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FORMULATION OPTIMIZATION OF SILYMARIN MICROPARTICLES AND EVALUATION OF ITS HEPATOPROTECTIVE ACTIVITY

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Abstract

Purpose: Theaim of the present study was to develop sustained release polymeric microparticles of silymarinso as to reduce its frequent dosing, minimize the adverse effects and maximize the therapeutic efficiency, in terms of its hepatoprotective activity.

Materials and methods: Silymarin loaded microparticles were prepared by Emulsification and Ionic gelation technique. Evaluation ofoptimised batch of microparticlesincluded determination of yield, entrapment efficiency, morphological studies, DSC, FT-IR, *in vitro* dissolution studies and *in vivo*study on Swiss Albino mice using CCL_4 induced hepatotoxicity as an experimental model.

Results: In vitro dissolutionstudies illustrated sustained, zero order drug release from optimized formulation also its therapeutic potential was amplified during in vivo studies on swiss Albino mice using CCL_4 induced hepatotoxicity model.

Conclusion: Microparticles could provide a promising stratagem to combat various biopharmaceutical problems of silymarin through its oral sustained release drug delivery.

Key Words: Hepatoprotection, Sustained release, Silymarin Microparticles, Chitosan and CCL_4

Abbreviations: Carbon tetrachloride (CCL_4),Chitosan (Ch), Na Tripolyphosphate (TPP), Microparticles (MPs), Particle size (PS) and Encapsulation efficiency (EE)

Introduction

Liver is the major site for drug elimination making it prone to drug induced liver injury (DILI). Many chemical substances may also lead to liver injury. Liver toxicity can range from elevated liver enzymes to hepatic failure to autoimmune liver diseases. Liver protective agents can protect liver from the ill-effects and could also repair the

damaged hepatocytes. Silymarin is a naturally occurring hepato-protective agent that has been of interest since ages [1]. Silymarin is a polyphenolic flavonolignan derived from Silybummarianum; also known as milk thistle [2]. Other pharmacological activities of Silymarin include anti-inflammatory, antioxidant, anticancer, treatment of gallbladder stones, protection against Amanita phalloides fungus [3, 4].

Active components of Silymarin extract include flavonolignans (silybin A & B, isosilybin A & B, silydanin, silychristin) and flavonoids (taxifolin and quercetin) comprising 70-80% of the extract [5-7].

Recommended dose of Silymarin in adults is 240-800 mg/day in 2-3 divided doses, orally. Water solubility of Silymarin is low and it is prone to degradation in gastric environment leading to low absorption hence poor bioavailability. It is rapidly absorbed (2-4 hours) and t1/2 is 6 hours. It is metabolized via phase I and II biotransformation in liver and is eliminated from the body in free and conjugate form (through bile and urine) [8-9].

There is a constant emphasis to develop particulate drug delivery systems to increase bioavailability hence therapeutic efficacy. Sustained and controlled drug delivery systems can provide the desired level of therapeutic efficacy so that the drug is delivered in a sustained manner and therapeutic concentration is maintained to provide the minimum required therapeutic effect for a stipulated duration.

Microspheres are the formulations that can deliver drug in a sustained manner.

