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RESEARCH ARTICLE

Formulation of Microspheric Form of Silymarin and its Anticancer Potential on Cancercell Lines

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ABSTRACT

This present study was aimed to formulate microspheric form of Silymarin by using various polymers like Chitosan, Pectin, Carbopol 934P, Ethyl cellulose, Eudragit RS100. Microspheres were successfully prepared by emulsion solvent evaporation and ionic cross linking method. The prepared microspheres were evaluated for various parameters like encapsulation efficacy, swelling index, particle size, percentage yield, angle of repose, *invitro* release and evaluated for their *invitro* cytotoxicity potential. The obtained yields were varies from 46-91% on various polymers and encapsulation efficacy is up to 91.12%. Results from *Invitro* release studies indicated that, the microspheres of silymarin exhibited prolonged drug release for at least 12 h, and, therefore, could potentially improve the bioavailability of the silymarin. Results from invitro cytotoxic studies shows that, maximum percentage of cell viability is reduced up to 25 percentages at 12 hour which clearly indicated that 75 percentage cell deaths occurred in HT-29 colon carcinoma cells and DU145 Prostate carcinoma cells.

KEYWORDS

Microspheres, Silymarin, Polymers, Invitro release study, Cytotoxicity

INTRODUCTION

Cancer is a common term for a group of more than 100 diseases that can affect any part of the body. The main feature of cancer is the rapid formation of abnormal cells which grow beyond their usual limits and spread within the body. The most common rationale for the use of chemotherapy by cell-kill mechanism. Drug administrated by either oral or parenteral route is distributed to all the tissues without any selectivity to tumurs[1]. This leads to toxic effects of anticancer drugs on rapidly proliferating cells and also on normal cells. Silymarin is a flavonolignan, extracted from the dried seeds of Silybum marianum (Compositae), where it is present in higher concentrations than in other parts of the plant. Silymarin is a mixture of four flavonolignans namely silybin, isosilybin, silydianin and silychristin. The major and the most active component of the silymarin complex is silybin (50-60% of silymarin), Silymarin has many pharmacological activities such as antioxidant**,** anti-inflammatory, antifibrotic, anticarcinogenic and immunomodulatory effect on diseased liver. Silymarin is poorly soluble in water and, therefore, an acidic medium is essential for its dissolution. Its dose is 70 - 140 mg three times a day and has low bioavailability. The low bioavailability of the drug is due to rapid biotransformation in the liver, and has a biological half-life of 4-6 h. Its relatively short half-life, poor bioavailability and lipophillic

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nature make it a suitable candidate for gastro retentive drug delivery system[2].

The objective of this work was to develop and characterize microspheres of silymarin which, following oral administration, would exhibit prolonged gastric residence time and, hence increase the bioavailability of the drug. Anticancer activity of silymarin has been observed against colon cancer cell-lines and prostate cancer cell lines. Failure of conventional treatment modalities, especially of hormone refractory PCA has led to increased efforts in identifying new approaches to improve the outcome of this disease.

Silymarin showed potential anticancer activity against both colon and prostate cancer celllines[3]. Recent research is mainly focused on the delivery of anticancer drugs at the target site, thus maximizing the therapeutic efficacy of the drug and reducing its side effects. The microspheres drug delivery system in cancer chemotherapy is promising approach for both passive and active targeting. Microspheres have shown significant promise as vehicles for the delivery of small molecular drug to tumors. Microencapsulation is described as a process of enclosing micron sized particles of solid or droplets of liquid or gases in an inert shell, The products obtained by this process called microparticles, microcapsules and microspheres with different morphology and internal structure. Microencapsulation for oral use has been employed to control the drug release and to reduce or eliminate the GIT irritation.[4]

MATERIALS AND METHOD

Silymarin was received as a gift sample from Micro labs Pvt. Ltd. Bangalore. Ethyl cellulose, Carbopol 934, Pectin, was received from SD fine chemicals, Mumbai, Eudragit RS100 was received from Vikram Thermonik Pvt. ltd, Hyderabad, Chitosan received from Cochin marine Pvt. Ltd and all other chemicals used were analytical grade.

