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Gel formulation of silymarin loaded microsponge: Development and Evaluation

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ABSTRACT

Wound healing in a short period with minimum side effects is one of the major goals of modern medicinal sciences. Aim of the present study was to develop and evaluate the microsponge-based topical delivery system of Silymarin for sustained release and enhanced drug deposition in the skin for the treatment of wounds. Microsponge containing silymarin were prepared by quasi-emulsion solvent diffusion method. The effect of silymarin microsponges on *human neonatal foreskin fibroblast* proliferation rate was determined by MTT assay and was seen that the fibroblast proliferation rate increased with increase in concentration of silymarin. Formation of spherical and porous microsponges were confirmed by scanning electron microscopy and they were incorporated in the Carbopol 934 (1%) gel base and further evaluated by determination of pH, viscosity, spreadability etc. From *in vitro drug* release studies, silymarin microsponge loaded topical gel formulation has shown sustained release till 24 hrs whereas plain drug topical gel has shown an immediate burst release within 5 hours. From ex-*vivo* skin permeation studies, flux and permeability was found to be lowest for silymarin microsponge loaded topical gel formulation, indicating that with this kind of formulation systemic permeation of drug can be avoided. Therefore, Silymarin microsponge loaded topical gel prepared in this study is promising as being useful than conventional formulation in therapy for wound healing.

INTRODUCTION

In recent years, there has been considerable emphasis given to the development of novel microsponge based drug delivery systems, to modify and control the release behaviour of the drugs[1].

Silymarin, a flavonoid obtained from Silybum marianum (milk thistle plant), has been used for centuries to treat liver, spleen and gallbladder disorders. It is an effective agent on skin tissue regeneration and wound healing due to its anti inflammatory and antioxidant activities[2].

Silymarin gel and ointment were also been reported to stimulate fibroblast proliferation, tissue regeneration, collagen bundle synthesis, hair follicle population and consequently wound closure in full thickness skin wound[3,4]. The conventional formulations showed rapid releases of the drug causing high accumulation producing side effects, irritation, and toxicity.

Hence an attempt was made to formulate silymarin loaded microsponges to fulfil enhanced wound healing through fibroblast proliferation. Thus, formulation provided additional advantage of microsponges that prolonged drug release due to entrapped form in porous structure and maximum amount of time that an active ingredient is present either on the skin surface or within the epidermis, while minimizing its transdermal penetration into body.

MATERIALS

Silymarin was purchased from Yarrow Chem Products, Mumbai, India. Both polyvinyl alcohol and ethylellulose was purchased from central drug house (P) Ltd, New Delhi, India. Carbopol 934 and Triethanolamine were purchased from Loba Chemie, Mumbai, India. Propyl Paraben and Propylene Glycol was purchased from CDH, New Delhi. Ethyl Alcohol was purchased from Jebsen & Jessen GmbH & Co. Germany. Human neonatal foreskin fibroblast cells were procured from National Centre for Cell Sciences (NCCS), Pune, India. 3-(4, 5-

Table 1 : Optimization constraints selected for optimization of silymarin microsponge

Variables	Constraints		
Independent variables	**Lower limit**	**Upper limit**	**Goal**
A=Amount of ethyl cellulose (mg)	100	300	In range
B=Amount of ethanol(ml)	3	7	In range
Dependent variables			
Particle size(µm)	58.89	88.76	Minimize
Entrapment efficiency (%)	61.12	78.23	Maximize

dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (Seelze, Germany).

METHODS

PREPARATION AND OPTIMIZATION OF MICROSPONGES

Microsponges were prepared by Quasi-emulsion solvent diffusion method [5] using ethyl cellulose as polymer and ethanol as solvent. A response surface central composite statistical design with 2 factors, and 5 levels and 13 runs was selected for the study using Design-Expert ®software 7 trial version (state-Ease Inc,Minneapolis,USA.) [6, 7].The dependent variables were particle size and entrapment efficiency and independent variables are amount of ethyl cellulose and amount of ethanol (table 1).

CHARACTERISATION OF OPTIMIZED MICROSPONGE FORMULATION

Scanning Electron Microscopy (SEM)

Surface morphology of microsponge formulation was determined using a scanning electron microscope by JOEL: Model JSM-6390 LV.

