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THYMOQUINONE LOADED SOLID LIPID NANOPARTICLE: FORMULATION, CHARACTERIZATION AND IN-VITRO CELL VIABILITY ASSAY.

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ABSTRACT

The aim of the present research is to formulate and characterize solid lipid nanoparticles (SLNs) encapsulated Thymoquinone (TQ) to improve the bioavailability of the hydrophobic drug. Solid lipid nanoparticles loaded Thymoquinone (TQ-SLNs) were prepared using hot homogenization followed by micro emulsion method. TQ-SLNs were characterized for particle size, zeta potential, entrapment efficiency, transmission electron microscopy, X- ray diffraction and in-vitro cytotoxicity. Optimized TQ-SLNs with appropriate characteristics (Particle size = 172.10 ± 7.41 nm; Zeta potential = -45.40 ± 2.68 mV; Entrapment efficiency = 84.49 ± 3.36%) were fabricated. The in-vitro drug release data revealed a maximum of 86.15 ± 2.76% for TQ-SLNs in 72 hrs. TEM images showed a spherical surface of TQ-SLNs and uniform particle size distribution. The state of formulation was characterized by XRD and it exhibited the reduced crystallinity of the entrapped drug. In addition, TQ-SLNs showed a concentration-dependent increase in cytotoxic activity with IC_{50} value of 35.5 ± 10.5 µg/ml. The results of the present study demonstrate that TQ-SLNs are promising nanoformulations with significant characteristic nature and can be a potential delivery system for the treatment of various diseases.

KEYWORDS: Solid lipid nanoparticles, Thymoquinone, Characterization, Cytotoxicity, Release kinetics.

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INTRODUCTION

Several approaches have been investigated to develop nanosized drug delivery systems in recent years. These systems can generally be divided into two groups: polymeric and colloidal systems. The polymeric nanoparticles have many limitations because of its toxic degradable products and in-efficient large-scale production[1]. In order to overcome these problems, a great deal of interest has been focused on lipid-based carriers such as micelles[2], nanoemulsions[3], liposomes[4], solid lipid nanoparticles[5] (SLNs), those are considered as emerging potential drug carriers. Solid lipid nanoparticles (SLNs) have increasing attention as suitable colloidal carriers for delivery of hydrophobic drugs. SLNs are nanospheres made from physiological lipids, fatty acids, phospholipids, mono-, di-, triglycerides and they undergo biodegradation[6, 7]. SLNs are generally constituted by a matrix of lipids that remain solid at room and body temperature[8]. SLNs with a particle size ranging 120–200 nm rarely undergo blood clearance by the reticuloendothelial system[9], thereby improves the retention time of drugs in the blood stream. Consequently, they also provide controlled release of therapeutic agents and have low toxicity[10]. SLNs found to stabilize and promote oral absorption of drugs by improving lymphatic transport and enhancement of gastro intestinal permeability[11]. Furthermore, sufficient drug loading[12], long-term shelf stability[13], and easy large-scale production[14] were its advantageous. Hence, SLNs are very attractive carriers to enhance bioavailability and sustained delivery of drugs. Thymoquinone (TQ) (2-isopropyl-5-methyl-1,2-benzoquinone) is the major active compound of the volatile oil of Nigella sativa seed[15]. TQ has been demonstrated to possess antioxidant and anti-inflammatory effects by ameliorating membrane lipid peroxidation and eicosanoid generation. Several studies have attributed the free radical attenuation efficacy of TQ and provided evidence of increased expression of antioxidant genes[16]. TQ also improved the morphological abnormalities of neurons and prevents its degeneration caused by chronic toluene exposure[17]. It significantly modulates the levels of NO and GABA[18] and suppresses nuclear factor kappa B (NF-KB) activation in brain and spinal cord[19]. Furthermore, TQ has a protective role on cortical neurons[20] and PC12 cells[21, 22] against MPP+ and rotenone neurotoxins. In addition, Thymoquinone has already been reported for its effectiveness in protecting mice against acetaminophen-induced hepatotoxicity[23], gentamicine[24] and cyclosporine[25] induced nephrotoxicity. Moreover, it has been reported that Thymoquinone suppresses inducible NO synthase expression[26] in lipopolysaccharide-stimulated rat peritoneal macrophages. Although, Thymoquinone have been studied for its various therapeutics effects, they have a poor bioavailability due to its lipophilic nature. Thus, the drug was formulated in such a way to improve its absorption rate using a suitable carrier's preferably solid lipid nanoparticle (SLNs). Hence, the objective of this investigation is to formulate the solid lipid nanoparticles encapsulated Thymoquinone (TQ-SLNs) and the characteristic evaluation of its particle size, surface charge, morphology, encapsulation efficiency, in-vitro release potential, cytotoxicity were performed aiming to treat neurodegenerative disorder (Huntington's disease).

MATERIALS AND METHODS

Chemicals and Reagents

Thymoquinone was obtained from Sigma Aldrich. Stearic acid, Lecithin and Sodium taurocholate were purchased from Hi-Media chemicals. All the other chemicals used in the present study were of analytical grade and purchased from Merck (India) and Sisco Research Laboratories Pvt. Ltd.

Vero cell line

The vero cell culture was obtained from National centre for cell sciences Pune (NCCS). The cells were maintained in Minimal Essential Media supplemented with 10% FBS, penicillin

(100 µg/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37°C.