Preparation of Microspheres

By Emulsion Solvent Evaporation Technique

Preparation of Silymarin microspheres by emulsion solvent evaporation technique by using various polymers like ethyl cellulose, chitosan, carbopol 934P. A mixture of Silymarin and polymer in ratios of 1:1, 1:2, 1:3, 1:4 were prepared. The drug and polymer (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 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 overnight in an oven at 40 ± 2^0C and stored in a desiccator[5].

By Ionic Cross Linking Technique

Preparation of Silymarin microspheres by emulsion solvent evaporation technique by using various polymers like Eudragit RS100, Pectin using TPP as cross linking agent. Polymer solutions of varying concentrations were prepared by dissolving them in dilute acetic acid (1% v/v). Tween 80 was added into the solution as a surfactant. The Silymarin, dissolved in CH_2Cl_2 (2:10), was mixed with the aqueous phase (Eudragit solution) in a homogenizer speed at 5000 rpm for 20min. The volume ratio of CH_2Cl_2: Eudragit solution 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 12 h[6].

Evaluation of Microspheres

Identification of Silymarin

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.

Percentage Yield of Microspheres

The prepared microspheres were calculated and weighed. The percentage of yield was calculated by using the following formula[7].

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

Drug Entrapment Efficiency

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 make up the volume with 0.1N HCl. The resulting solution is then filtered through whatman's filter paper No: 44, 10ml was taken out and diluted up to 100ml with 0.1N HCl. Again from this solution 2ml was taken out and diluted up to 10ml with 0.1N HCl and absorbance was measured at 287nm against blank. The percentage drug entrapment was calculated as follows [7].

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

Angle of Repose

Flow property of silymarin microspheres is usually assessed by determining its angle of repose. It is maximum angle that can be obtained between the free flowing surfaces of silymarin microspheres[5].

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 and calculated by using the following formula[8].

$$\text{The angle of repose } \phi = \tan^{-1} h/r$$

Particle Size Determination

Particle size of prepared microspheres was determined by following optical microscopy method[6]. The mean of 100 microspheres and particle size were determined. All the studies were carried out in triplicate[8].

Degree of Swelling

Swelling properties of the microspheres were studied by soaking the microspheres at pH 1.2 in a glass beaker. Microspheres were removed at different time intervals and weighed after drying and the ratio of water uptake was calculated[8].

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

Scanning Electron Microscopy Study

SEM photographs were taken with JSM 5600 scanning Microscope (Japan) to examine the morphology and surface structure of the microspheres at room temperature. [7]The microspheres were deposited on brass hold on sputtered with a thin coat of gold under vacuum. Acceleration voltage used was 20k with the secondary electron as a detector[7].

In vitro Dissolution Studies

The dissolution studies were carried out using 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 for two hour and phosphate buffer pH 6.8 from 3 to 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[8].

Study of Kinetics of Drug Release

The drug release data of controlled-release microspheres was fitted to kinetics models i.e., zero order, first order and Higuchi to find out drug release pattern and mechanism.

In vitro Cytotoxicity Studies

The formulation which showed better *in vitro* release profile was taken further for cytotoxicity studies was performed on HT29 cells (colon cancer cells), DU145 cells (prostate cancer cells). And the percentage of cell inhibition was determined by MTT assay[10].

RESULTS AND DISCUSSION

Drug Entrapment Efficiency

Formulations ED3 & ES3 exhibited highest drug loading and % entrapment efficiency values were 72.34 ± 0.55 %,73.12 ± 0.22 and 90.45 ± 1.45% & 91.12 ± 1.25% respectively, whereas SC, PS, CPS formulation code of Silymarin microsphere showed the least value of drug loading and % entrapment efficiency the range were between 36 % to 78%.

Angle of Repose, Degree of Swelling, % Yield

Formulations ED3 & ES3 exhibited highest percentage yield values of 91.12 ± 0.25 %, 90.80± 0.12 respectively, whereas SC, PS, CPS Silymarin microsphere showed the least value of 40% - 78% respectively. Angle of repose values of ED3 & ES3 were 22º.61' to 31º.60' respectively. Average degree of swelling silymarin microspheres values 0.7916 to 0.9227.