Transmission Electron Microscopy (TEM)

The morphology, structure and particle size of Silymarin loaded microsponge were examined by transmission electron microscopy by JOEL Model JSM-6390LV an electronic transmission microscope at 70 KV.

Product yield

The product yield was determined by the equation

$$Product\ Yield = \frac{Practical\ mass\ of\ microsponges}{Theoretical\ mass(polymer + drug)}$$

Entrapment efficiency

The entrapment efficiency of microsponges was assayed spectrophotometrically at 288 nm (UV visible spectrophotometer, model UV-1601 PC, Shimadzu). The amount of entrapped drug was calculated from the equation.

$$EE\ (\%) = \frac{Total\ drug - Unentraped\ drug}{Total\ drug} \times 100$$

Particle size

Particle size of microsponge was determined using optical microscope.

IN VITRO DRUG RELEASE STUDY

In vitro drug release studies were performed by dialysis membrane method using phosphate buffer of pH 7.4. The receptor compartment was filled with buffer and kept for stirring on a magnetic stirrer. Microsponge equivalent to 100 mg of drug was loaded in membrane. Temperature was maintained at 37± 0.5° C and the speed of stirring was kept constant (600 rpm) for 24hrs. Aliquots of drug sample (5ml) were withdrawn at regular time intervals. The drug analysis was done using UV Spectrophotometry at 288nm [8, 9].

KINETIC MODELLING

The drug release data obtained was further studied for their fitness of data in different kinetic models like Zero order plot, First order plot, Higuchi plot and Korsmeyer-Peppas plot [10, 11].

IN-VITRO WOUND HEALING STUDY ON HUMAN SKIN FIBROBLAST CELLS [12]

- **MTT ASSAY**

Cell lines and maintenance

Human neonatal foreskin fibroblast cells were procured from National Centre for Cell Sciences (NCCS), Pune, India.

Cell culture media and maintenance

The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM-Himedia), the test sample were further diluted in DMEM media and seeded to the wells containing cultured cells at a final concentration of 12.5 µg, 25 µg, 50 µg, 100 µg respectively. Untreated wells were kept as control. After sample addition, the treated as well as the control wells were observed at regular intervals up to 24hrs in an inverted phase contrast tissue culture microscope (Labomed TCM-400 with MICAPSTM HD camera) and the observations were photographed.

Fibroblast Proliferation assay by MTT Method

MTT solution was added to all test and control wells, and incubated for 4 hours. Treatment with 100 µL of DMSO for 1h to solubilize formazan violet crystals in the cells. The absorbance of each well was determined by spectrophotometer at 570nm with a microplate reader.

$$Proliferation\ rate\ (\%) = \frac{(A(sample)-A(b))}{(A(c)-A(b))} \times 100$$

A (b) = Absorbance of blank A(c) = Absorbance of control

PREPARATION OF SILYMARIN MICROSPONGES LOADED TOPICAL GEL

Silymarin microsponge loaded topical gel was prepared by using carbopol 934 as the gelling agent. Amount of microsponge equivalent to 100 mg of drug was added to 10 gm of topical gel formulation [13].

CHARACTERIZATION OF SILYMARIN MICROSPONGES LOADED TOPICAL GEL

pH - The pH of the gel was determined using digital pH meter

Homogeneity - The formulated gel was tested for homogeneity by visual inspection

Spreadability - A sample of 0.5g of gel was placed between two slides; weight applied. Diameters of spread circles were measured in cm.

Extrudability - The extrudability test carried out using hardness tester.

Viscosity - Brookfield viscometer DV-E at 37°C with S 06 spindle at 10 rpm and the viscosity was measured in cP (centipoises).

Drug content - Drug content determined by taking 100 mg of topical gel formulation was made up to 100 ml using pH 7.4 phosphate buffers. Then drug concentration was determined by measuring the absorbance at 288nm using UV Spectrophotometer.

EX-VIVO SKIN PERMEATION STUDY

Permeation study was conducted using Franz diffusion cell. The goat abdominal skin is mounted between two cell compartments. Topical gel formulation (equivalent 10 mg drug) was kept in the donor compartment and the receptor compartment was filled with 10 ml of phosphate buffer pH 7.4. Sample collected at regular interval of time and replaced with equal amount of fresh media to maintain a sink condition. The samples were analyzed for the drug content using UV- Visible spectrophotometer at 288nm.