Preparation of TQ-SLNs

The Solid lipid nanoparticleswere prepared as described by Frautschy SA and Cole GM[27], with slight modifications. Briefly, stearic acid mole fraction 0.710, lecithin mole fraction 0.210, taurocholate mole fraction 0.069 and Thymoquinone mole fraction 0.011 were used to produce the Solid lipid nanoparticles encapsulated Thymoquinone. Stearic acid was maintained at ~75 °C to melt completely, simultaneously distilled water was heated up to ~75 °C in a separate beaker. Typically, surfactants were added to distilled water on a magnetic stirrer and allowed to equilibrate at ~75 °C. The water–surfactant solution containing Thymoquinone was then added to the melted lipid and again allowed to equilibrate at ~75 °C. The mixture was then homogenized at 24,000 rpm for 150 s to form the emulsion. Then the aliquot was continuously stirred near ice cold water (~2 °C), at a ratio of 1:20 (warm microemulsion/cold water) resulting in the formation of solidified solid lipid nanoparticles. The final product was centrifuged at 20,000×g for 15 min, and nanoparticle pellet was resuspended in distilled water. The preparation was stored sterile at 4 °C, until delivery by gavage.

Formulation characterization

The nanoparticles obtained were characterized in various parameters described below.

Particle size analysis, Zeta potential and Transmission electron microscopy (TEM)

The mean diameter of TQ-SLNs was determined using the differential light scattering (DLS) technique. In this technique, fluctuation in light scattering due to Brownian motion of the particles was analyzed. Zeta potential of formulated TQ-SLNs was also determined using the same instrument. TQ-SLNs were observed microscopically using TEM for uniformity of size, shape and physical stability characteristics, i.e., aggregation or irregularity.

Total drug content and Entrapment efficiency

Total amount of drug per unit volume present in TQ-SLNs formulation was determined by suitably disrupting SLN dispersion using a mixture of chloroform: methanol (1:1) volumetrically and centrifuged. The pellet containing drug was analyzed spectrophotometrically at 254 nm, using a respective blank described by Kakkar[28]. Each experiment was performed in triplicate. TDC was determined by a following equation:

$$TDC = \frac{\text{Amount of drug/ml of SLN dispersion}}{\text{Total amount of drug/ml of SLN dispersion}} \times 100$$

Entrapment efficiency was calculated by the formula mentioned by Rohit B and Pal KI[29]:

$$EE = \frac{TDC - D_f}{TDC} \times 100$$

Where, D_f = amount of drug in clear supernatant fluid

In-vitro drug release

The drug release from TQ loaded SLNs and TQ suspension (TQ-S) were carried out using the dialysis bag method[30]. The dialysis bags (MWCO 12KD, Hi-Media) were soaked in deionized water for 12 hrs before use. 1 ml of each samples were added into the dialysis bags individually and the bags were placed in a beaker containing a mixture of distilled water and ethanol (50:50, v/v) as the dissolution medium and placed at 37 °C at a stirring rate of 100 rpm for 72 hrs. Aliquots of the dissolution medium were withdrawn at different time intervals and were replaced with same volume of fresh medium to maintain the sink conditions. TQ in the sample solution were analyzed spectrophotometrically at 254 nm. All the operations were carried out in triplicate and precautions were taken to protect the drug from heat and light.

X-ray diffraction (XRD)

Powder X-ray diffraction patterns were traced for drug, SLNs and TQ-SLNs employed by RIGAKU, RV200 using monochromatic Ni-filtered Cu–K radiation, a voltage of 40 kV, a current of 30 mA radiation scattered in the crystalline regions of the sample, which was measured with a vertical goniometer. Patterns were obtained by using a step width of 0.04° with a detector resolution in 2θ (diffraction angle) between 10° and 70° at ambient temperature.

In vitro assay for Cytotoxicity activity

The cytotoxicity activity of sample on VERO cells was determined by the MTT assay[31]. Cells (1×10^5/well) were plated in 0.2 ml of medium/well in 96-well plates. Incubate at 5% CO_2 incubator for 72 hours. Then, added various concentrations of TQ-SLNs in 0.1% DMSO for 24 hrs at 5% CO_2 incubator. After removal of the sample solution and washed with phosphate-buffered saline (pH 7.4), 20 µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) in phosphate- buffered saline solution was added. After 4 hrs incubation, 1 ml of DMSO was added. Viable cells were determined by the absorbance at 540 nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC_{50}) was determined graphically. The effect of the samples on the proliferation of VERO cells was expressed as the % cell viability, using the following formula:

$$\% \text{ cell viability} = A540 \text{ of treated cells} / A540 \text{ of control cells} \times 100\%$$

Statistical analysis

Data from the individual experiments are expressed as the mean ± SD. All statistical analysis were performed using SPSS 20.0. One-way analysis of variance (ANOVA) was used, followed by the Student's t-test. Differences were considered to be statistically significant at $P<0.05$.

