

Original Research Article

PREPARATION OF SILYMARIN–QUERCETIN LOADED NANOPARTICLES BY SPONTANEOUS EMULSIFICATION SOLVENT DIFFUSION METHOD USING D-ALPHA-TOCOPHERYL POLY (ETHYLENE GLYCOL) 1000 SUCCINATE

ABSTRACT

Silymarin, a flavonolignan, derived from *Silybum marianum*, family *Asteraceae* has long been used as a hepatoprotective remedy. Silymarin has cytoprotective activities due to its antioxidant property and free radical scavenging activity. It inhibits the binding of hepatotoxins to receptor sites, protects hepatocyte membranes, enhances liver parenchyma regeneration, and increases glutathione levels. The pharmacokinetic studies of past three decades revealed that Silymarin has poor absorption, rapid metabolism especially by Phase II metabolism and ultimately poor oral bioavailability. Quercetin, a flavonoid present in edible vegetables and fruits, It is a potent antioxidant and shows a wide range of biological functions. Quercetin improves blood levels and efficacy of number of drugs since it is P-Glycoprotein inhibitor and also inhibits drug metabolising enzymes. Both Silymarin and Quercetin were, poorly soluble in the water shows low bioavailability. D-alpha-tocopheryl poly (ethylene glycol) 1000 succinate (TPGS) is a widely used form of vitamin E that has been used as a solubilizer, an emulsifier and as a vehicle for drug delivery formulations. In this study, poly lactide-co-glycolide (PLGA) nanoparticles of Silymarin and Quercetin were prepared by spontaneous emulsification solvent diffusion (SESD) method. TPGS as an emulsifier and further as a matrix material blended with PLGA was used to enhance the encapsulation efficiency and improve the drug release profile of nanoparticles. The effect of formulation parameters such as drug/polymer ratio, volume and surfactant content was evaluated. The surface morphology and size of the nanoparticles were studied by scanning electron microscopy (SEM) and laser light scattering. Drug encapsulation efficiency and in vitro drug release profiles of nanoparticles were determined using High Performance Liquid Chromatography (HPLC). The nanoparticles prepared in this study were spherical with size range of 150–250 nm. It was shown that TPGS was a good emulsifier for producing nanoparticles of hydrophobic drugs and improving the encapsulation efficiency and drug loading and drug release profile of nanoparticles.

KEYWORDS: Silymarin; Quercetin; D-alpha-tocopheryl poly (ethylene glycol) 1000 succinate; poly lactide-co-glycolide ; spontaneous emulsification solvent diffusion

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1. INTRODUCTION

Silymarin is a mixture of flavonolignans isolated from *Silybum marianum*. Even if, it is having a potent antioxidant and safest hepatoprotective drug, the problem associated with its use is poor oral bioavailability. Hence dose of silymarin given needs to be large in order to achieve therapeutic plasma levels [1]. At recent time, Silymarin have other valuable activities such as antidiabetic [2], hypolipidemic [3], anti-inflammatory, neuroprotective, cardioprotective [4] and nephroprotective effects [5]. In addition, silymarin has been shown to be safe in animal models and no significant adverse reactions are reported in human studies[6]. Quercetin is a flavonoid present in various edible vegetables and fruits. It is a potent antioxidant and exhibits a wide range of biological functions[7].

Quercetin improves blood levels and efficacy of number of drugs since it is P-Glycoprotein inhibitor and also inhibits drug metabolising enzymes[8] .

Several formulations of Silymarin are currently available in the markets for oral administration and their pharmacodynamic and pharmacokinetic properties are well characterized[9]. Both Silymarin and Quercetin were, poorly soluble in the water and only 20–50% of Silymarin extract is absorbed in the intestine after administration[10] .