COMPARATIVE *IN-VITRO* DRUG RELEASE STUDIES

In vitro drug release studies were conducted for silymarin microsponge loaded topical gel and plain silymarin gel using dialysis membrane method. Graph was plotted for cumulative amount of drug released v/s time for two formulations and extend of drug release was compared.

RESULTS AND DISCUSSION

PREPARATION AND OPTIMIZATION OF MICROSPONGE

Microsponges prepared by the quasi emulsion solvent

Table 2 : Central composite experimental design data for process optimization.

Experimental Runs	Independent Factors		Dependent Factor	
	Amount. of Ethyl cellulose(mg)	Amount .of Ethanol(ml)	Particle size (µm)	Entrapment efficiency (%)
1	300	7	66.76	78.23
2	300	3	86.36	67.11
3	100	7	60.24	67.76
4	200	5	72.12	74.64
5	200	2.17	88.76	65.56
6	200	5	72.84	73.31
7	200	7.83	58.89	76.44
8	58.58	5	68.99	61.12
9	200	5	72.66	74.11
10	200	5	72.28	74.18
11	100	3	78.94	61.13
12	200	5	74.86	73.78
13	320	5	76.81	76.14

diffusion method. All 13 batches proposed by the experimental design yielded microsponge preparations and were characterized for particle size and entrapment efficiency. The data obtained for experimental design is shown in Table 2.

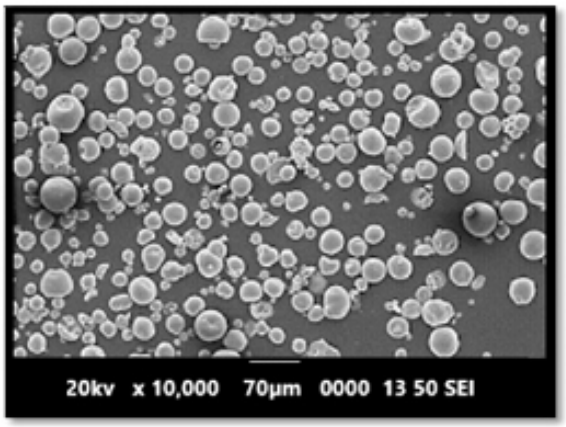

Fig 1: SEM images of optimized microsponge

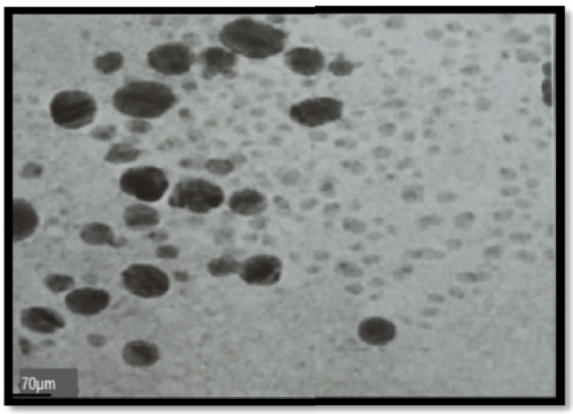

Fig 2: TEM images of optimized microsponge

EVALUATION OF OPTIMIZED SILYMARIN MICROSPONGES

- The SEM analysis shows (fig.1) prepared optimized microsponge formulation was roughly spherical in shape and porous in nature. The size of the optimized microsponge was found to be 70µm.
- The TEM images of the Silymarin microsponge shows (fig.2) spherical shaped particle having particle size of 70µm.
- The percentage yield and entrapment efficiency was found to be 88.45% and 75.34% respectively.
- The mean particle size was found to be 66.16µm.

IN-VITRO DRUG RELEASE STUDY

The results obtained in *in-vitro* release studies were plotted as percentage cumulative drug release v/s time (fig 3). After 24 hours the cumulative percentage drug release of silymarin from microsponges were found to be 82.67%.

KINETIC MODELLING

Different plots of kinetic modelling are constructed such as zero order, first order,Higuchi, Korsmeyer-Peppas plot(fig.4-7). The *in-vitro* drug release of Silymarin from microsponge at pH 7.4 was best explained by Higuchi Model, indicate the sustained release with high R^2 value (table 3).