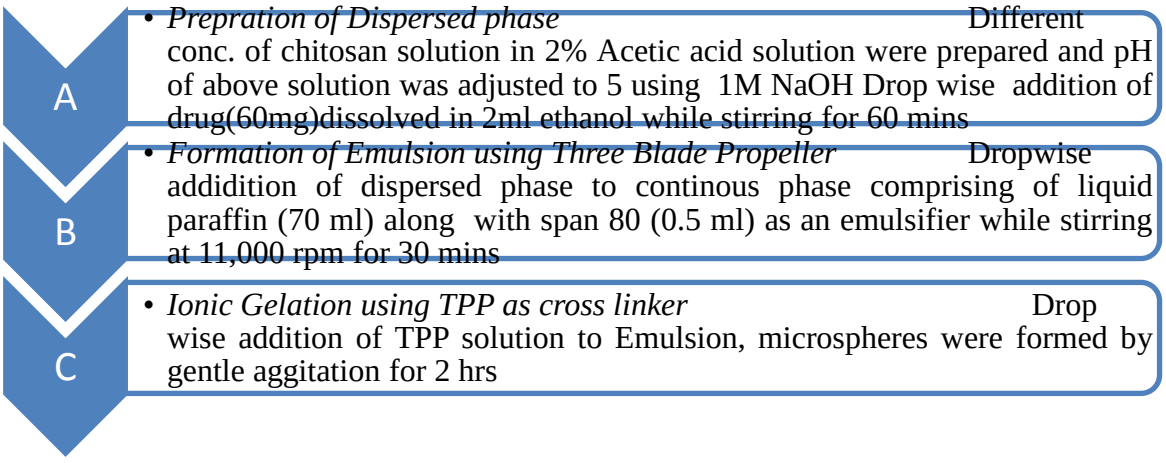
Materials and Methods

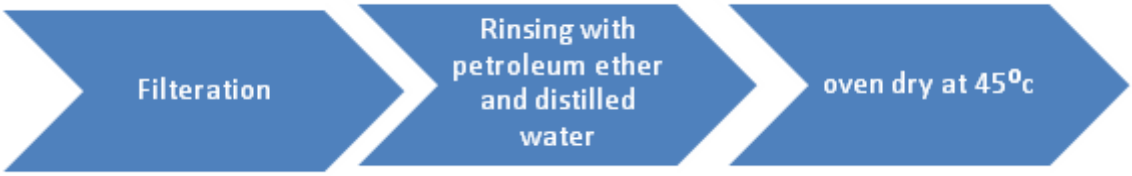
Materials

Silymarin was generously gifted by Micro Labs (Bangalore), chitosan was procured from Central institute of fisheries and technology (Kochi), sodium tripolyphosphateand span 80 were purchased from Himedia laboratories Pvt. Ltd.(Mumbai). All other chemicals were of analytical grade.

METHODS

Preparation of silymarin- chitosan Microparticles by Emulsification and Ionotropic Gelation [10, 11].





Experimental Design

The preparation of Silymarin loaded chitosan microparticles was optimized using a 2-factor 3-level $(3)^2$ central composite experimental design including –α and +α values for formulation variables (independent variables). Concentration of Chitosan(X1) and ratio of Chitosan: TPP(X2) were selected as independent variables on the basis of preliminary trials. Release rate(Y1) and encapsulation efficacy(Y2) were selected as dependent variables. Independent variables along with low, medium and high levels is shown in Table I.

Table 1: Experimental design with dependent and independent variables.

Trial Run	Coded factor levels X1	X2	EE (%) Y1	RR (%) Y2
1	0	0	63.02	63.34
2	0	0	74.32	69.86
3	0	0	58.13	55.14
4	0	+α	44.4	59.31
5	0	0	68.6	65.52
6	+α	0	66.26	40.45
7	0	-α	64.32	31.75
8	-α	0	61.4	40.45
9	-1	+1	40.51	32.18

Factors	Unit	High	Median	Low	-α	+α
Chitosan(X1)	mg	400	350	300	279.29	420.71
Chitosan:TPP(X2)	-	1.25:1	1:1	0.75:1	2.66:1	4.34:1

10	+1	-1	61.4	30.82
11	-1	-1	67.59	41.79
12	+1	+1	65.6	80.32
13	0	0	65.13	64.42

Independent variables in CCD

Determination of Encapsulation Efficiency (EE)

Microspheres (20 mg), soaked in methanol (10 ml) was placed at flask shaker for 24 hours at 37℃. The solution was filtered using 0.45 mm membrane filters and after suitable dilution (10 times) with phosphate buffer samples were analysed by UV detector at wavelength of 287nm. The amount of drug entrapped was calculated using the following equation:

$$\%\mathbf{EncapsulationEfficiency} = \frac{\mathbf{Amountofdruginmicrospheres}}{\mathbf{Totalamountofdrugadded}}\mathbf{X\ 100}$$

Morphological Study

The morphological examination of prepared microspheres was carried outusing SEM (S-3400N, Hitachi, Tokyo, Japan) at an accelerating voltage of 20 kV. AnAppropriate amount of microspheres was placed on aluminium stubs, using double sidedadhesive tape and were coated with gold film using sputter coater.

Differential Scanning Calorimetry (DSC)

The physical state of drug inside the MPs was investigated by Differential Scanning Calorimetry (DSC). The thermogram of the drug loaded MPs were obtained using DSC (TA instruments, Model no. Q10). For this, the small amount (2-7 mg) of sample was sealed in the aluminium pan and the temperature was raised at 10℃/min from 40 to 300℃.

FT-IR (Fourier Transform Infrared Spectroscopy) Spectral Analysis

Infrared spectroscopy of the different formulations was studied to confirm the drug loading and drug-excipient interaction by KBr pellet method at moderate scanning speed between 4000-400 cm^{-1} was carried out using FT-IR (Perkin-Elmer Life and Analytical Sciences).

Percentage Yield

The yield of the chitosan microspheres was calculated using the following equation

$$\% \textbf{ Yield} = \frac{\textbf{Weightofdriedmicrospheres}}{\textbf{Totalweightofdrug, polymerandcrosslinker}} \textbf{X 100}$$

***In vitro* Drug release Study**

The drug release rate was studied using USP XXII dissolution apparatus II (Paddle type). Solid microspheres containing drug equivalent to 3.5 mg, suspended in 1ml of dissolution medium in a dialysis membrane bag (Himedia, MWCO, and molecular mass cut off 12,000–14,000, pore size 2.4 nm), was placed into a flask containing 250 ml phosphate buffer with 0.1% tween 20 (pH 7.4). Tween 20 had been used in dissolution media for poorly soluble drugs [12].

Temperature maintained was 37±1^{O}C at 50 rpm. At specific intervals (0.25, 0.50, 1, 2, 3, 4, 5, 6, 8, 9, 11 & 24 hours) 5 ml aliquots were withdrawn and was replenished by fresh dissolution medium.