SEM Analysis

The result of SEM revealed that microspheres of silymarin using Eudragit RS 100, were discrete and spherical in shape with cracks on rough outer surface which may be due to cross linking of polymer. Microspheres of silymarin using ethyl cellulose were spherical and their surface was smooth and devoid of cracks giving them good appearance.

Particle Size Analysis

The mean size increased with increasing polymer concentration which is due to a significant increase in the viscosity, thus leading to an increased droplet size and finally a higher microspheres size. Microspheres of Silymarin using Chitosan, Pectin, Carbopol, Ethyl cellulose and EudragitRS100 had a size range of 796.04µm to 944.65µm, 1.675 -1.783, 696-846, 812-939 and 786-962. The particle size of the microspheres increased with increase in the polymer concentration.

In Vitro Dissolution Study

The highest cumulative drug released by the ethyl cellulose microspheres after 12 h was found to be 92.45%, Eudragit microspheres were found to be 91.32%. The formulation with the lowest drug release was found to be ranges from 48.12 to 75.81 % of formulation code of SC, PS, and CPS Batches. Best formulation batch selected on the basis of Drug entrapment efficiency, Percentage yield, Degree of swelling, angle of repose, Particle size analysis and *In vitro* dissolution study. To be selected for ED3 and ES3 for further *in vitro* cytotoxicity studies. To check the anticancer activity by using colon carcinoma cell lines and prostate carcinoma celllines[10,11]

In Vitro Cytotoxicity Studies

In vitro cytotoxicity results show that formulation batch of ES3, ED3 showed anti-cancer activity. Most importantly, 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 free drug maximum activity was observed only upto 6 hrs, then after the activity was reduced. Thus, were able to make a formulation, which will have controlled release of the drug and hence this would be more beneficial in the treatment in order to reduce the number of daily doses.[12,13]

Although all formulations were found to be beneficial, but ES3, ED3 formulations showed better activity. Silymarin, Ethyl cellulose microspheres(ES3) and Eudragit RS100(ED3) showed cytotoxicity against HT-29 cells & DU145.

The results show that, pure silymarin maximum percentage cell viability is reduced up to 40-50% which means that maximum of 50 % cell death occurred in HT-29 cells and DU145 cells. Ethyl cellulose and Eudragit RS100 (ES3, ED3) microspheres showed very significant results compared with the pure drug. The silymarin microspheres showed maximum release at 8 to 12 hours in pH 1.2 Maximum percentage cell viability is reduced up to 25% at 12 hour means that 75% cell death occurred in HT-29 cells and DU145 Prostate carcinoma cells which shows significant cytotoxicity.[14,15]

Table 1: Preformulation Characteristics

S. No	Parameter	Standard
1	Physical Appearance	Yellow Crystalline powder
2	Solubility	Methanol/ Polyethylene glycol
3	Melting Point	158°C-165°C
4	UV Spectra	λmax 287nm (Methanol)

Table 2: Standard Calibration Curve of Silymarin

S. No	Concentration (µg/ml)	Absorbance Mean ± SD
0	0	0.0000000000
1	2	0.045±0.006
2	4	0.092±0.008
3	6	0.132±0.006
4	8	0.176±0.005
5	10	0.218±0.006

Table 3: Silymarin Microspheres Comparison of Evaluation Study

Batch code	DEE (%)	% Yield	Swelling Index	Angle of repose	Particle size	*In Vitro* Release
SC	55.12-76.34	46-72	0.080-2.24	22º 62'-40º 38'	791.90-944.64	48.12-66.87
PS	36.24-58.52	46-68	1.24-2.34	22º.36'-40º 38'	1.675-1.783	58.26-75.81
CPS	41.36-74.12	56-78	0.84-1.36	25º 36'-37º 76'	696-840	52.44-73.29
ES	76.42-91.12	68-91	0.871-1.34	25º 20'-32º 65'	812-939	72.28-92.45
ED	66.16-90.45	66-90	0.791-1.26	22º 61'-32º 14'	786-980	66.28-91.32