The spontaneous emulsification solvent diffusion is used for the formulation of biodegradable nanoparticles[11]. In this method nano-sized particles of PLGA can be produced by pouring the polymeric organic solution into an aqueous phase with continuous mechanical stirring. Here a binary mixture of a water-miscible organic solvent such as acetone and a water immiscible solvent such as dichloromethane as the solvent of the polymer is used for the preparation of nanoparticles. The nanoparticles are formulated by an emulsification process, then subsequently by solvent evaporation process. Fabrication parameters such as type and quantity of the emulsifier can affect the nature of the nanoparticles obtained. PLGA nanoparticles are usually prepared by using chemical emulsifiers such as poly vinyl alcohol (PVA) [12]. PVA have some disadvantages including low emulsification efficiency and difficulties to wash away in the formulation process. TPGS have reported high emulsification efficiency several times higher than PVA. TPGS is a succinyl derivative form of vitamin E that differs from other vitamin derivatives in that TPGS itself does not act as an antioxidant [13]. The present work investigated the use of TPGS as emulsifier with PLGA for the preparation of nanoparticles containing Silymarin and Quercetin. Therefore, this study was conducted to formulate and optimize a stable formulation of Silymarin-Quercetin by incorporating in PLGA nanoparticles (SL-QR NPs) using TPGS for passive targeted delivery, thereby prolonging its retention time.

2. MATERIALS AND METHODS

MATERIAL

The Silymarin and TPGS were purchased from Sigma Aldrich, India. Poly Lactic-co-Glycolic Acid (PLGA) (75:25) was obtained as gift sample from Hindustan latex limited, Akkulam, Trivandrum. Quercetin was purchased from Sisco research laboratories, Mumbai. DCM and Ethanol (Analytical grade) were purchased from SD Fine, Nashik, Poly Vinyl Alcohol, Akshar Enterprises, Mumbai. All chemicals used in the study were of analytical grade and used without further purification. Deionized water was used throughout the experiment.

METHODS

PREPARATION OF SILYMARIN- QUERCETIN LOADED PLGA-TPGS NPS (SL- QR PLGA-TPGS NPS)

Nanoparticles were prepared using spontaneous emulsification solvent diffusion method[14]. Briefly, known amounts of polymer and drug were added into the mixture of DCM/ Ethanol (1:1) and stirred for 15 minutes to ensure that all material was dissolved. This solution of organic phase was slowly poured into an aqueous solution containing emulsifier using a high speed homogenizer at 14000 rpm for 5 min. stirring continued for the evaporation of the internal phase. The polymer was then precipitated, and the nanoparticles were isolated by using a centrifuge at 10000 RPM for 15 min and washed thrice with deionized water. The suspension was then freeze-dried for 48 hrs to obtain a fine powder of nanoparticles, which was then kept in a desiccator[15]

SATURATION SOLUBILITY STUDIES

The saturation solubility studies were carried out for both the unprocessed pure drug and different batches of lyophilized nanosuspension. 10mg of unprocessed pure drug and nanosuspension equivalent to 10 mg of Silymarin were weighed and separately introduced into 25 ml stoppered conical flask containing 10 ml distilled water. The flasks were sealed and placed in rotary shaker for 24 hours at 37°C and equivalent for 2 days. The samples were collected after the specified time interval and were filtered and analyzed. The samples were analyzed using UV spectrophotometer at 287nm[16]

CHARACTERIZATION OF NANOPARTICLES

SIZE AND SIZE DISTRIBUTION

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The particle size and size distribution of the nanoparticles were measured by laser light scattering (Zetasizer ZS, Malvern, UK). The samples were prepared by suspending the freeze-dried nanoparticles in 10 ml deionized water (10 mg/ml).

MORPHOLOGY.

Scanning electron microscopy was employed to determine the shape and surface morphology of the produced nanoparticles. To examine the morphology of nanoparticles, the formulations were gently sprinkled on a double adhesive tape stuck on an aluminium stub. Further, the stubs were coated with gold using a polaron sputter coater and the samples were examined at an acceleration voltage of 30 kV. The photomicrographs were taken at suitable magnification.

IN-VITRO DRUG RELEASE

The release of Silymarin and Quercetin entrapped in NPs was determined by dialysis tube diffusion technique. The prepared formulations (5 mL) were separately filled into the dialysis tube (MWCO 10 KDa; Hi Media, India), hermetically tied at both the ends and suspended in recipient media of 40 mL of Phosphate buffer solution (PBS) (pH 7.4) in different beakers under sink conditions, while maintaining study temperature at $37 \pm 1^\circ\text{C}$ throughout. At definite time intervals, samples were withdrawn and replaced with same volume of PBS. The samples were then analyzed by HPLC for drug content. Drug release data was normalized by converting drug concentration in solution to a percentage of the cumulative drug release.