Fig 1A

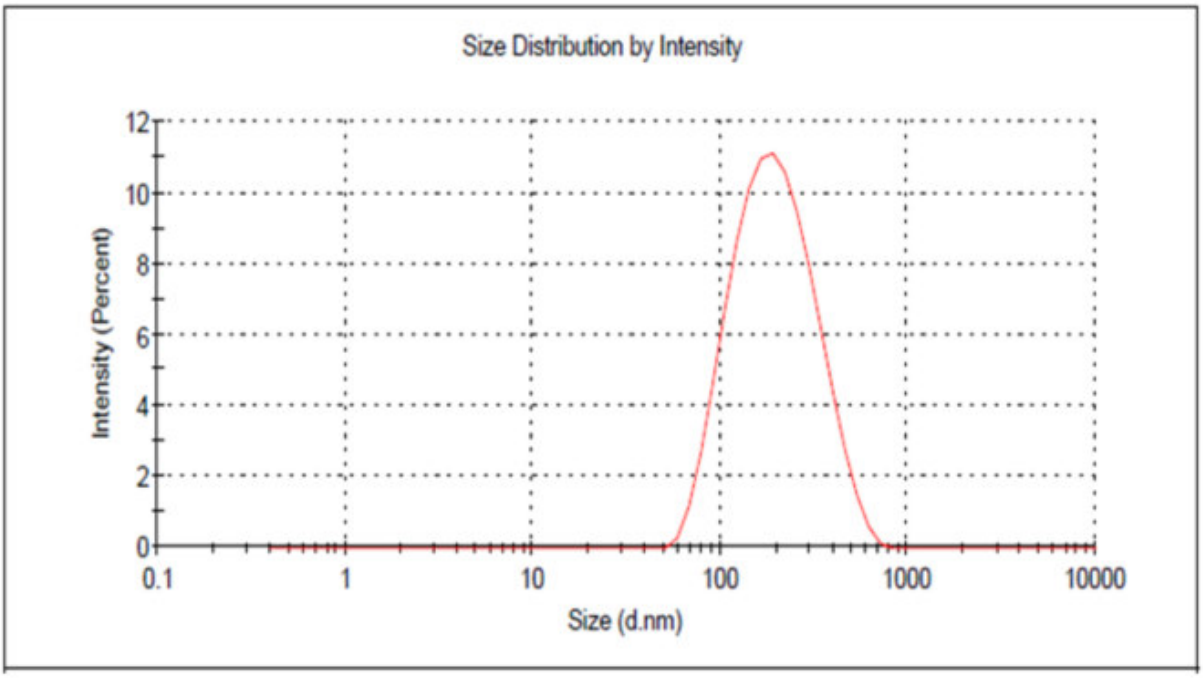
Size Distribution by Intensity
Intensity (Percent)
12
10
8
6
4
2
0
0.1
1
10
100
1000
10000
Size (d.nm)

Fig 1B

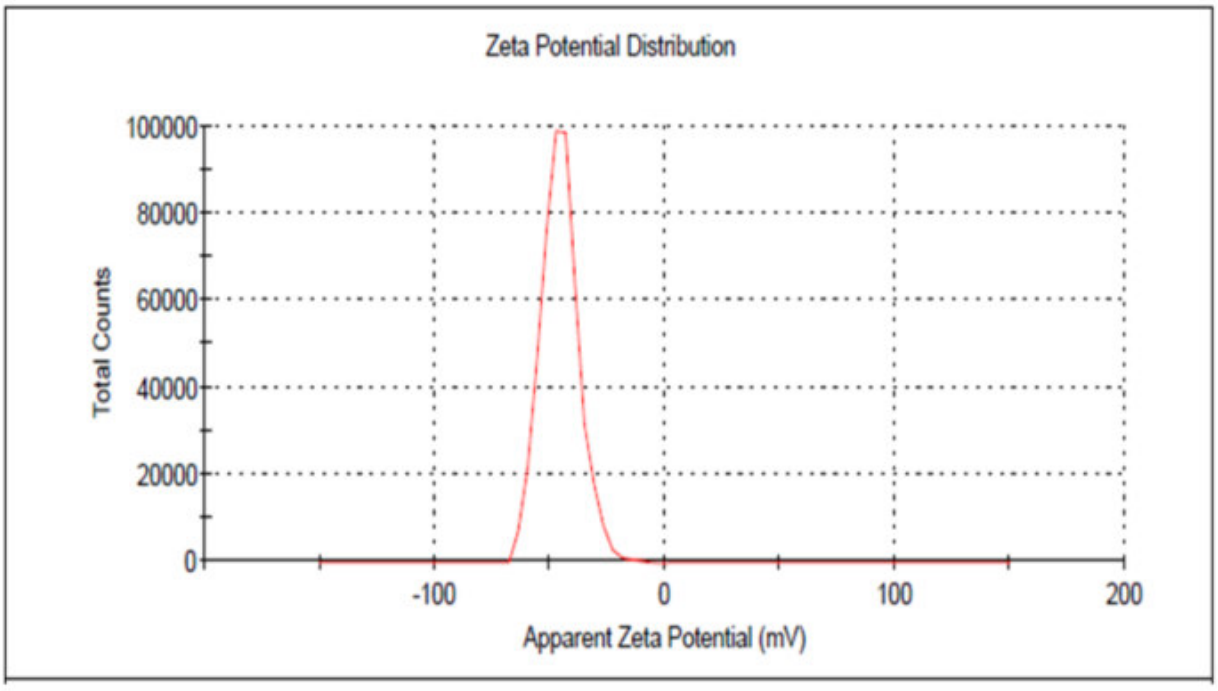
Zeta Potential Distribution
Total Counts
100000
80000
60000
40000
20000
0
-100
0
100
200
Apparent Zeta Potential (mV)