Table 4: Kinetics of Drug Release

Batch code	Zero order	Higuchi	Papas	n	Batch code	Zero order	Higuchi	Papas	n
ES1	0.9714	0.8845	0.9926	1.064	ED1	0.9686	0.8411	0.9988	1.429
ES2	0.9912	0.8534	0.9996	1.364	ED2	0.9916	0.8525	0.9618	1.316
ES3	0.9824	0.8816	0.9912	1.286	ED3	0.9847	0.8865	0.9864	1.234
ES4	0.9816	0.8564	0.9918	1.344	ED4	0.9846	0.8515	0.9991	1.216
ES5	0.9612	0.8234	0.9624	1.262	ED5	0.9914	0.8612	0.9912	1.229
ES6	0.9214	0.8642	0.9214	1.284	ED6	0.9812	0.9912	0.9984	1.326
ES7	0.9436	0.8434	0.9936	1.362	ED7	0.9926	0.9918	0.9826	1.364
ES8	0.9816	0.8965	0.9928	1.242	ED8	0.9948	0.8824	0.9834	1.438

Table 5: *In vitro* Cytotoxicity study of HT29 Colon Carcinoma Celine

Time (Hour)	Pure Silymarin		ES3 (1:3)		ED3 (1:3)	
	Conc^n	%Cell Viability	Conc^n	%Cell Viability	Conc^n	%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 6: *In vitro* Cytotoxicity study of DU145 Prostate Carcinoma Celline

Time (Hour)	Pure Silymarin		ES3 (1:3)		ED3 (1:3)	
	Conc^n	%Cell Viability	Conc^n	%Cell Viability	Conc^n	%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

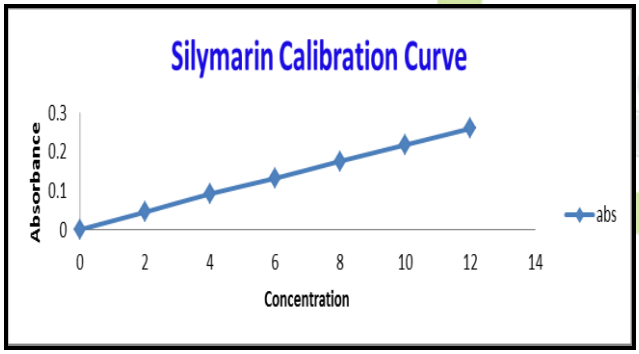

Figure 1: Standard Calibration Curve of Silymarin

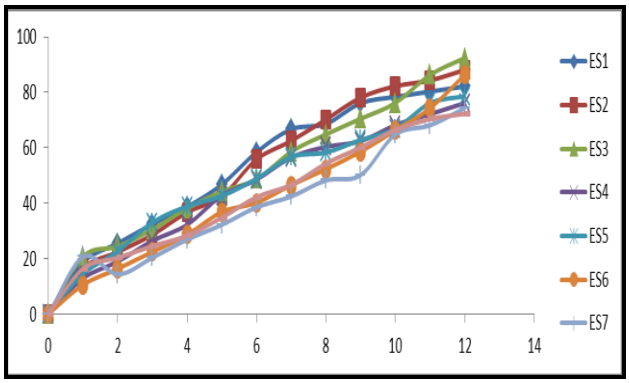

Figure 2: *In vitro* Dissolution study of Silymarin Microspheres using Ethyl cellulose

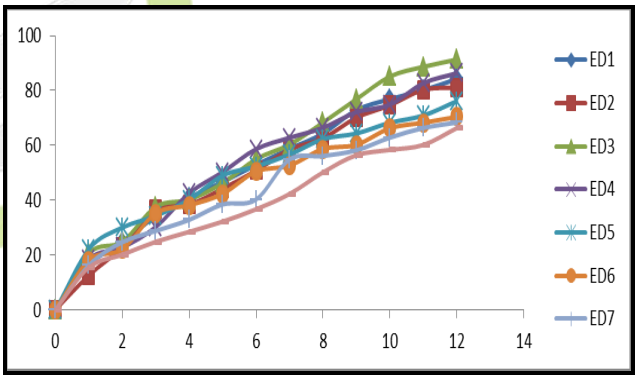

Figure 3: *In vitro* Dissolution study of Silymarin Microspheres using Eudragit RS100