The Korsmeyer-Peppas plot shows the mechanism of release of silymarin from silymarin loaded microsponge that follows a non-fickian or anomalous diffusion.

EFFECT OF SILYMARIN MICROSPONGE ON PROLIFERATION OF HUMAN FIBROBLAST CELLS

- **MTT ASSAY**

The effects of silymarin microsponge on the proliferation of human neonatal foreskin fibroblast cells were examined by the MTT assay (table 4). Morphological changes of cells with different concentration of drug are shown in (fig.8).

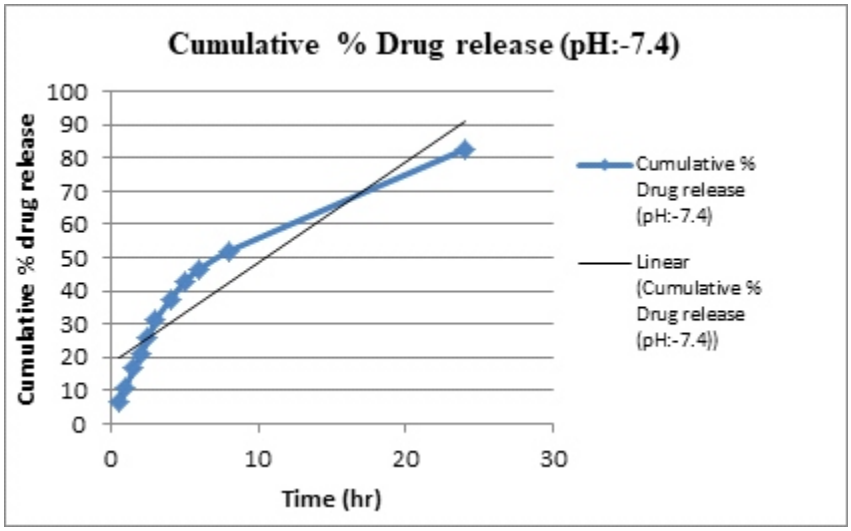

Fig 3: *In Vitro* Drug Release Studies Plot for Microsponge Formulation

Table 3 : Release kinetics of silymarin from microsponge

pH	Zero order	First order	Higuchi	Korsmeyer- Peppas	
	R^2	R^2	R^2	R^2	n
7.4	0.942	0.967	0.994	0.987	0.782