Fig 1C

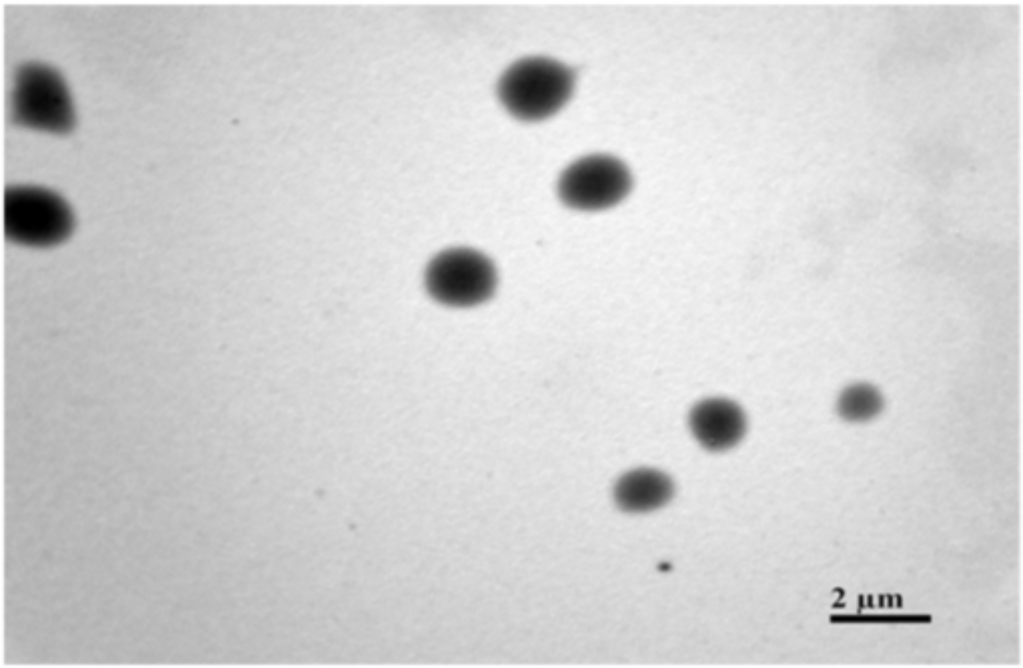
2 µm

Fig 2

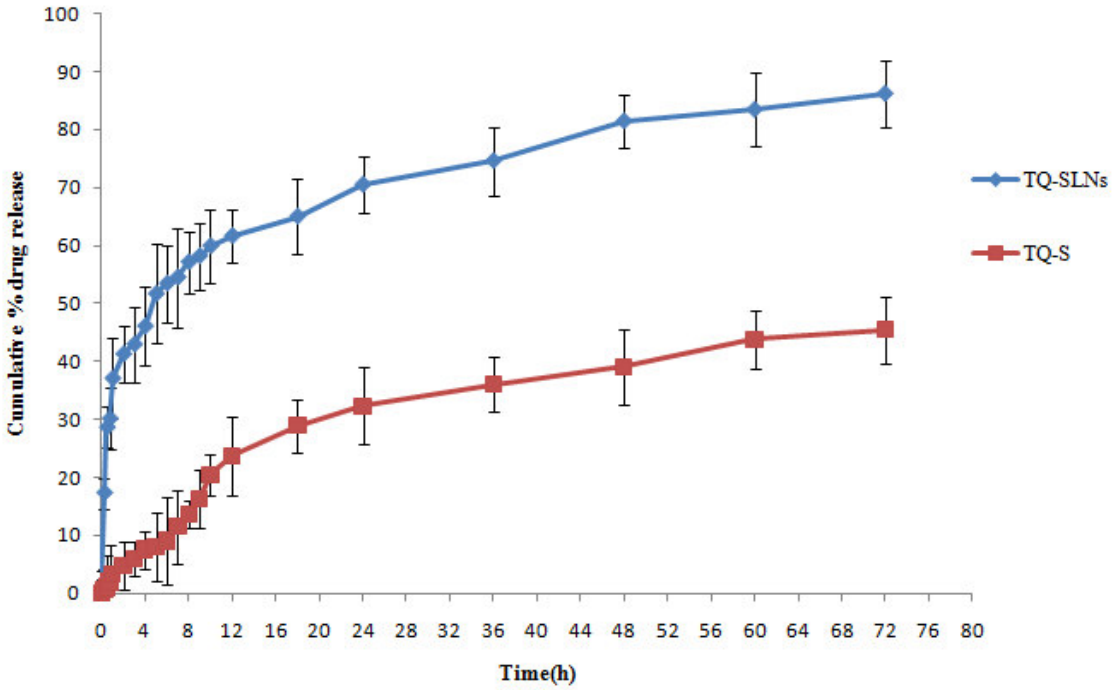
Cumulative % drug release
Time(h)
TQ-SLNs
TQ-S
0
10
20
30
40
50
60
70
80
90
100
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72 76 80

Fig 2A

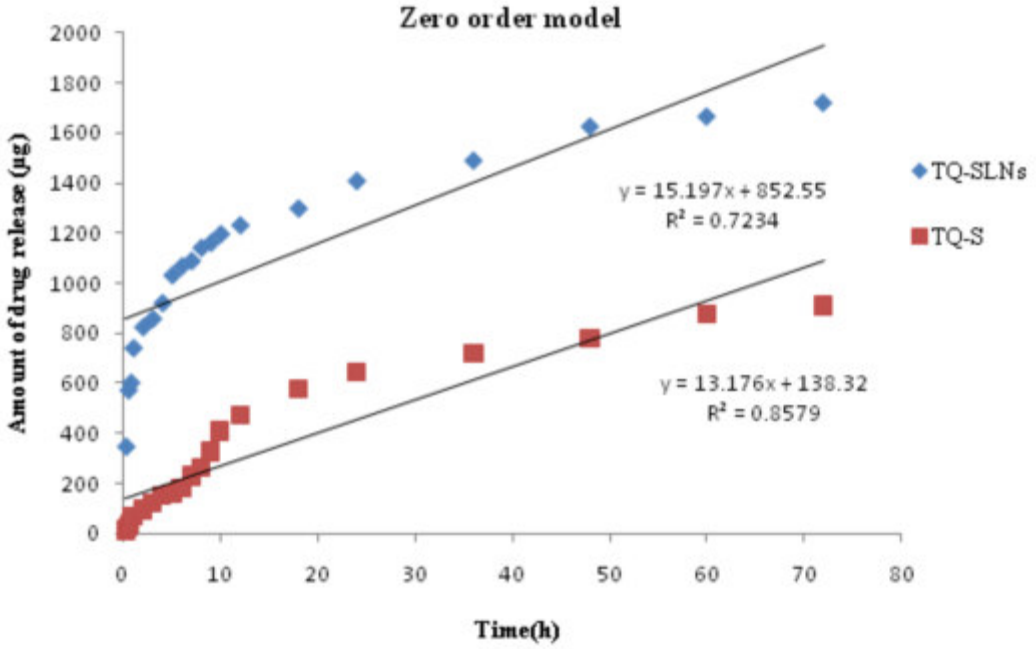
Zero order model
Amount of drug release (µg)
Time(h)
y = 15.197x + 852.55
R² = 0.7234
y = 13.176x + 138.32
R² = 0.8579
TQ-SLNs
TQ-S

