



Preparation of Silymarin–quercetin Loaded Nanoparticles by Spontaneous Emulsification Solvent Diffusion Method Using D-alpha-tocopheryl Poly (Ethylene Glycol) 1000 Succinate

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Authors' contributions

This work was carried out in collaboration among all authors. Authors SS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors PMK and PV managed the analyses of the study. Author PV managed the literature searches. All authors read and approved the final manuscript.

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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. 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 metabolizing enzymes. Both silymarin and quercetin were, poorly soluble in the water shows low bioavailability. The advanced type of formulation like polymeric nanoparticles (PNPs) can be

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successfully utilised for bioavailability enhancement and targeting the Silymarin-quercetin to hepatocytes. A controlled release PNPs of silymarin-quercetin were prepared by spontaneous emulsification solvent diffusion (SESD) method using Poly Lactic-co-Glycolic Acid (PLGA) as biodegradable polymer, D-alpha-tocopheryl poly (ethylene glycol) 1000 succinate (TPGS) used as a solubilizer, as an emulsifier. 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. Different formulations with various drug: polymer ratios and volume and concentration of surfactant, centrifugation time were evaluated. The effect of formulation parameters such as drug/polymer ratio, volume and surfactant content were evaluated. The surface morphology and size of the nanoparticles were studied by scanning electron microscopy (SEM) Transmission electron microscopy (TEM). Drug encapsulation efficiency and in vitro drug release profiles of nanoparticles were determined using UV spectrophotometry. The nanoparticles prepared with combination of both the drugs in this study were spherical with size range of 100–200 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. Although the amount of the TPGS used had a significant effect on the nanoparticle size and morphology, the drug loading and release profile of nanoparticles

Keywords: *Bioavailability enhancement; cytoprotective; polymeric nanoparticles; poly lactide-co-glycolide; scanning electron microscopy; transmission electron microscopy.*

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 beings [6]. Quercetin is a flavonoid present in various edible vegetables and fruits. It is a potent antioxidant and exhibits a wide range of biological activities [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. Both silymarin and quercetin were, poorly soluble in the water and only 20–50% of Silymarin extract is absorbed in the intestine after administration [9].

The spontaneous emulsification solvent diffusion is used for the formulation of biodegradable nanoparticles [10,11]. 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 E 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

2.1 Materials

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.

2.2 Methods

2.2.1 Drug polymer interaction studies by FT-IR

Drug-polymer interaction was studied by FT-IR spectroscopy using the instrument Shimadzu FT-IR-8400S. The spectra were recorded for Silymarin, PLGA, and physical mixture of Silymarin: PLGA (1:1). Samples were prepared in KBr disks (2 mg sample in 200 mg KBr) with a hydrostatic press and pressed for 2 for 3 minutes. The scanning range was 400 - 4000 cm^{-1} and the resolution was 4 cm^{-1} .

2.2.2 Preparation of silymarin-quercetin loaded PLGA-TPGS nanoparticles (SI-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 desiccators [15].

2.3 Saturation Solubility Studies

The saturation solubility studies were carried out for both the unprocessed pure drug and different batches of lyophilized nanosuspension. 10 mg 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 of 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 (UV-160IPC, Shimadzu, Japan) at 287 nm [16].

2.4 Characterization of Nanoparticles

2.4.1 Size and size distribution

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)

2.5 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 aluminum stub. Further, the stubs were coated with gold using a polaron sputter coater and the samples were examined at an acceleration voltage of 30kV. The photomicrographs were taken at suitable magnification. Transmission electron microscopy was used to analyze the surface morphology of the synthesized nanoparticles. To this end, the analysis was performed on a Hitachi, Japan H-7650 120 kV Automatic transmission electron microscope (Hitachi, Tokyo, Japan). The Fourier transform infrared spectrum of the powdered nanoparticles also been recorded.

2.6 In-vitro Drug Release

The release of silymarin and quercetin entrapped in nanoparticles 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 analyzed by UV Spectrophotometry for drug content. Drug release data was normalized by converting drug concentration in solution to a percentage of the cumulative drug release [16].