KINETIC MODELLING

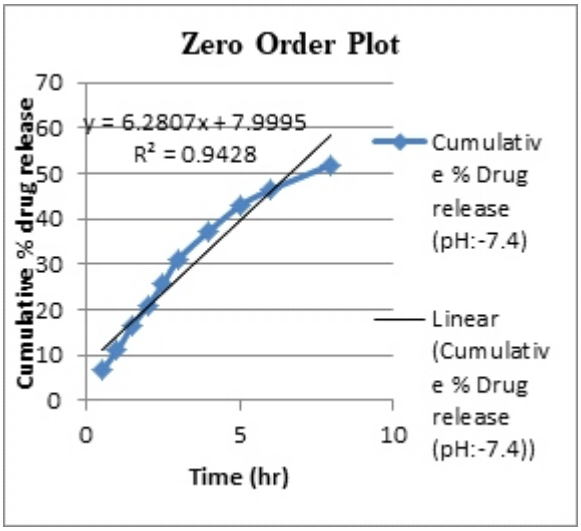

Fig 4: Zero order plot of optimized microsponge

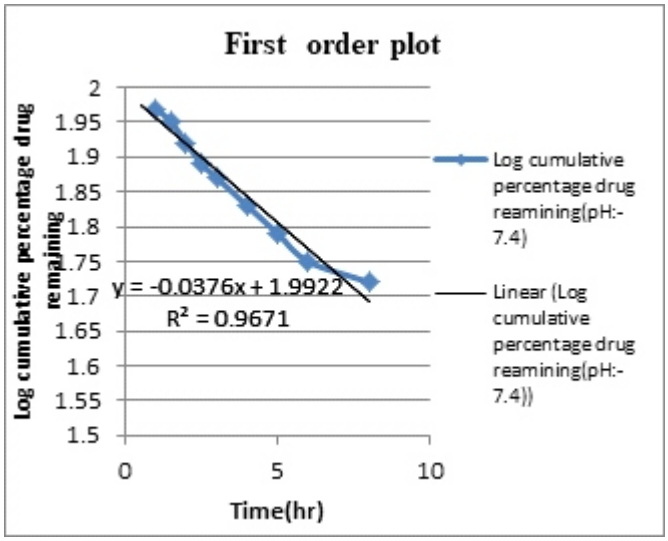

Fig 5: First order plot of optimized microsponge

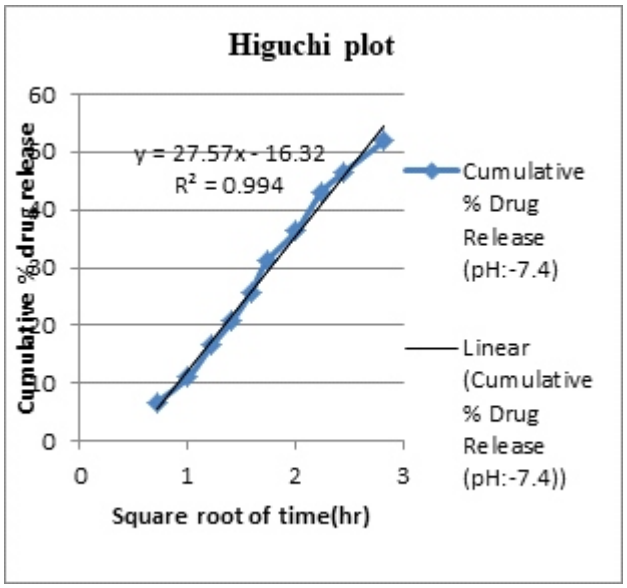

Fig 6: Higuchi plot of optimized microsponge

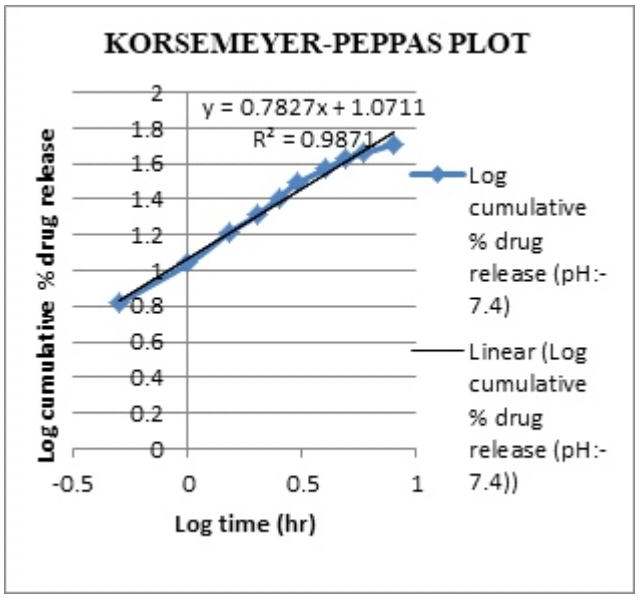

Fig 7: Korsemeyer Peppas plot of optimized microsponge

Table 4 : Proliferation rate data of Silymarin from MTT assay

Concentration of Silymarin loaded microsponge (µg/ml)	Absorbance at 570nm	Proliferation Rate (%)
12.5	0.568	104.99
25	0.591	109.24
50	0.658	121.62
100	0.782	144.54