Fig 2B

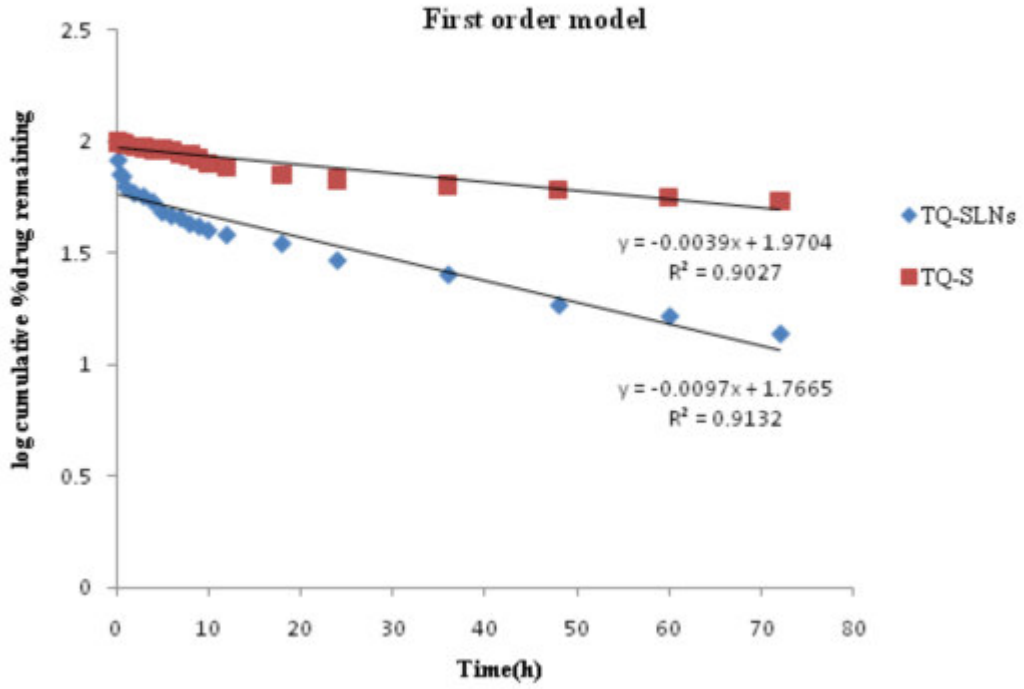
First order model
log cumulative %drug remaining
Time(h)
y = -0.0039x + 1.9704
R² = 0.9027
y = -0.0097x + 1.7665
R² = 0.9132
TQ-SLNs
TQ-S

Fig 2C

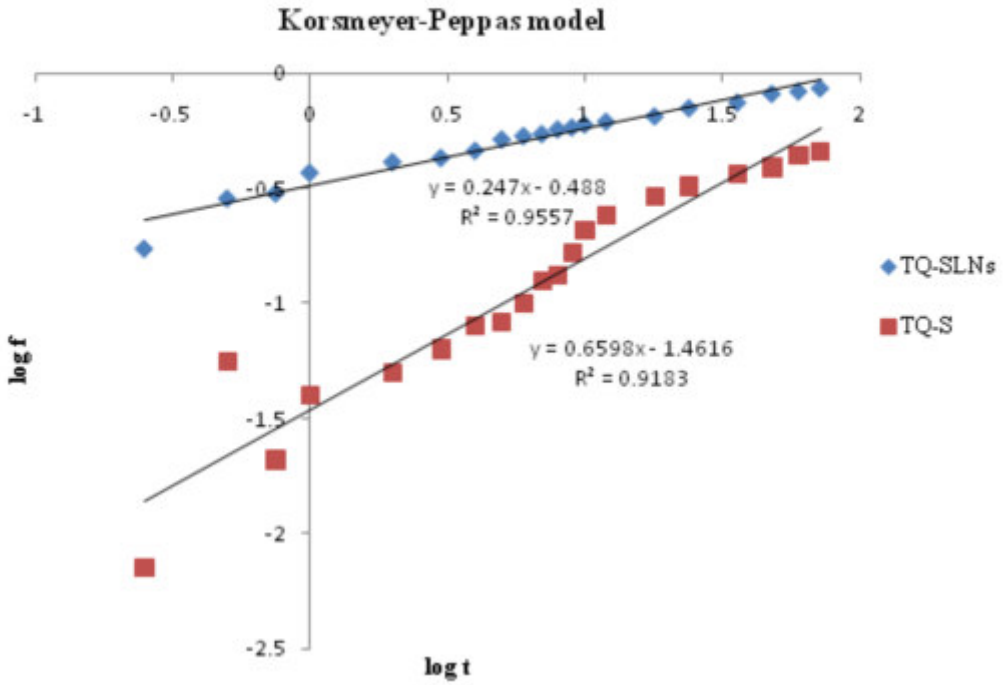
Korsmeyer-Peppas model
y = 0.247x - 0.488
R² = 0.9557
y = 0.6598x - 1.4616
R² = 0.9183
TQ-SLNs
TQ-S
log f
log t