3. RESULTS

3.1 DRUG-excipient Compatibility Studies By FTIR

The drug polymer compatibility study is done by FTIR. Here IR spectra of physical mixture when compared with individual spectra of drug and polymer showed no significant change in position of IR absorption peaks which conforms that the drug and polymer have no compatibility problems.

3.2 Preparation of Nanoparticles

Number of formulations used for the preparation of Silymarin and Quercetin nanoparticles are presented in Tables 1 and 2 respectively.

3.3 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.

3.4 UV-Visible Spectrum Analysis

UV-Visible spectra recorded during analysis of pure drug-silymarin and quercetin and nanoparticles were shown given in Figs. 2 & 3. The calibration curve of both the drugs in medium such as 0.1N HCl, 6.8 Phosphate Buffer 7.4 buffer were performed.

3.5 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 1.025 mg/ml in ethanol and Silymarin shows maximum solubility 2.024 mg/ml in methanol.

3.6 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. 4.

3.7 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 concluded that PLGA polymer and TPGS emulsifier are compatible with the drug Silymarin and Quercetin to formulate Sil-Que PLGA-TPGS Nanoparticles. It's shown in Fig. 5.

3.8 In Vitro 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 optimized formulation, Sil-Que PLGA-TPGS NPs was 93.16% (Silymarin Fig. 7) and 92.01% (Quercetin) Fig. 6.

3.9 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).

3.10 Morphology-Scanning Electron Microscopy

SEM 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 Figs. 8 & 9. It indicated that NPs have a discrete spherical structure without aggregation. No crystals of drug were observed on the surface of nanoparticles.

3.11 Morphology-Transmission Electron Microscopy

Transmission electron microscopy analysis (TEM) was carried out to investigate the morphology of the synthesized nanoparticles regarding their shape and size. The TEM image (Figs. 10 & 11) shows that the synthesized Nanoparticles have mostly spherical in shape and have a determined size in the range less than 100 nm.

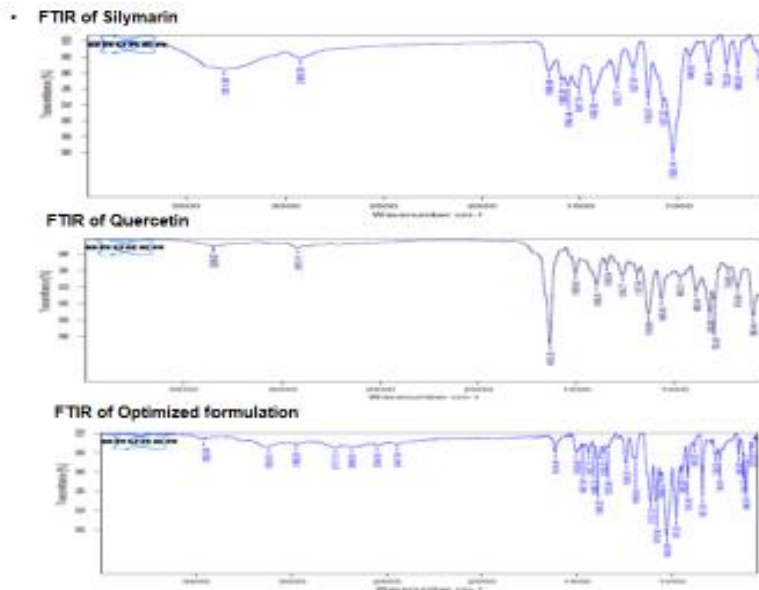


Fig. 1. FTIR analytical study for detecting drug polymer

Table 1. Formulation trails of Sil-Que PLGA-TPGS Nanoparticles centrifugation (10000rpm for 15min)

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
Poly Vinyl Alcohol (%w/v)	1	1	1	1	1	1	1	1	1
DCM:Ethanol	20	20	20	20	20	20	20	20	20

Table 2. Formulation of Sil-Que PLGA-TPGS Nanoparticles based on concentration of PVA& nanoparticles based on centrifugation

Ingredients	Nanoparticles based on concentration of PVA			Nanoparticles based on centrifugation		
	F10	F11	F12	F13	F14	F15
Drug:PLGA:TPGS	1:1:3	1:1:3	1:1:3	1:1:3	1:1:3	1:1:3
Poly Vinyl Alcohol (%w/v)	0.5	1.5	1	1	1	1
DCM:Ethanol (1:1)	20	20	20	20	20	20
Centrifugation (10000rpm) /Time (min)	15	15	5	25	35	45