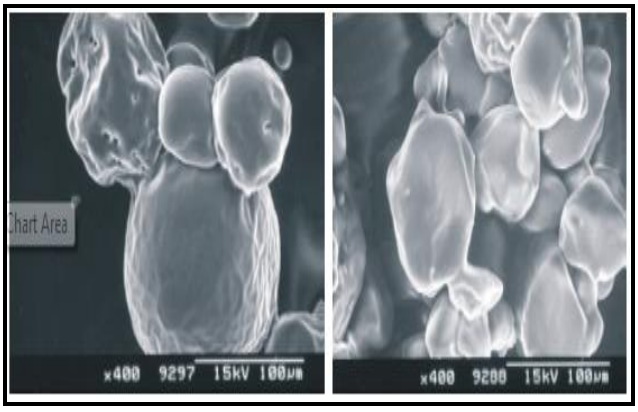

Figure 4: SEM Analysis

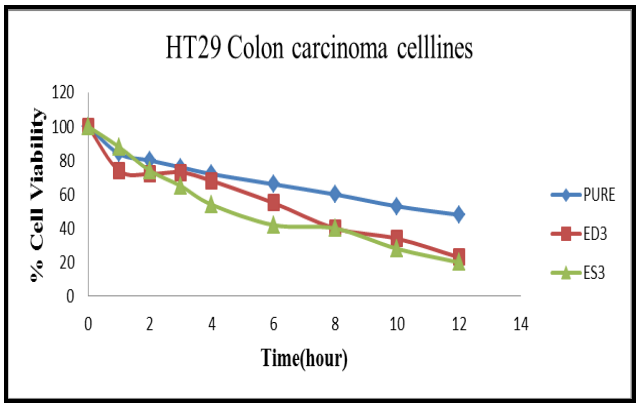

Figure 5: *In vitro* cytotoxicity studies on HT29 Colon carcinoma cell lines

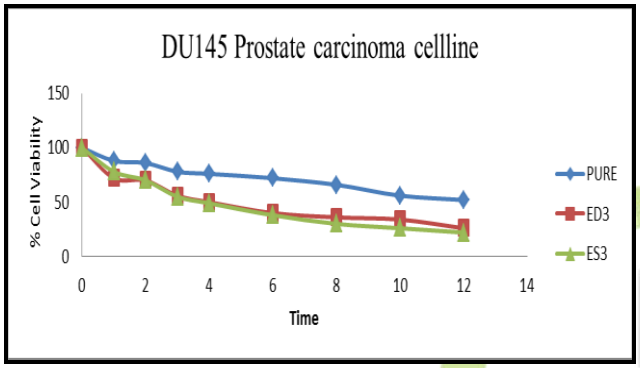

Figure 6: *In vitro* cytotoxicity studies on DU145 Prostate carcinoma cell lines

CONCLUSION

Among the five polymers studied, Ethyl cellulose and Eudragit RS100 were found to be better polymers for silymarin microspheres. We could successfully develop controlled release silymarin microspheres. Results of Preformulation studies, Angle of repose, entrapment efficiency, *and in vitro* dissolution study have shown satisfactory results. The results also indicated that the amount of drug release decreases with increases in the concentration of Eudragit RS100, Ethyl cellulose concentration which Follows Zero order kinetics and the mechanism of drug release was diffusion controlled. *In vitro* cytotoxicity results showed that the formulations show significant anti-cancer activity. Most importantly, the formulations were able to release the drug slowly and hence we could achieve sustained release of silymarin on cancer cells up to 12hrs.Thus our formulation might be beneficial in reducing the number of daily doses.

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