Fig 2D

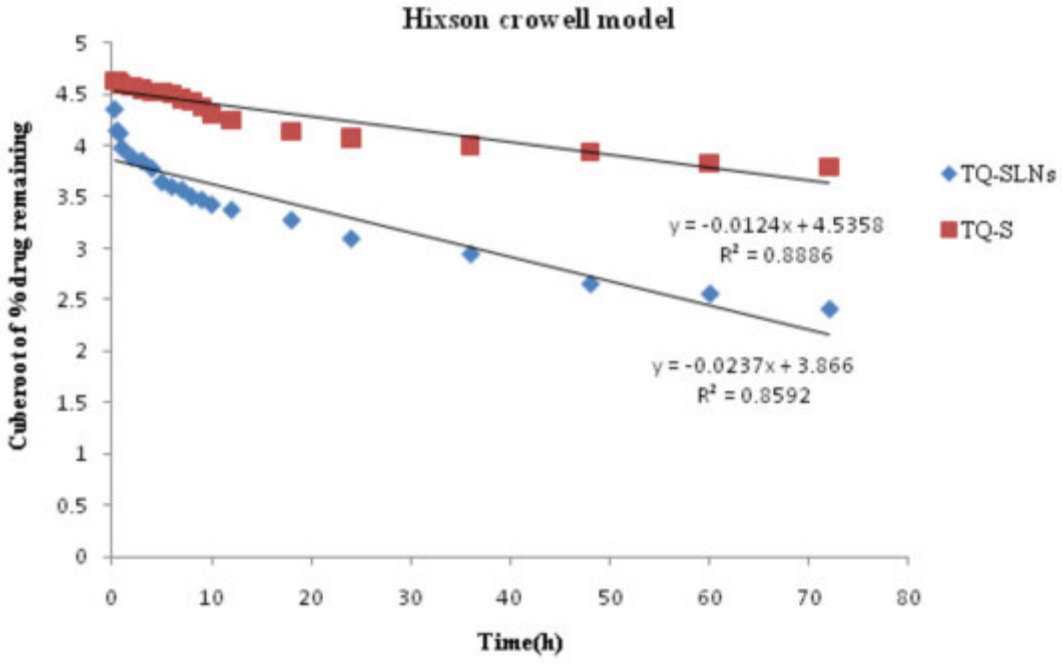
Hixson crowell model
y = -0.0124x + 4.5358
R² = 0.8886
y = -0.0237x + 3.866
R² = 0.8592
TQ-SLNs
TQ-S
Cuberoot of % drug remaining
Time(h)

Fig 2E

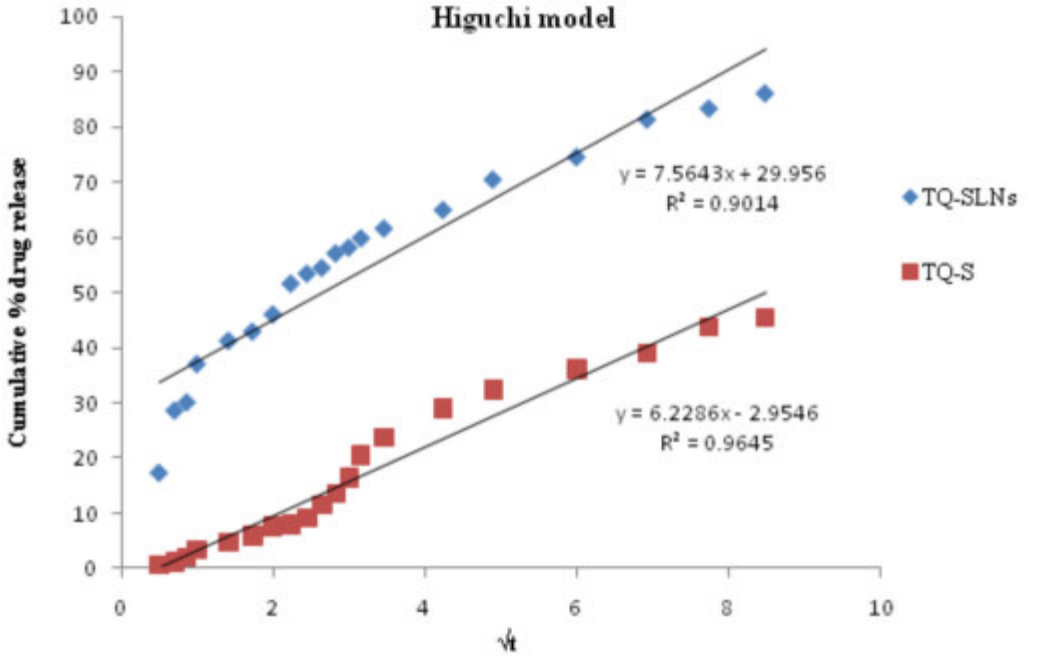
Higuchi model
Cumulative % drug release
√t
y = 7.5643x + 29.956
R² = 0.9014
y = 6.2286x - 2.9546
R² = 0.9645
TQ-SLNs
TQ-S