3. RESULT AND DISCUSSION

PREPARATION OF NANOPARTICLES

COMPOSITION OF NANOPARTICLES:

Different formulations used for the preparation of Silymarin and Quercetin nanoparticles are presented in tables 1, 2 and 3 respectively.

Table 1: Formulation trails of Sil-Que PLGA-TPGS Nanoparticles:

Ingredients	F1	F2	F3	F4	F5	F6	F7	F8	F9
Drug:PLGA:TPGS	1:1:1	1:1.5:1	1:2:1	1:2.5:1	1:3:1	1:1:1.5	1:1:2	1:1:2.5	1:1:3
PVA (%w/v)	1	1	1	1	1	1	1	1	1
DCM:Ethanol	20	20	20	20	20	20	20	20	20
Centrifugation (10000rpm)	15	15	15	15	15	15	15	15	15

Table 2: Formulation of Sil-Que PLGA-TPGS Nanoparticles based on concentration of PVA:

Ingredients	F10	F11
Drug:PLGA:TPGS	1:1:3	1:1:3
PVA (%w/v)	0.5	1.5
DCM:Ethanol (1:1)	20	20
Centrifugation (10000rpm)	15	15

Table 3: Formulation of Sil-Que PLGA-TPGS Nanoparticles based on centrifugation:

Ingredients	F12	F13	F14	F15
Drug:PLGA:TPGS	1:1:3	1:1:3	1:1:3	1:1:3
PVA (%w/v)	1	1	1	1
DCM:Ethanol (1:1)	20	20	20	20

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Centrifugation(10000rpm) / Time (min)	5	25	35	45
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CHARACTERISATION OF SILYMARIN-QUERCETIN LOADED PLGA-TPGS NPS

Silymarin-Quercetin loaded PLGA –TPGS nanoparticles (Sil-Que PLGA-TPGS NPs) were characterized by particle size analysis UV–Vis spectrophotometer analysis, XRD technique, DSC and FTIR spectrophotometer analysis was performed.

UV-VISIBLE SPECTRUM ANALYSIS

UV-Vis spectra recorded during analysis of Pure drug-Silymarin and Quercetin and nanoparticles were shown given in Fig.1&Fig 2.The calibration curve of both the drugs in medium such as 0.1N HCl, 6.8 Phosphate Buffer,7.4 buffer are shown in Fig.3 to Fig.8.

Fig.1: UV of Quercetin at 257nm (10µg/mL) Fig.2: UV of Silymarin at 288nm (10µg/mL)

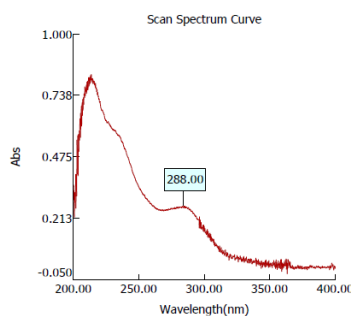
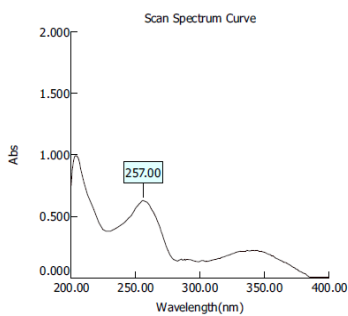