The absorbance of the samples was determined by UV Spectrophotometer at ʎmax=287 nm. Absorbance for the samples aliquots was recorded and % drug release at different time intervals was plotted against time. The cumulative % drug release at different time is shown in Fig 8.

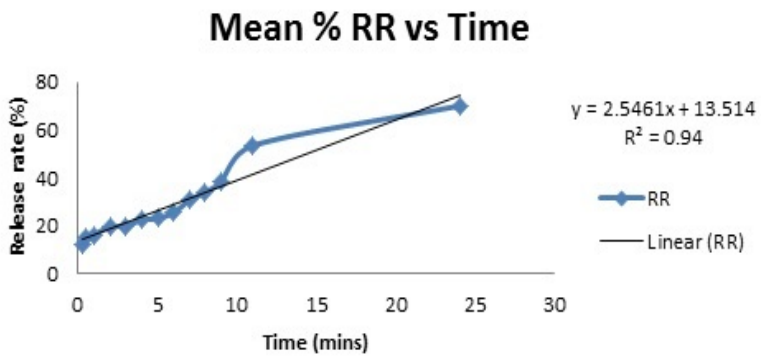


Figure 8: Dissolution profile of ch MPs.

***In vivo* Study**

The efficacy of Silymarin loaded MPs were compared with pure drug Silymarin, by evaluating *in vivo* Hepatoprotective activity in mice.

Experimental Animals

Albino mice of either sex (weighing 25-30 gm) were procured from LLRUVAS, Hisar (INDIA). The mice were placed in a group of 6 mice per cage (cage size = 29x22x14) under standard environmental conditions (25±2^{O}C and relative humidity 50±5%) with alternating dark and light cycle of 12hours each. The animals were maintained on standard pellet food and water *ad libitum*, in order to acclimatize them to laboratory environment for two weeks before experiment was carried out. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) and animal care was taken as per guidelines of Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India (Registration No. 0436). Efforts were made throughout to minimize animal discomfort.

Animal Model for evaluation of hepatoprotective activity (mice)

CCl_4 induced Hepatotoxicity [13-15].

The mice weighing 25-30 gm were used for CCl_4 induced hepatotoxicity Mice were randomly divided into four groups ofsix mice each:

a) Group I: Control group

b) Group II: 1ml/kg CCl_4 and olive oil[1:1](Toxic Control group)

c) Group III: 50mg/kg body weight Silymarin (Standard group)

d) Group IV: 50mg/kg body weight Silymarin loaded Microparticles (test group)

The doses were administered to test animals through gavage using gastric tube. The animals of group I received distilled water for 4 days and served as normal control. The second group animals received distilled water for all four

days and a single dose of CCl_4 (1 ml/kg intraperitoneally, i.p.) on 4th day, and served as the toxic control. The third group served as the standard and animals were treated with an oral suspension of standard drug silymarin in Tween 20 (1%, v/v) at a dose of 50 mg/kg/day on all 4 days and CCl_4 (1 ml/kg, i.p.) on day 4, 1 hr after the administration of the standard drug. Group four served as the test group for treatment with MPs suspension in distilled water with Tween 20 (1%, v/v) at a dose of 100 mg/kg/day on all 4 days and was intoxicated with CCl_4 (1 ml/kg, s.c.) on day 4, 1hr after the administration of formulation. The blood was collected after 24 hours of CCl_4-intoxication treatment via cardiac puncture in glass tubes. The blood and liver samples were assessed for their biochemical changes including levels of SGPT, SGOT and ALP.

Statistical Analysis: The values are expressed as mean ± S.D. The statistical analysis was carried out by one way analysis of variance (ANOVA) followed by Dunnett's't'-test. P values <0.01 were considered to be significant.

Results

Data analyses and validation of optimization model for MPs (using design expert 7.1.6)

Design Expert 7.1.6 was used for the study of response surface methodology of microparticles. The goal of optimization was to maximize entrapment efficiency and target the release rate of prepared formulation. Quadratic model was selected for given response variables (EE and RR), polynomial equation using multiple linear regression analyses were generated. Model was found to be significant (*P* value <0.05) with non-significant lack of fit (*P* value>0.05). Response variable entrapment efficiency (Y_1) and release rate(Y_2) both fitted best into the quadratic model after none transformation.

Response Surface Analyses

3-D response surface graphs were obtained using Design Expert software as shown below in Fig 1 and Fig 2.

From above figure EE was observed to be decreasing with increase in chitosan: TPP ratio as well as chitosan concentration at lower levels of the other factor where as max. EE was observed at lower levels of both the factors and formulation optimization yielded predictability of 67.41 for the solution proposed.

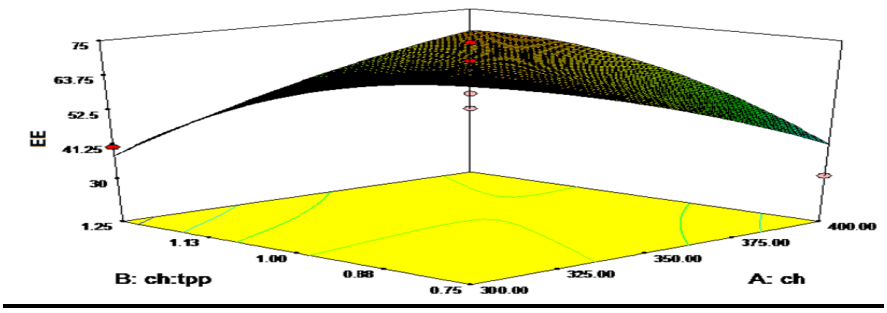


Figure 1: 3-D response surface curve depicting the influence of ch and ch:tpp on EE of given formulation.