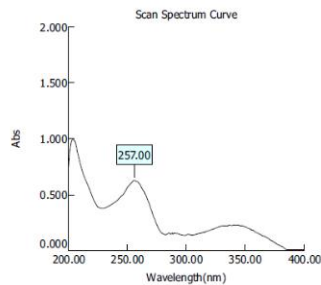


Fig. 2. UV of Quercetin at 257nm (10 µg/mL)

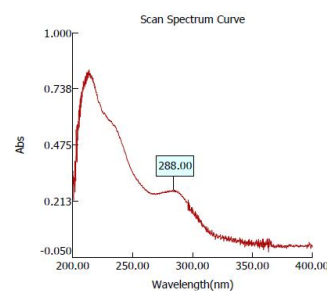


Fig. 3. UV of Silymarin at 288nm (10 µg/mL)

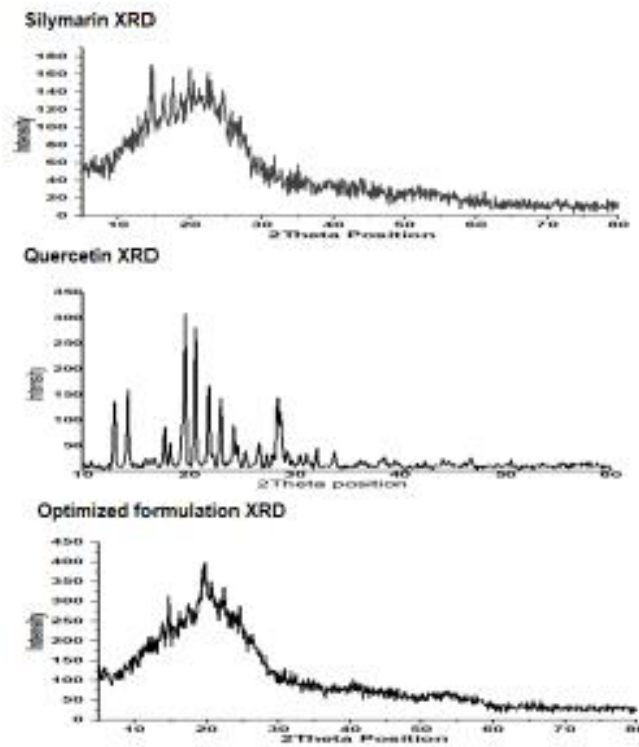


Fig. 4. XRD graph pure drugs and the optimized formulation

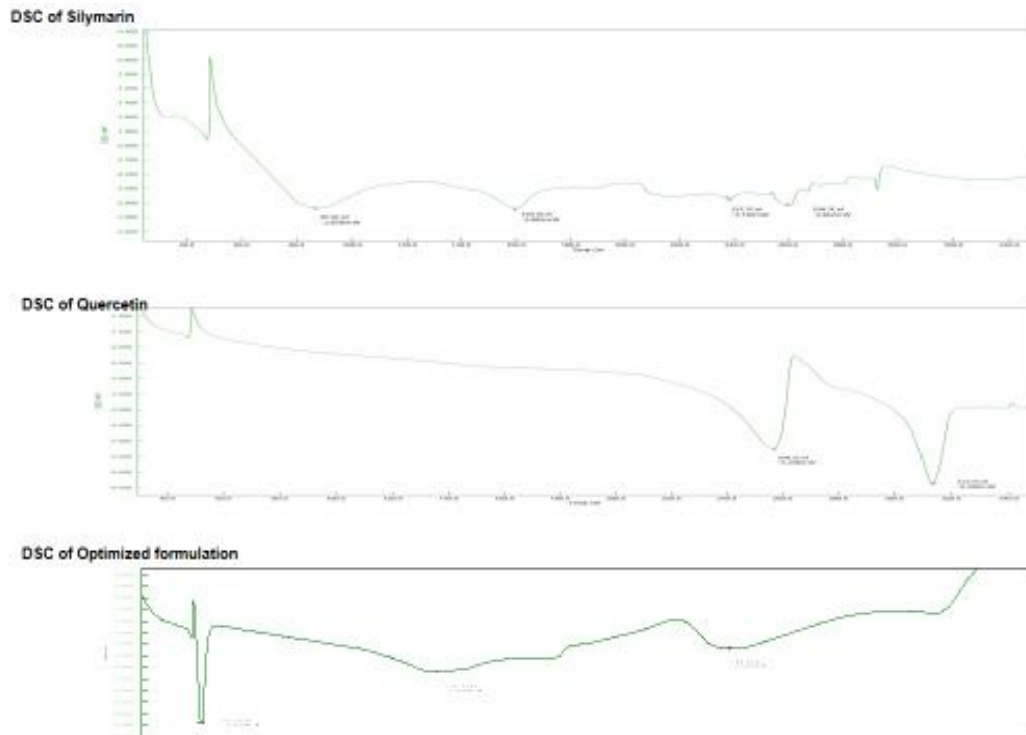


Fig. 5. DSC graph pure drugs and the optimized formulation