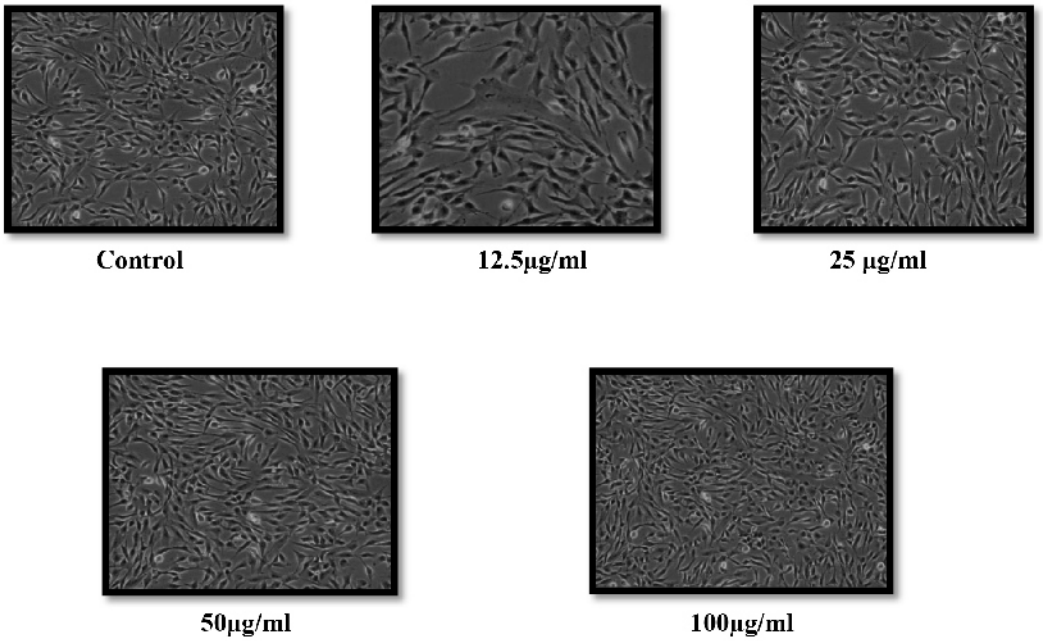

Fig 8: Morphological changes of human neonatal foreskin fibroblast cells when treated with 12.5µg/ml, 25µg/ml, 50µg/ml, 100µg/ml of silymarin loaded microsponge.

The fibroblast cell proliferation rate increase with increase in concentration of silymarin microsponge. Highest proliferation rate obtained in concentration of 100µg/ml.

EVALUATION OF SILYMARIN MICROSPONGE LOADED TOPICAL GEL

The prepared silymarin loaded microsponge gel subjected to following studies.

pH - pH value of the prepared gel formulation was found to be 6.97±1.8.

Homogeneity - The prepared gel formulation was clear and transparent without any aggregates.

Spreadability - The Spreadability of the prepared gel formulation was found to be 8.24±.36 cm.

Extrudability - Suitable consistency is required in order to extrude the gel from the tube. The extrudability of the prepared gel formulation was found to be excellent.

Viscosity - Viscosity of the prepared gel formulation was found to be 1370±1.5Cp.

Drug content - The percentage drug content in the topical gel formulation was found to be 92.21% and the drug was uniformly distributed in the formulation.