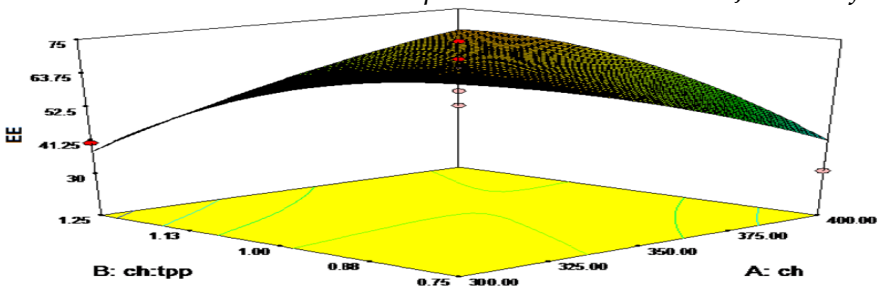


Figure 2: 3-D response surface curve depicting the influence of ch and ch:tpp on PS of given formulation.

It is evident from the above 3–D curve (Fig 1) that EE is linearly dependent on ratio of chitosan: TPP i.e. its value increases on increasing the ratio from 3:1 to 4:1while, it is nonlinearly dependent on chitosan concentration i.e. non linear decrease from the maximum value.

From Fig 2 release rate was found to be related with both the factors and extreme values for the parameters selected i.e. (chitosan: TPP 1.25 and chitosan concentration 400mg) yielded maximum release rate. The absence of any one factor decreases the release rate drastically from 70.32 to 32.86.

The optimization criteria with goals, limits and importance used for optimization of silymarin microparticles are shown in Table II. The criteria for obtaining optimized solution of MPs were set so as to maintain maximum EE and targeted RR. Based on desirability approach for optimization one solution was obtained, which suggested Chitosan level at 400 mg and chitosan: TPP ratio at 1.17:1 should yield entrapment efficiency of 67.41 and Drug release in 24 hrs at 70.32% with higher level of desirability (0.897) as indicated in given Table III and represented in Fig 3. Table III outlines predicted values of EE and RR, as suggested by the software, which were similar to their observed values obtained upon preparation and characterization of the optimised solution. This negligible error certainly approves the success of optimization tool and technique. Above graph (Fig 3) clearly depicts that maximum desirability (0.897) can be achieved at higher values of chitosan : TPP(1.17:1) and around mid value of chitosan concentration(400mg).

Table 2: Criteria used for formulation optimization of silymarin MPs.

Name	Goal	Lower limit	Upper limit	Weight	Importance
Chitosan (mg)	In range	300	400	1	3
Chitosan:TPP	In range	0.75:1	1.25:1	1	3
EE	Max.	30.82	74.34	1	5
RR	Target =70.32	30.82	80.32	1	3

Table 3: Solution of numerical optimization.

Optimized formulation	Ch (mg)	Ch:TPP	EE			PS		
			Predicted	Observed	Difference	Predicted	Observed	Difference
1	**400**	**1.17:1**	**67.41**	**66.58**	**-1.21**	**70.32**	**70.008**	**-0.312**

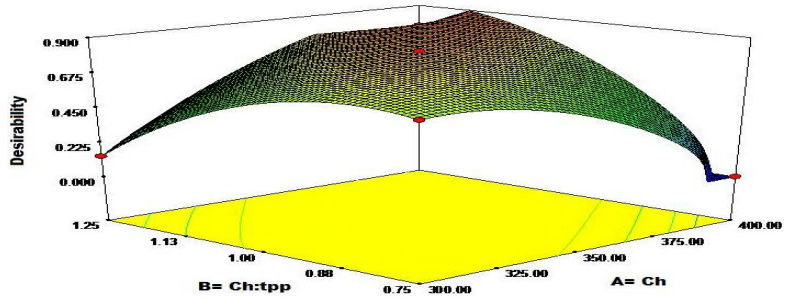


Figure 3: 3-D response surface curve depicting the influence of ch and ch:tpp on desirability of given formulation.

Evaluation of Microparticles

Yield and Particle size of MPs

Yield and Particle size of the given formulations anticipated as per experimental design along with final optimized batch is been represented with the help of bar diagram in Fig. 4.