Table 3. Solubility studies of quercetin

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

Table 4. Solubility studies of silymarin

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

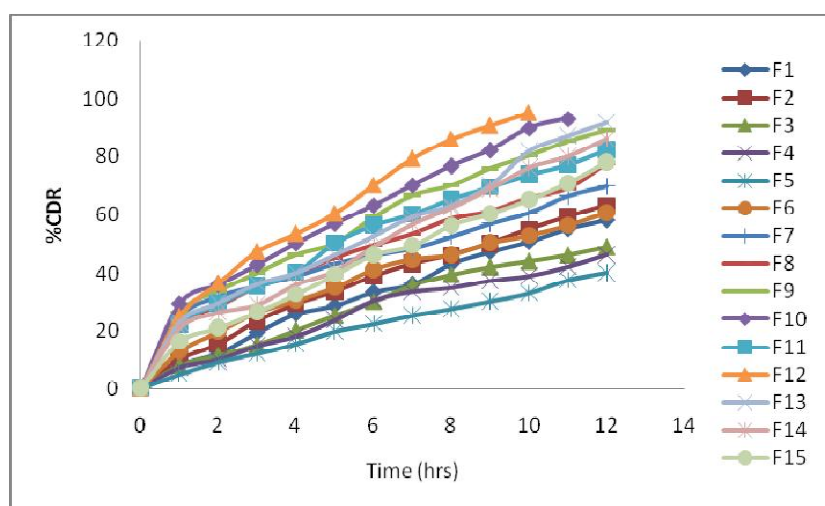


Fig. 6. In-vitro drug release of quercetin (F1-F15)

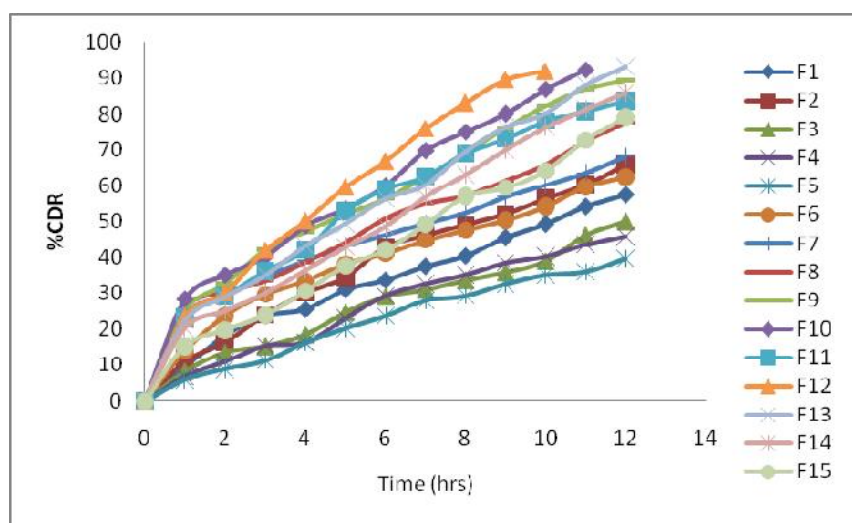


Fig. 7. In-vitro drug release of silymarin (F1-F15)