Fig 2F

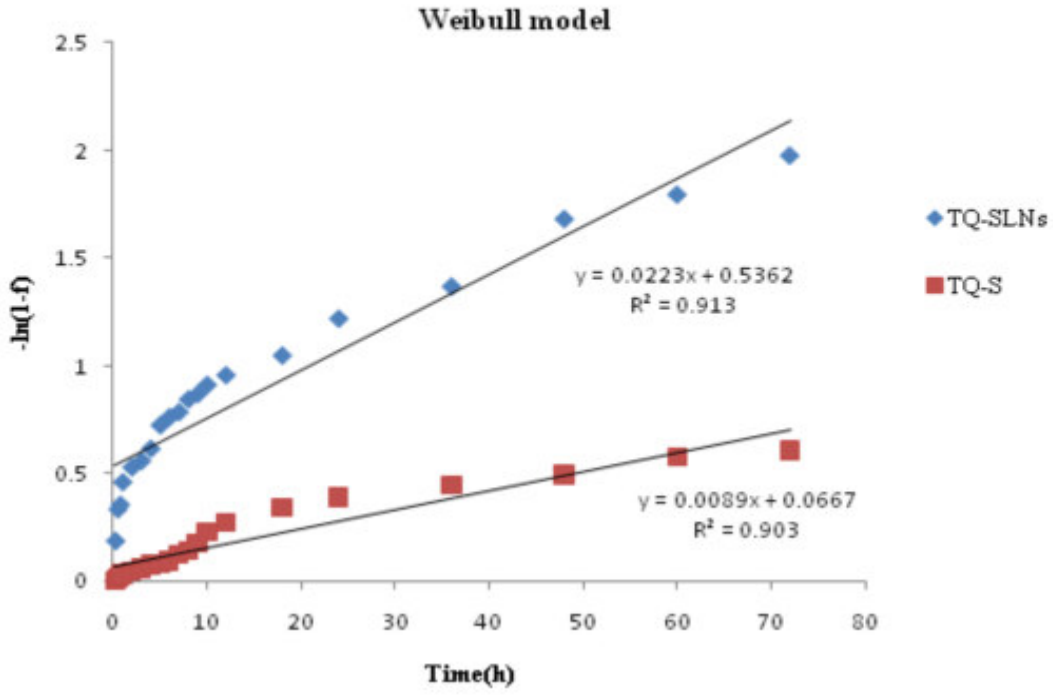
Weibull model
-ln(1-f)
Time(h)
y = 0.0223x + 0.5362
R² = 0.913
y = 0.0089x + 0.0667
R² = 0.903
TQ-SLNs
TQ-S

Fig 3A

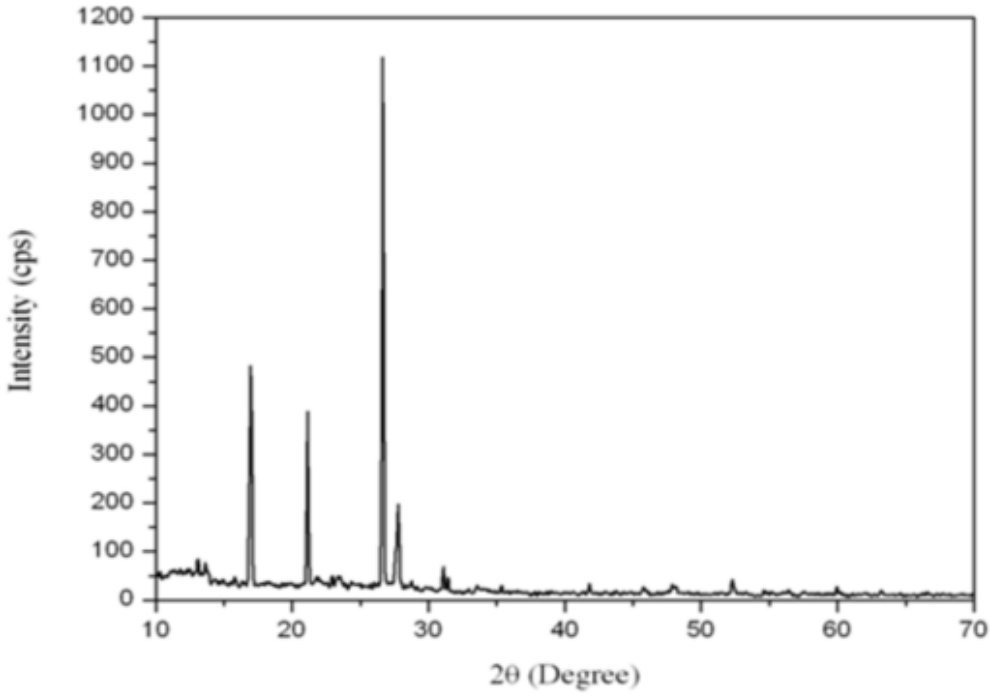
Intensity (cps)
2θ (Degree)
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
10
20
30
40
50
60
70

Fig 3B

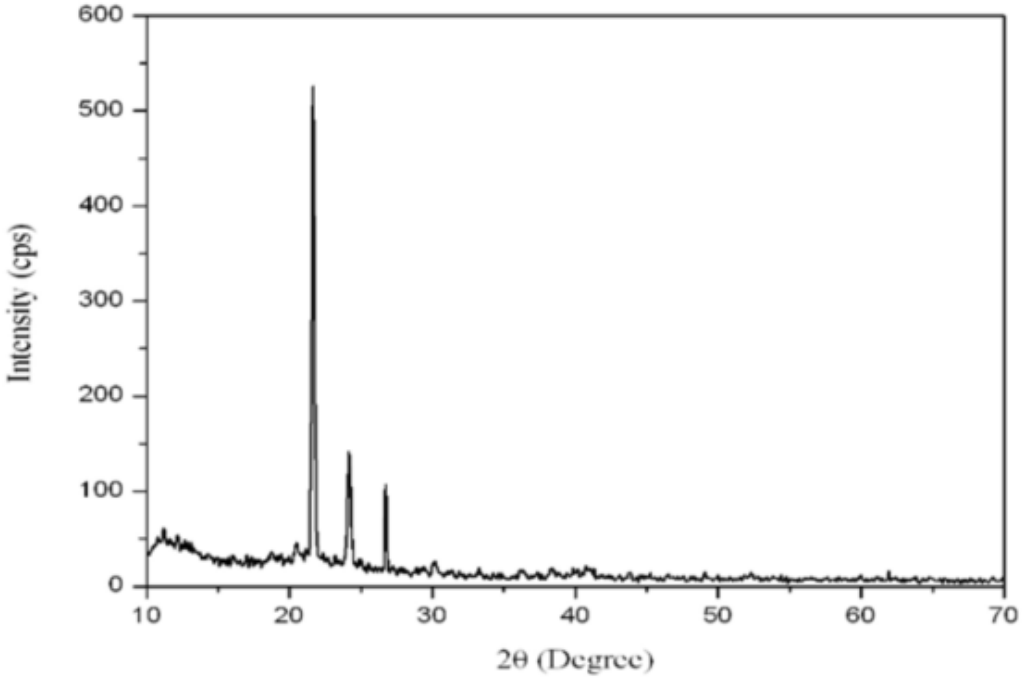
Intensity (cps)
2θ (Degree)
0
100
200
300
400
500
600
10
20
30
40
50
60
70