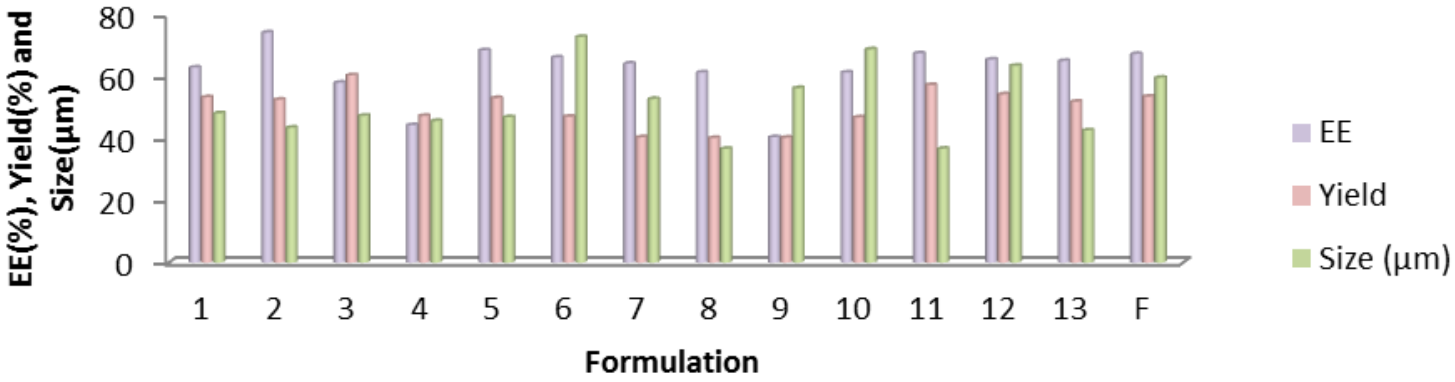


Figure 4: EE, Yield and Size of various Formulations.

Entrapment Efficiency (EE) of Microparticles

Drug entrapment efficiency (EE) of the prepared MPs was determined using the method described earlier. The EE of MPs ranges from 40.51% to 74.32% as shown in Fig 4 while that of optimized batch is 65.58%.

Morphological study of MPs by Scanning Electron Microscopy (SEM)

SEM image of silymarin loaded microparticles is shown in Fig 5, which reveals spherical microparticles with rough and porous surface.

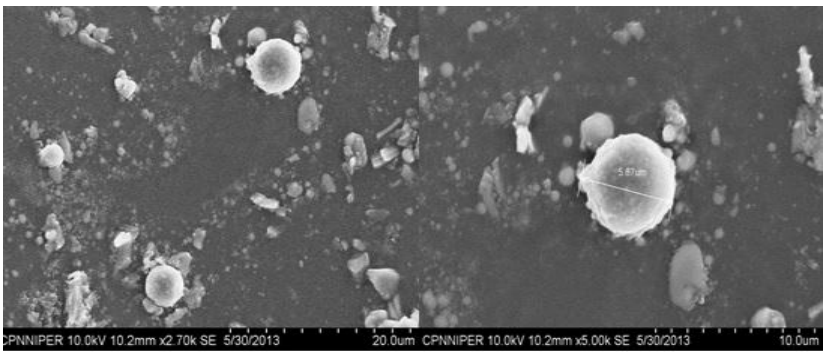


Figure 5: SEM image of silymarin MPs.

Differential Scanning Calorimetry (DSC)

The DSC thermo grams provide information about physical and chemical changes that involve endothermic or exothermic processes or change in heat capacity. An overlay was prepared using the thermograms of polymer (Chitosan), drug (silymarin), and optimized silymarin-loaded MPs, as shown in Fig.6.

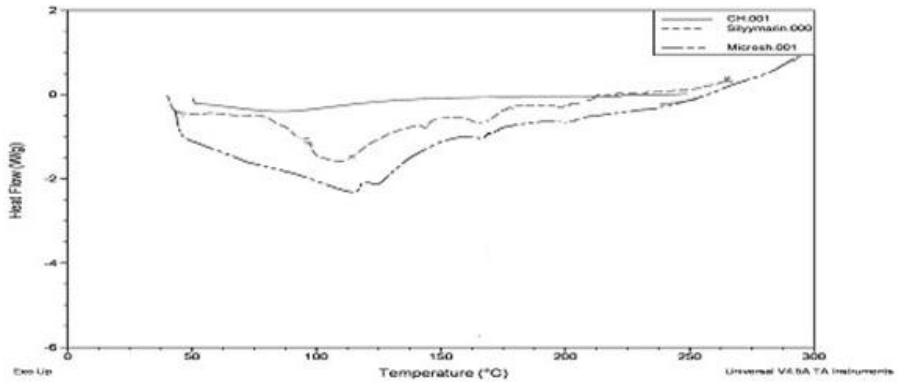


Figure 6: Overlay of differential scanning thermogram of polymer, drug, MPs of silymarin.

FT-IR Spectral Analysis

The FT-IR spectra of chitosan, sodium tripolyphosphate, silymarin, and drug loaded microparticles were analysed Fig 7. Optimised batch of MPs exhibited all characteristic peaks of silymarin. It may be concluded that the drug was present in formulation without any chemical interaction between drug and other excipients and the processing technique has not affected the chemical stability of drug.