Fig 3 : Calibration curve of Quercetin in 0.1N HCl

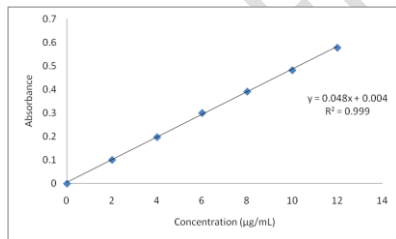


Fig .4: Calibration curve of silymarin in 0.1N HCl

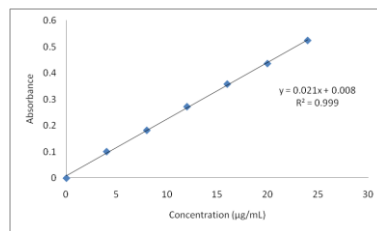


Fig 5: Calibration curve of Quercetin in 6.8 pH buffer

Fig.6: Calibration curve of silymarin in 6.8pH buffer

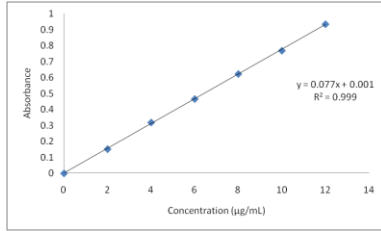


Fig 7: Calibration curve of Quercetin in 7.4 pH buffer

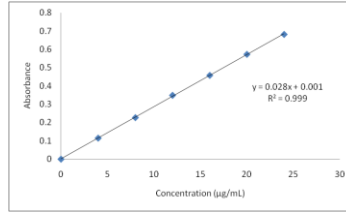
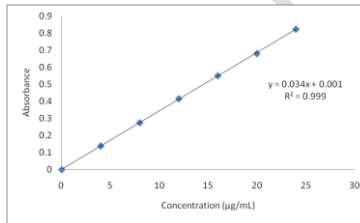
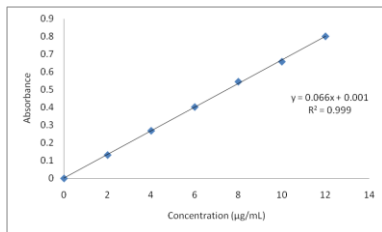


Fig 8: Calibration curve of silymarin in 7.4 pH buffer



SATURATION SOLUBILITY STUDIES

The saturation solubility studies were carried out for both the unprocessed pure drug and different batches of lyophilized nanosuspension. Quercetin shows maximum solubility 1.025mg/ mL in ethanol and Silymarin shows maximum solubility 2.024mg/mL in methanol.

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Table 5: Solubility studies of Quercetin

Table 6: Solubility studies of Silymarin

Solvents	Solubility (mg/mL)
0.1N HCl	0.098
6.8pH buffer	0.312
7.4pH buffer	0.276
Methanol	0.976
Ethanol	1.025

Solvents	Solubility (mg/mL)
0.1N HCl	0.124
6.8pH buffer	0.569
7.4pH buffer	0.798
Methanol	2.024
Ethanol	1.916

Fig.9: Solubility profile –Quercetin

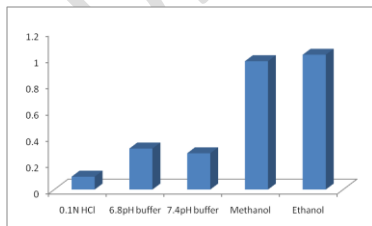
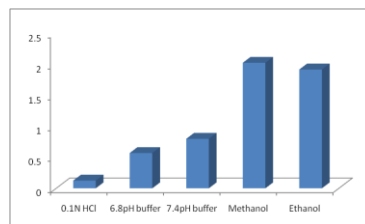


Fig.10: Solubility profile –Silymarin



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Fig.11: FTIR of Quercetin

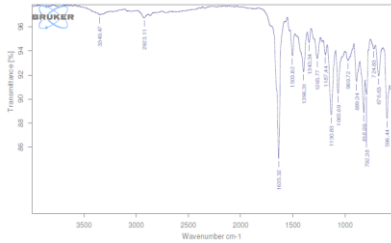


Fig.12 : FTIR of Silymarin

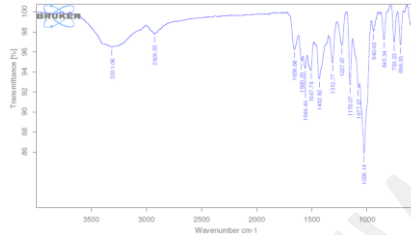
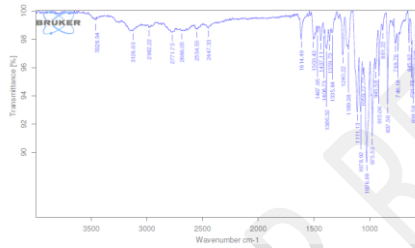


Fig.11: FTIR of Optimized formulation



XRD TECHNIQUE

X-ray diffraction (XRD) is one of the most extensively used techniques for the characterization of NPs. Here the XRD data provides information regarding the crystalline structure, nature of the phase, lattice parameters and crystalline grain size. It is shown in Fig.13, Fig.14 and Fig.15.