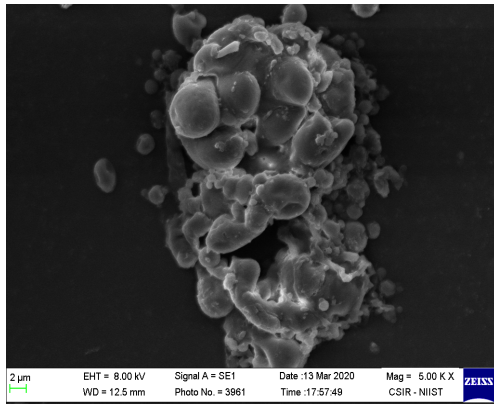


Fig. 8. SEM – Silymarin & Quercetin

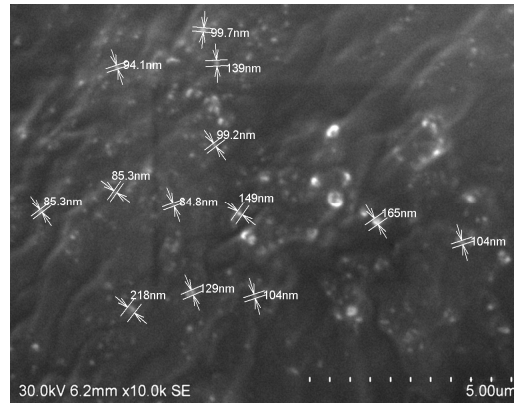


Fig. 9. SEM- Sil-Que PLGA-TPGS NPs

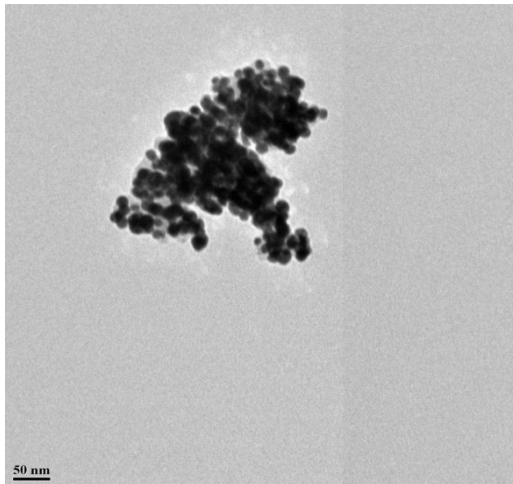


Fig. 10. TEM- Images of Sil-Que PLGA-TPGS NPs

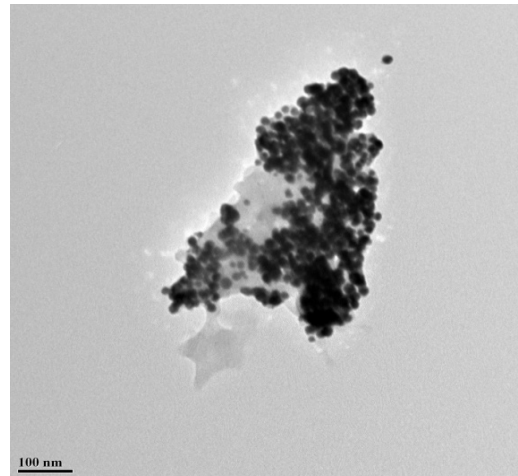


Fig. 11. TEM- Images of Sil-Que PLGA-TPGS NPs

3.12 Particle Size and Polydispersity Index

The optimized 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.12.

3.13 Zeta Potential

The zeta potential of optimized 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.

4. DISCUSSION

Silymarin and quercetin have have several limiting factors like low solubility in water, low bio

availability and poor absorption in GIT. So the present study is an effort for improving its features and characteristics, prolong its release to improve the therapeutic effects. In this present investigation we synthesized and characterized Silymarin-quercetin nanoparticles by incorporating in PLGA nanoparticles using TPGS as emulsifier. The synthesied nanoparticles shows a smoothly round shaped with an average particle size of 100nm.The particle size of the synthesized nanoparticles were similar to the literature collected but shape varies.