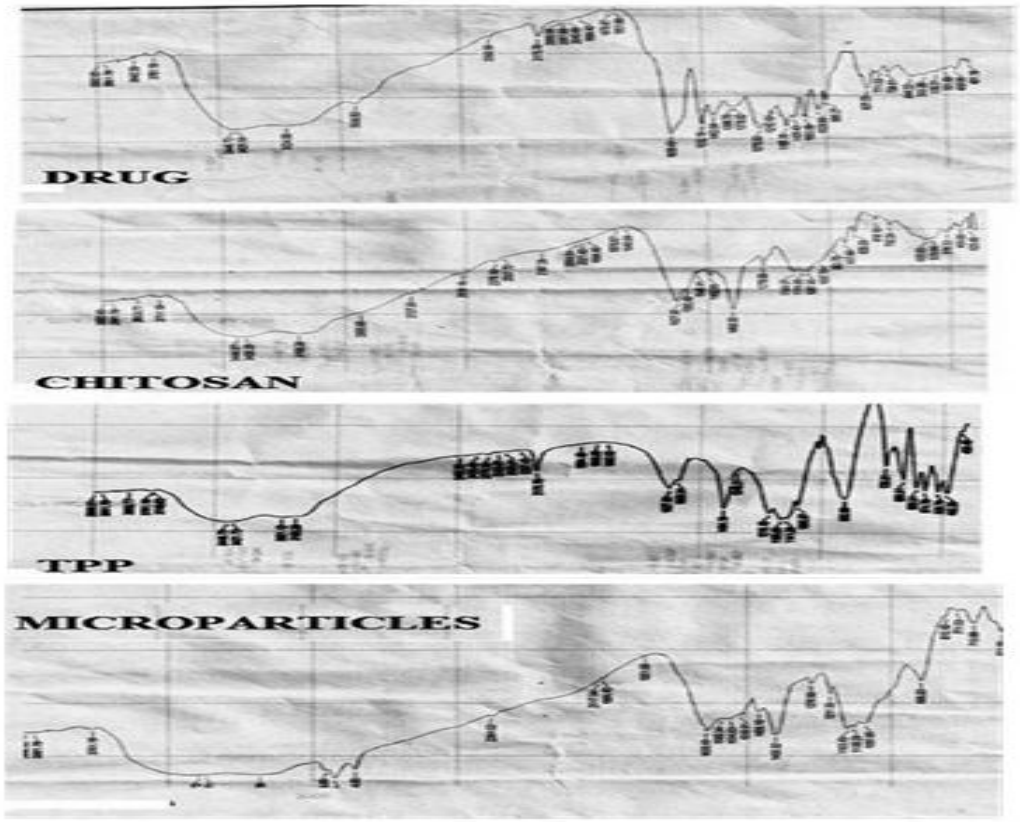


Figure 7: Comparative FT-IR spectra of silymarin, chitosan, TPP and MPs.

***In Vitro* Drug Release**

Optimized formulation:

The *In vitro* drug release behaviour of quoted optimized batch as per the experimental design and pure drug (silymarin) was performed (Fig.8). Only 70.10% of drug was released from chitosan microparticles in 24 hours while 99.94% of drug was released from pure silymarin thereby, showing sustained release behaviour of formulated MPs.

The release data was of silymarin loaded microparticles is fitted into various modelsas presented in Fig 9. Different models (Zero-order kinetics, First-order kinetics, Peppas and Higuchi equation) were employed to fit the release profiles of silymarin-loaded MPs in Phosphate buffer Their corresponding R^2 and n values are shown in Table 4. Interestingly, the in vitro release pro- files of silymarin-loaded MPs can be best fitted to Zero order. Which is a typical sustained-release model, indicating the sustained-release effect of silymarin formulation. Hence drug release rate is independent of amount of drug present in sustained release formulation which is an ideal drug delivery for sustained release dosage form.