Fig.13: Silymarin XRD

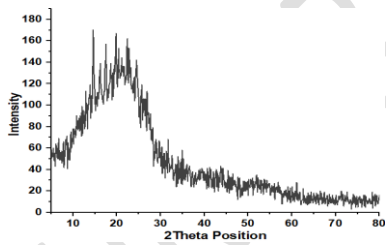


Fig.14: Quercetin XRD

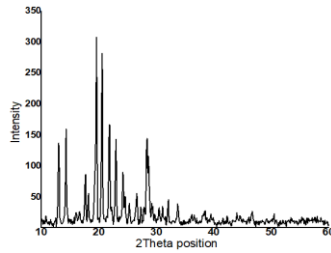
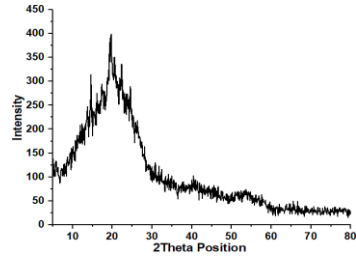


Fig.15: Optimized formulation XRD



DIFFERENTIAL SCANNING CALORIMETRY

DSC studies confirm the chemical inertness of the PLGA and TPGS Concentration with the drug Silymarin and Quercetin. From the results obtained we can conclude that PLGA polymer and TPGS emulsifier are compatible with the drug Silymarin and Quercetin to formulate Sil-Que PLGA-TPGS NPs. Its shown in Fig 16, Fig.17 &Fig 18.

Fig.16: DSC of Silymarin

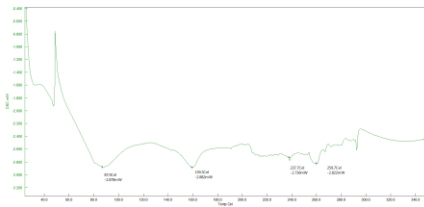


Fig.17: DSC of Silymarin

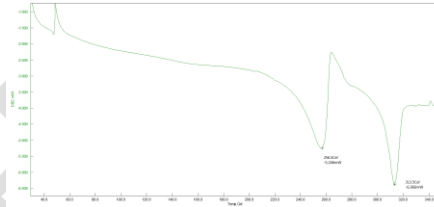
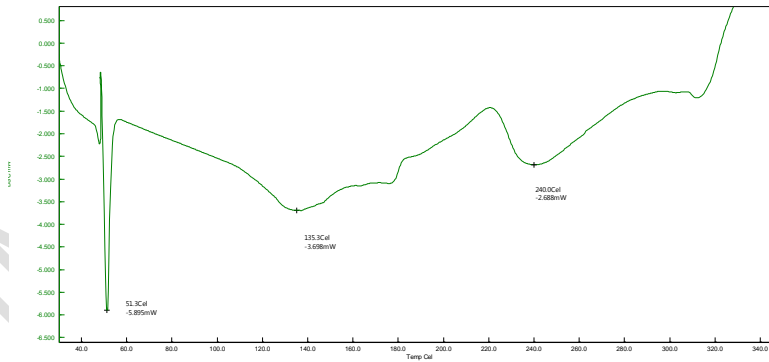


Fig.18: DSC of Optimized formulation



INVITRO DRUG RELEASE DATA OF NANOPARTICLES

In *in vitro* studies, Sil-Que PLGA-TPGS NPs exhibited an initial 50% release at 5th hr. This may be due to burst release of drug absorbed at the surface or present in the outermost layer just beneath the surface of nanoparticles. The drug release followed a characteristic sustained pattern until the end of 12hr. The cumulative drug released over 12 hr from optimised formulation, Sil-Que PLGA-TPGS NPs was 93.16 % (Silymarin) and 92.01% (Quercetin) (table.7).

