0	100	0	100
1	110	88	90	72	115	78
2	122	86	110	70	120	70
3	145	78	130	56	130	55
4	210	76	145	50	160	49
6	235	72	180	40	190	38
8	249	66	220	36	240	30
10	274	56	280	34	270	26
12	280	52	320	26	310	22

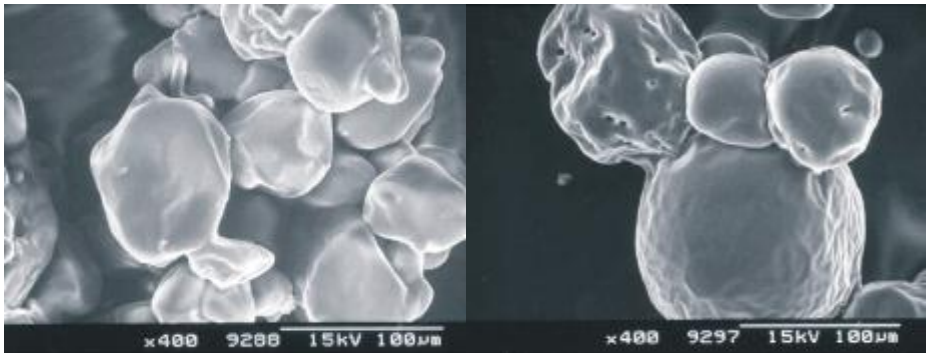

Fig. 1a **Fig. 1b**

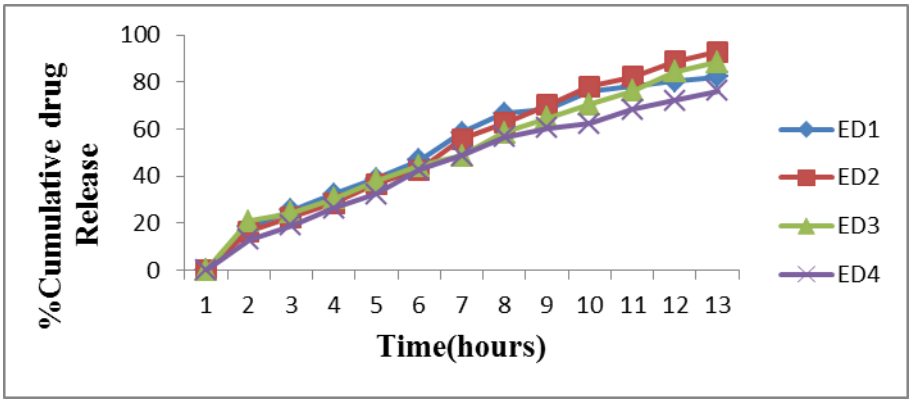

Fig. 2: In vitro dissolution study of silymarin microspheres using Eudragit RS100

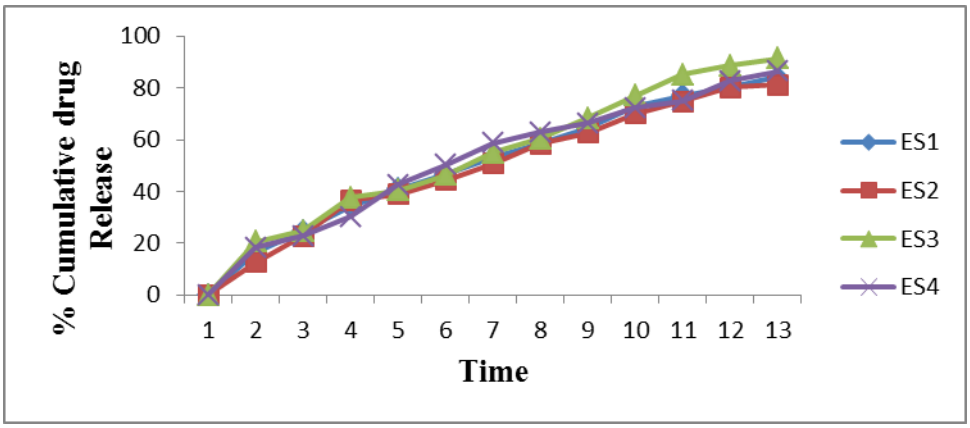

Fig. 3: In vitro dissolution study of silymarin microspheres using Ethyl cellulose

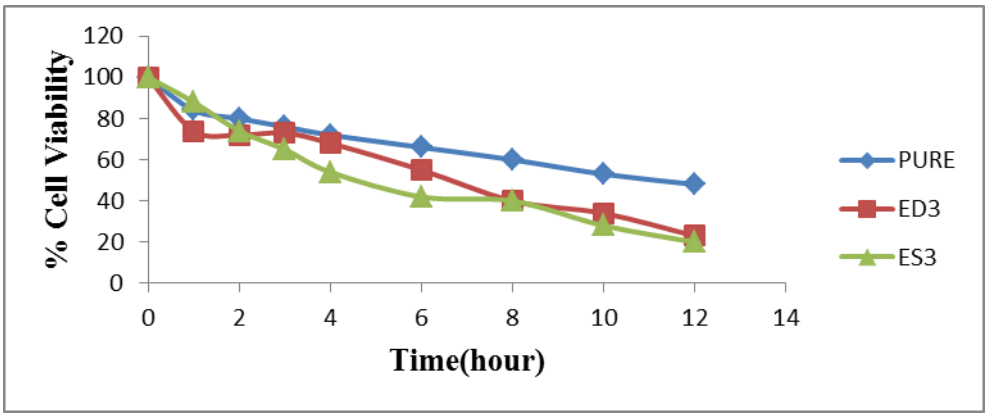

Fig. 4: In vitro cytotoxicity studies HT29 Colon carcinoma cell line

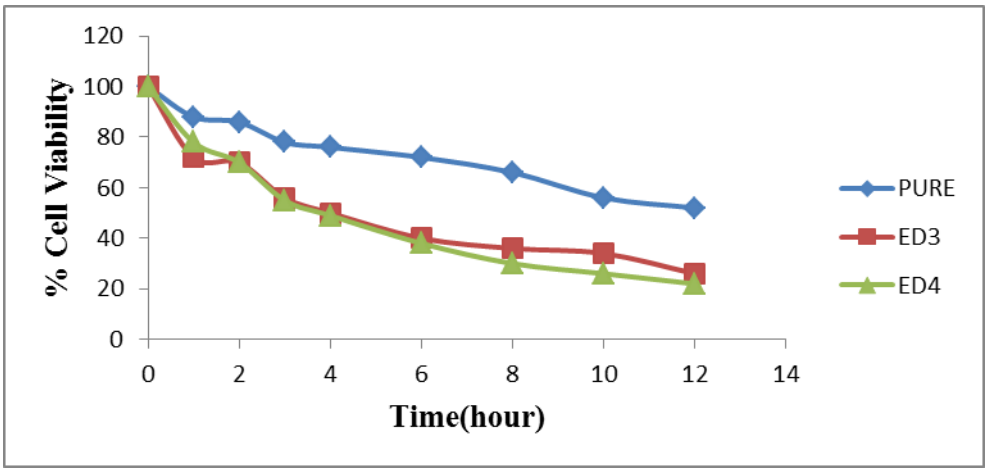

Fig. 5: *In vitro* cytotoxicity studies DU145 Prostate carcinoma cell line

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