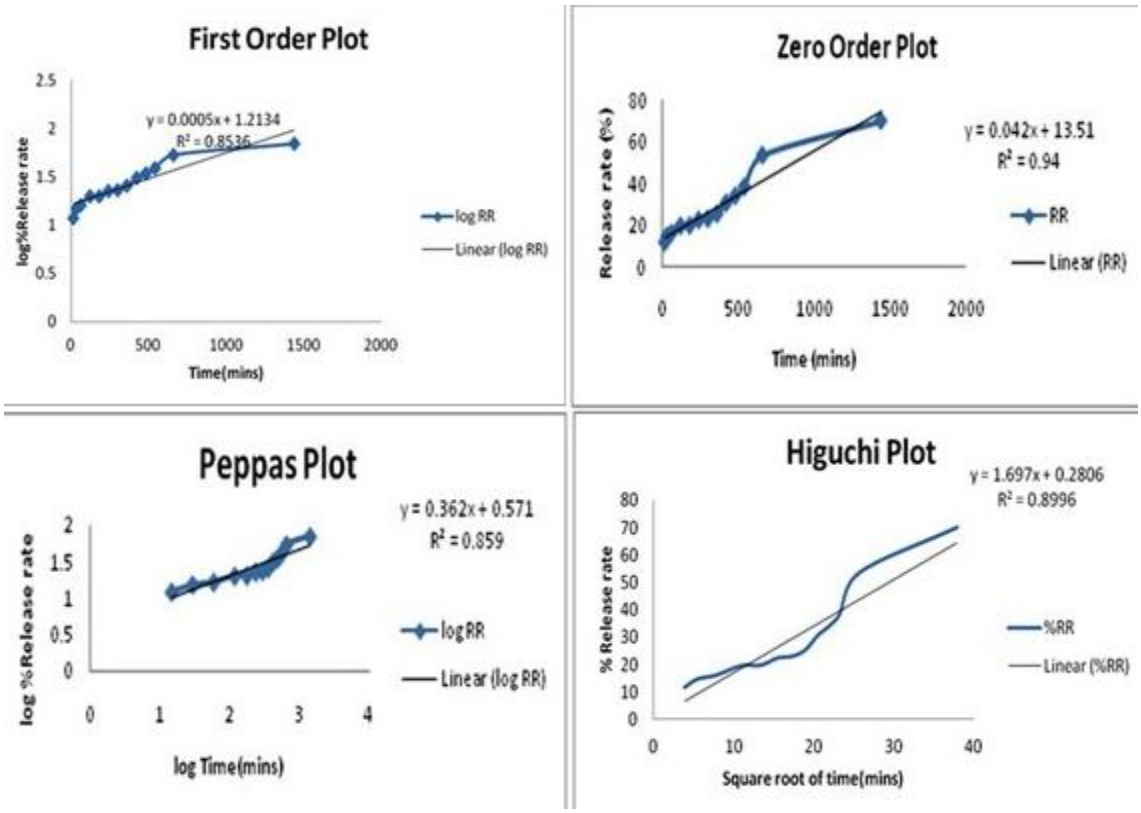


Figure 9: First order, Zero order, Peppas and Higuchi plot of optimized batch.

Table 4: Various models with their R^2 and n value.

Model	R^2 Value	n value
Zero order	0.94	0.0424
First Order	0.8536	0.0005
Higuchi	0.8996	1.697
Korsmeyer-Peppas	0.8597	0.3621
Best Fit Model	Zero order	

***In Vivo* Study**

The efficacy of silymarin loaded microparticles compared to pure drug silymarin, were evaluated by *in vivo* study in mice for hepatoprotective activity.

Biochemical Estimations

The results of hepatoprotective activity of silymarin microparticles on CCl_4 treated mice are shown in Table 5. The formulation significantly ($p<0.01$) reversed the level of various hepatic enzymes (SGOT, SGPT and ALP) when compared to CCL_4 treated animals**.** The results have also been exhibited in Fig 10.

Table 5: Effect of silymarin and its formulations on CCL_4 induced liver damage.

Parameters	**SGOT**	**SGPT**	**ALP**
Control	12.20 ± 0.53	26.68 ± 0.6	31.96 ± 1.43
Toxic control CCl_4	42.89 ± 0.9$^{\#}$	88.4 ± 0.76$^{\#}$	91.44 ± 1.25$^{\#}$
Silymarin (pure drug)	19.1 ± 0.65**	38.80 ± 0.79**	42.04 ± 0.7**
Silymarin NPs	14.53 ± 0.38$^{** \$}$	31.55 ± 0.43$^{** \$}$	34.84 ± 0.66$^{** \$}$

Values are mean±SD (*n*=6).**p<0.01 compared to CCl4-treated mice, #p<0.01 compared to control group, $p<0.01 compared to silymarin pure drug group.