Table 7: Invitro drug release data of Nanoparticles (F1-F9)

Time(hrs)	%CDR of Quercetin								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
0	0	0	0	0	0	0	0	0	0
1	8.21	10.25	8.06	7.41	5.02	13.02	24.61	22.01	26.32
2	12.34	15.65	11.65	10.25	9.25	19.68	31.85	28.63	33.94
3	19.35	23.45	15.36	14.96	12.32	26.35	35.67	35.46	40.18
4	25.76	29.35	20.24	18.24	15.63	30.42	38.95	40.12	46.32
5	28.63	33.72	25.32	23.64	19.85	35.12	43.16	45.36	50.25
6	33.41	39.04	30.14	30.52	22.36	41.36	46.02	49.78	59.08
7	36.12	43.26	35.97	33.65	25.32	44.78	48.36	53.61	66.73
8	43.02	46.21	39.52	35.01	27.63	46.21	52.36	58.76	70.24
9	47.12	50.24	42.15	37.45	30.24	50.36	56.74	61.36	76.09
10	50.63	55.02	44.12	39.02	33.15	52.95	60.51	65.96	80.42
11	54.96	59.24	46.25	42.16	37.65	56.32	66.35	69.35	85.35
12	57.95	63.24	49.02	46.28	40.28	60.79	69.74	77.96	89.24

Table 8: Invitro drug release data of Nanoparticles (F10-F15)

Time (hrs)	%CDR of Quercetin					
	F10	F11	F12	F13	F14	F15
0	0	0	0	0	0	0
1	29.35	22.02	25.02	23.02	20.96	16.34
2	36.12	29.36	36.58	29.78	26.37	20.97
3	42.86	35.46	47.35	36.41	29.04	26.41
4	50.25	40.25	53.46	40.25	36.12	32.65
5	56.92	50.25	60.35	46.31	40.75	39.42
6	63.21	56.34	70.21	52.79	49.02	46.18
7	70.24	60.12	79.36	59.35	56.75	49.37
8	76.95	65.42	86.02	63.24	62.31	56.32
9	82.34	69.58	90.78	70.25	69.05	60.25
10	90.04	73.95	95.27	82.04	76.34	65.13
11	93.27	77.25	---	87.35	80.15	70.96
12	---	82.46	---	92.04	86.24	78.24

Table 9: Invitro drug release data of Nanoparticles (F1-F9)

Time (hrs)	%CDR of silymarin								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
0	0	0	0	0	0	0	0	0	0
1	9.52	11.02	8.32	6.96	6.02	14.02	23.61	25.61	25.12
2	18.24	16.35	13.62	11.02	8.96	23.62	29.35	30.36	32.63
3	23.65	23.96	15.35	15.42	11.25	29.65	34.72	33.45	42.15
4	25.41	30.12	18.52	16.32	16.35	33.45	38.96	38.67	47.36
5	31.02	34.52	24.63	22.96	20.25	38.12	43.16	44.25	52.09
6	33.65	42.96	29.12	29.34	23.61	41.06	46.35	50.87	56.32

7	37.45	46.32	31.02	32.63	27.96	44.78	49.52	54.91	62.46
8	40.35	49.25	33.64	35.12	29.32	47.69	52.36	57.04	69.25
9	45.63	52.16	35.86	38.46	32.64	50.36	56.72	61.35	76.12
10	49.35	56.32	38.95	40.25	35.12	54.09	60.06	65.82	82.36
11	54.12	60.24	46.32	43.61	36.02	59.62	63.49	72.36	86.94
12	57.36	65.96	50.14	45.98	39.68	62.41	68.07	77.61	89.42

Table 10: **Invitro** drug release data of Nanoparticles (F10-F15)

Time (hrs)	%CDR of silymarin					
	F10	F11	F12	F13	F14	F15
0	0	0	0	0	0	0
1	28.41	23.21	23.46	22.34	20.36	15.32
2	35.12	29.25	30.25	28.96	25.01	19.78
3	40.29	36.12	42.11	35.01	30.12	23.85
4	48.97	42.08	50.32	43.26	36.45	30.63
5	53.62	52.96	59.68	49.52	42.96	37.52
6	60.02	58.98	66.98	56.31	48.75	42.16
7	69.85	62.47	76.12	60.15	56.85	49.25
8	75.12	69.02	83.26	69.52	63.12	56.95
9	80.24	72.96	89.63	76.35	70.06	59.35
10	86.91	78.06	92.04	80.23	76.34	64.15
11	92.34	80.74	---	87.94	81.36	72.76
12	---	83.65	---	93.16	85.94	79.25