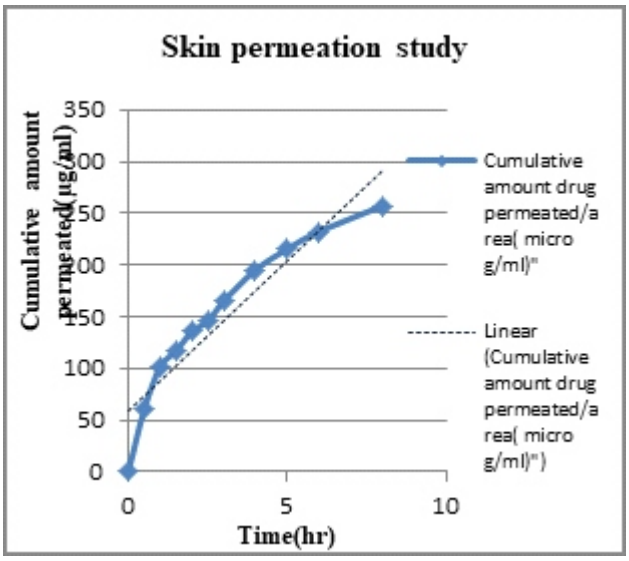

Fig 9: Plot of *Ex-vivo* Skin permeation

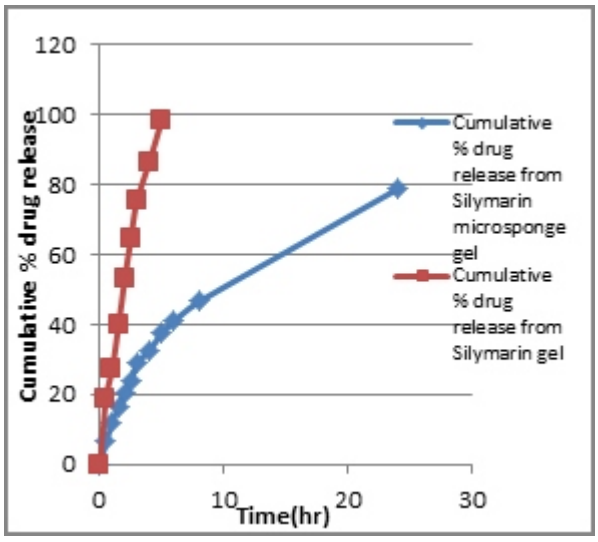

Fig 10: Plot of comparative *in-vitro* drug release

EX-VIVO SKIN PERMEATION STUDY

Ex-Vivo skin permeation of Silymarin microsponge loaded gel was studied by Franz Diffusion Cell using excised goat skin. The flux and permeability was found to be 29.15 µg/cm^2/ h and 0.0219 cm/h respectively, which indicate the lowest systemic permeation of silymarin microsponge loaded topical gel (fig 9). Most of the drug deposited on the surface of skin, rather than entering into systemic circulation.

COMPARATIVE *IN-VITRO* DRUG RELEASE STUDIES

In *in-vitro* drug release studies plain drug gel has shown an immediate burst release within 5 hrs whereas the silymarin microsponge loaded topical gel formulation has shown sustained release up to 24 hrs (fig 10).

DISCUSSION

In the present study Silymarin loaded microsponge were prepared by quasi emulsion solvent diffusion method. The formulation and process variables were optimized using Design of Experiments Software. Central composite study confirmed that the amount of ethyl cellulose and quantity of ethanol significantly influenced the dependent variables particle size and entrapment efficiency. The optimized microsponges were characterized by SEM, TEM, product yield, entrapment efficiency and particle size analysis. SEM analysis revealed that microsponges were spherical, porous with rough surface morphology without any aggregation. The pores were caused by the diffusion of solvent from the surface of microsponge [14]. The entrapment efficiency was found to be 75.34%. Particle size analysis confirmed that the optimized formulation had mean particle size of 66.16µm and uniformly distributed throughout the formulation.

The present study evaluates the effect of silymarin on human fibroblast cells in an *in vitro* model of wound healing. Carried out the MTT assay on *human neonatal foreskin fibroblast cells* and the results showed silymarin at concentration 100µg/ml significantly enhanced the proliferation of fibroblast compared to the untreated group which is in agreement with earlier reports [3].

S.A.Tabari *et al* [4] demonstrated the ability of silymarin ointment to improve fibroblast proliferation, inflammation and wound healing rates in diabetic rats, while in the present study microsponges were formulated and are superior over ointment. Ointments are aesthetically unappealing, greasiness, stickiness etc. That often results into lack of patient compliance. In microsponge system, maximum amount of time that an active ingredient is present either on the skin surface or within the epidermis, while minimizing its transdermal penetration into body.

The optimized microsponges were incorporated into Carbopol 934 gel base and evaluated for pH homogeneity, spreadability, extrudability, viscosity and drug content. pH of the gel shows 6.97± 1.8 pH which is near towards neutral. Spreadability of the gel was found to be 8.24±.36cm. Viscosity of formulation shows the pseudoplastic flow property of gel and percentage of drug content in the formulation was found to be 92.21% [15]. The *in-vitro* drug release study was found that silymarin microsponge gel show sustained drug release up to 78.67% in 24hr while plain silymarin gel shows immediate burst release within 5hr which indicates that microsponge formulation greatly affects release of entrapped drug. *Ex vivo* skin permeation study using excised goat skin was also done by Franz diffusion cell showed low systemic permeation of drug. Enhancement of drug residence time in the skin was achieved as the microsponges in the gel act as a reservoir of drug and slowly delivers the drug as the skin needs it [16].

CONCLUSION

Silymarin loaded microsponge gel was successfully developed for the treatment wounds. Microsponges were prepared by quasi-emulsion solvent diffusion method and further formulated into gel and characterized. Results indicated that the microsponges slowly released the entrapped drug on wound surface and gel provided moist environment for wound management. Microsponge system overcomes the problems of conventional silymarin formulations by improving sustained

drug release and minimizing transdermal penetration into body.

In future, microsponge can be used to prepare topical formulation and it's *in vivo* studies will throw more light into its utility as a novel drug delivery system.

CONFLICT OF INTEREST

There is no conflict of interest in the study.

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