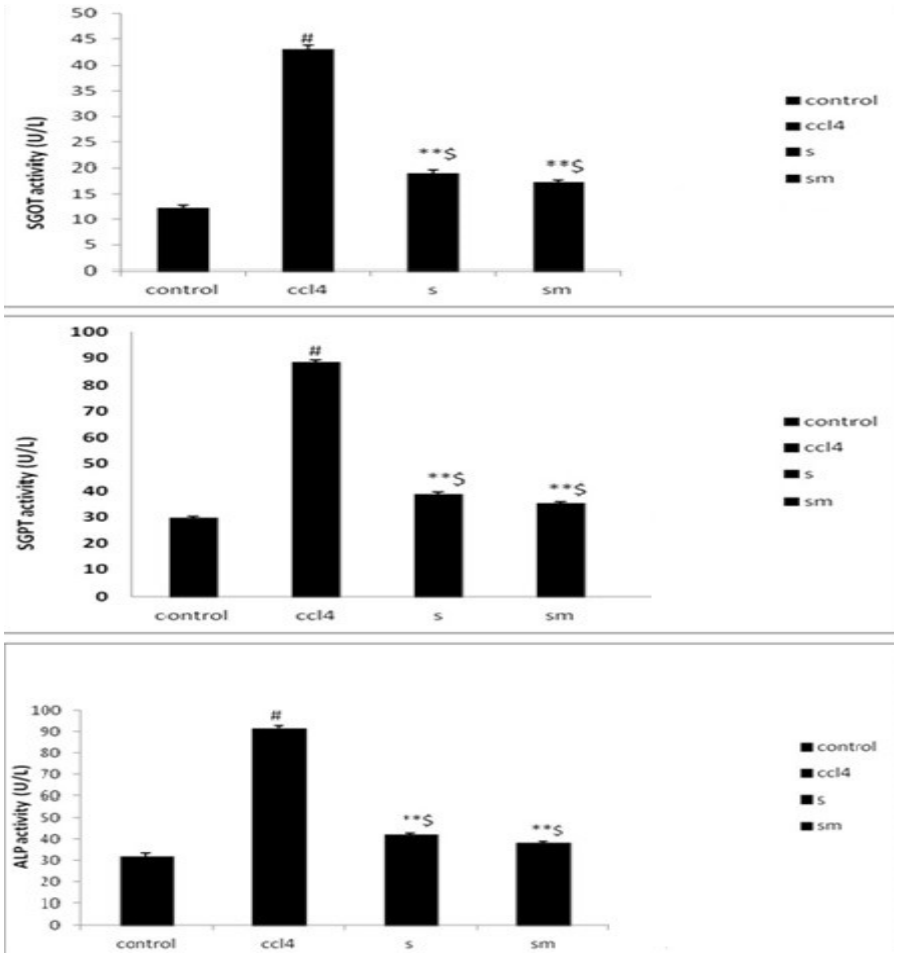


Figure 10: Effect of silymarin and silymarin loaded Nanoparticles on levels of liver markers (SGOT, SGPT and ALP) after 24h of CCl_4 administration.

With the animals treated with pure drug silymarin and silymarin loaded microparticles, there was a significant lowering of SGOT, SGPT and ALP levels in experimental animals after 24 hours of the CCl_4 administration in all the groups. The decrease in levels of SGPT, SGOT and ALP indicated the hepatoprotective activity of drug and formulation from CCL4 induced hepatotoxicity. Moreover, the hepatoprotective potential of microparticulate formulation was higher than the hepatoprotective action shown by pure drug dispersion of silymarin, which may be accredited to sustained release action of formulation

Values are mean ± SD (n=6)** $p<0.01$ compared to CCl_4-treated mice, $^{\#}p<0.01$ compared to control group, $^{\$}p<0.01$ compared to silymarin pure drug group.

Discussion

The current investigation was an attempt to improve the solubility associated bioavailability of silymarin by formulating micro formulation, and also to control the release of silymarin from the dosage form, to be able to achieve a prolong hepatoprotective effect. A plethora of literature have established that microparticles can be used *in vivo*to safeguardthe drug in the systemic circulation and deliver the drug at sustained rate at the site of action, which trim down the dosing frequency.

SEM study revealed formulation of spherical microspheres with rough and porous surface.DSC studies of drug and formulations confirmed no polymorphic changes in the drug loaded microspheres. FT-IR also confirmed successful formulation of colloidal particles without any significant chemical interaction. The *in-vitro* dissolution studies of the optimised formulation was carried out in phosphate buffer (pH=7.4). It exhibited only 70.10% drug release in 24 hrs from microparticles owing to sustained release formulation. Zero order drug release was found to be the most suitable best fit for sustained release of formulations. The value of dependent variables was close to predicted values of solution suggested by software which indicated success of optimization technique.

The therapeutic potency of silymarin loaded microparticles was evaluated through *in-vivo* studies on Albino mice using CCL_4 induced hepatotoxicity as an experimental model. The levels of serum SGPT, SGOT and ALP activities, generally considered as good markers of hepatic injury and hepatocellular integrity, were determined. An augmented activity of marker transaminases in the serum indicates liver damage. Aggravated potential of silymarin loaded microparticles was witnessed. This was also evidenced from the downturn in levels of marker enzymes of test groups compared with the toxic control group and pure drug. The improvement in the enzyme activity was due to sustained and targeted action of microparticles on the hepatocytes which due to their pronounced antioxidant effect may have

diminished the release of SGOT, SGPT and ALP enzymes from the liver cells and thereby eliciting hepatoprotective activity. The therapeutic strength of Silymarin loaded microparticles was assessed through *in-vivo* studies on Albino mice using CCL4 induced hepatotoxicity as an experimental model. The level of serum SGPT, ALP and SGOT activities, generally considered as good markers of liver damage and hepatocellular integrity were determined. An enhanced level of marker transaminases in the serum signifiesliver damage. Aggravated potential of silymarin loaded microparticles was witnessed. . This was also evidenced from the decline level of marker enzymes of test groups compared with the toxic control group and pure drug. The improvement in the enzyme activity was credited to sustained action of microparticles on the hepatocytes which due to their distinct antioxidant activity may have reduced the release of SGOT, SGPT and ALP enzymes from the hepatocytes and therefore eliciting hepatoprotective activity.

Conclusion

It could thus be concluded that the microparticulate methodology developed for silymarin can help to navigate silymarin molecules *in vivo* in an effective way for hepatoprotection. Its sustained hepatoprotective effect might be attributed to small size, higher surface area and coating the formulation with biodegradable and biocompatible polymer chitosan (matrix reservoir), which permitted improved bioavailability and better absorption when administered orally. However, further clinical studies are cardinal to brace the verdict of pharmacodynamic investigations and to certify the performance of developed MPs in vivo.

Acknowledgements:

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