FTIR measurement were performed to identify the biomolecules. The result of FTIR spectrum of PLGA, TPGS and drugs revealed that the presence of various functional groups in the optimized formulation. FTIR analysis revealed the successful entrapment of the drugs within the

polymer. The *in vitro* results showed that the release of both the drugs from the nanoparticles were sustained and continued till 12hrs. Slow release of drug from the nanoparticle may result from the interaction of drug and self-assembled micelles. The dissolution study results indicates the drug release from the formulation by diffusion and polymer erosion with dissolution media.

XRD pattern showed number of Bragg Reflections that may be indexed on the basis of the face centered structure of PLGA. Synthesized nanoparticles were morphologically evaluated by SEM and TEM .Results indicates

that nanoparticles have discrete spherical structure without any crystallization or aggregation.

Entrapment efficiency is influenced by characteristic of surfactant. Low entrapment efficiency is due to low affinity of drug and polymer. The result shows that highest entrapment showed by F13 formulation 90.24 (Silymarin) and 92.56% (Quercetin).The particle size distribution, poly dispersibility index and Zeta potential also shows were similar to the literature collected for individual drugs with slight numerical variation.

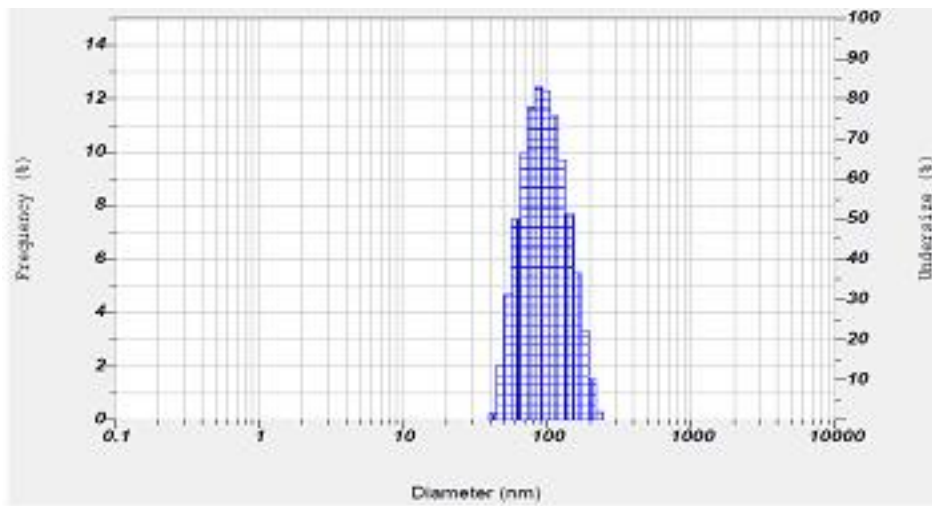


Fig. 12. Particle size and polydispersity index

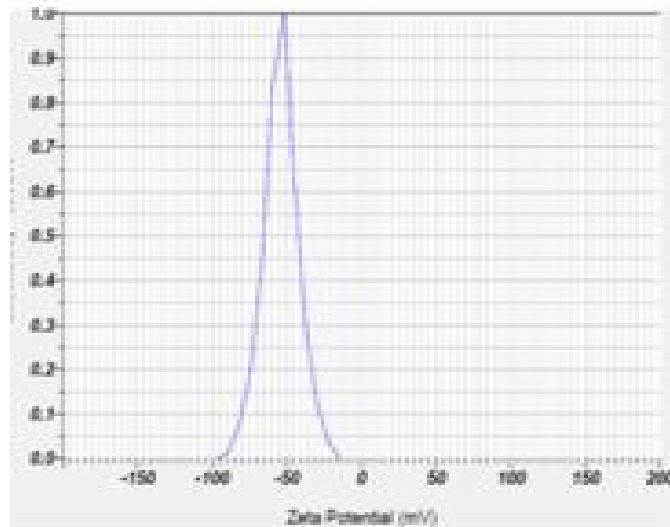


Fig. 13. Zeta potential

5. CONCLUSION

The present research proposed a novel nanoformulation of Silymarin and Quercetin 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 significant effect on the nanoparticle size and morphology, the drug loading and release profile of nanoparticles were highly influenced by the presence 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.

DISCLAIMER

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.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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