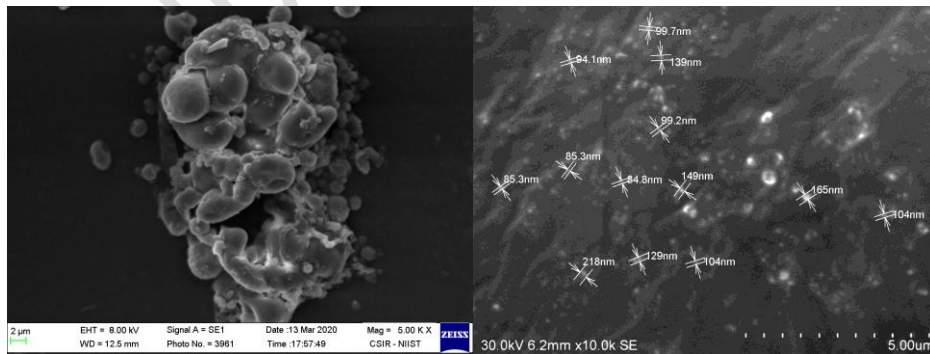
ENTRAPMENT EFFICIENCY & YIELD

Based on the yield and **entrapment** efficiency data, F13 formulation was found to be the better one compared to other formulations. Percentage entrapment efficiency of optimized Sil-Que PLGA-TPGS NPs was found to be 90.24 (Silymarin) and 92.56% (Quercetin).

MORPHOLOGY

SEM images confirmed the spherical nature of Sil-Que PLGA-TPGS NPs. The images also proved that the size of nanoparticles. The scanning electron microphotograph of Sil-Que PLGA-TPGS NPs was shown in Fig.20. It indicated that NPs have a discrete spherical structure without aggregation. No crystals of drug were observed on the surface of nanoparticles.

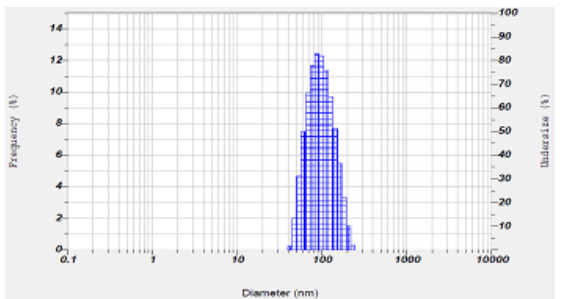
Fig.19: SEM –Silymarin & Quercetin Fig.20: SEM- Sil-Que PLGA-TPGS NPs.



PARTICLE SIZE AND POLYDISPERSITY INDEX

The size of Sil-Que PLGA-TPGS NPs was found to be 88.6 nm with PDI 0.231. The size distribution graph is given in Fig.21.

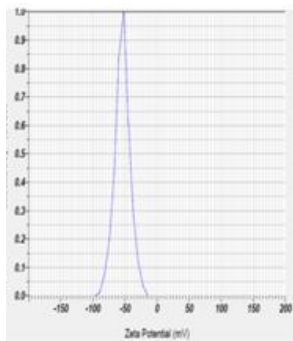
Fig.21 Particle size and polydispersity index



ZETA POTENTIAL

The zeta potential Sil-Que PLGA-TPGS NPs was -53.8 mV. Zeta potential of Sil-Que PLGA-TPGS NPs was negative due to terminal carboxylic functionalities of the PLGA used in the formulation.

Fig.22 Zeta potential



CONCLUSION

The present research proposed a novel nanoformulation of Silymarin and Quercein nanoparticles by applying the SEDS method using TPGS as emulsifier. Different formulations with various drug: polymer ratios and volume and concentration of surfactant, centrifugation time were evaluated. Although the amount of the TPGS used had no significant effect on the nanoparticle size and morphology, the drug loading and release profile of nanoparticles were highly influenced by the use of TPGS. Our results suggest that TPGS is a good emulsifier with PVA for producing nanoparticles of hydrophobic drugs with desired particle size, size distribution and morphological properties.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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