Fig 3C

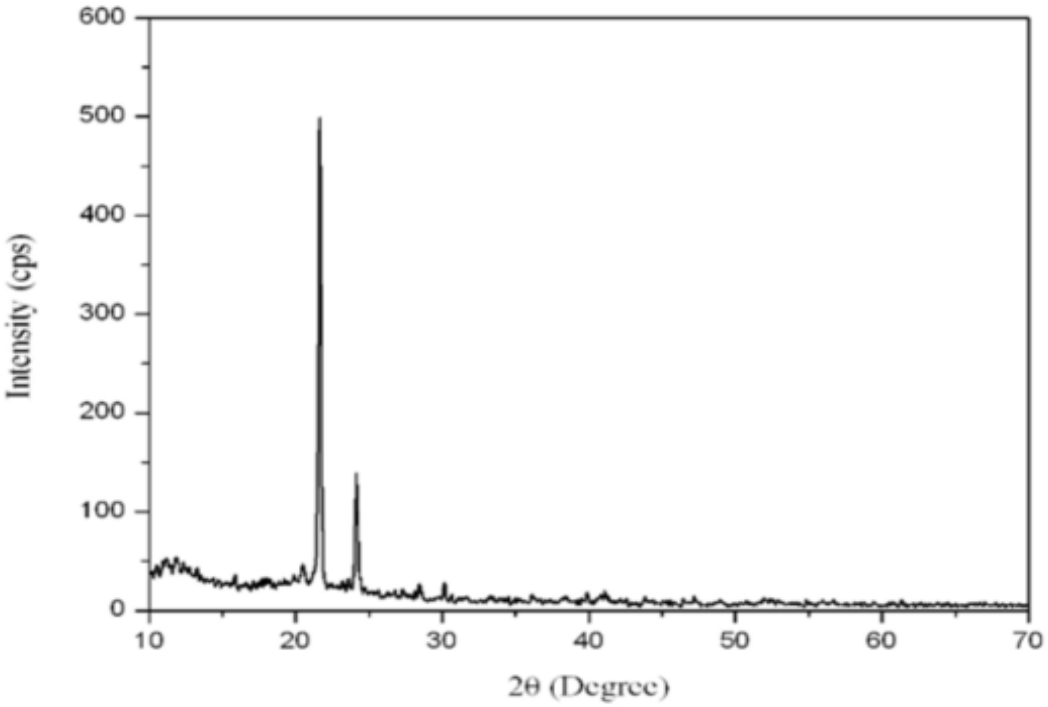
Intensity (cps)
600
500
400
300
200
100
0
10
20
30
40
50
60
70
2θ (Degree)

Fig 4

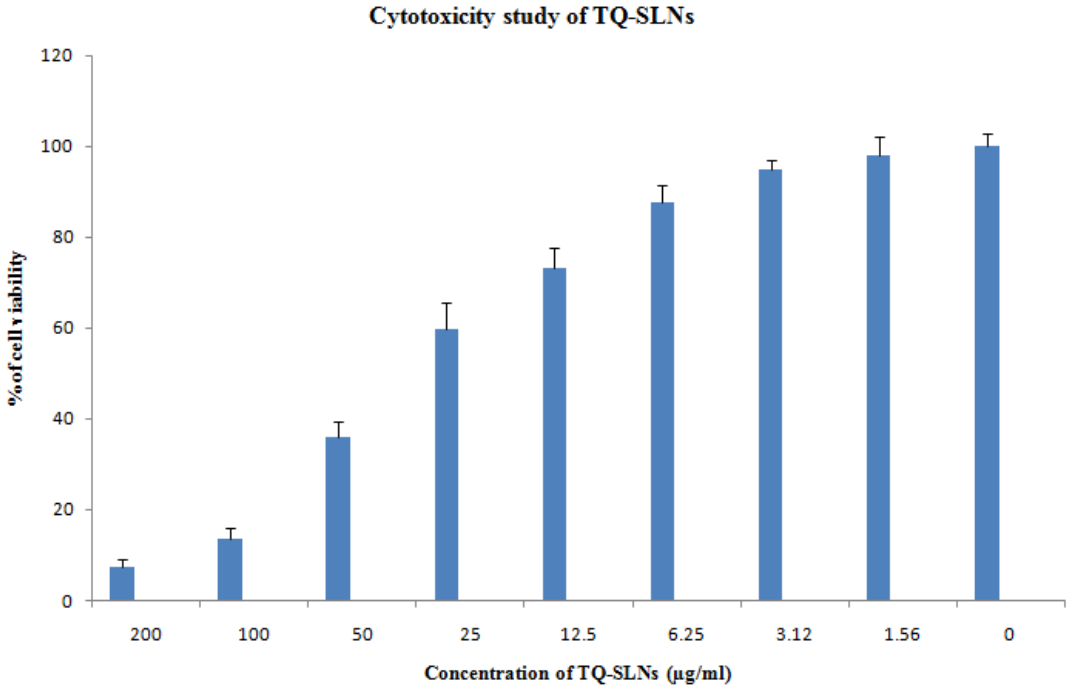
Cytotoxicity study of TQ-SLNs
%of cell viability
120
100
80
60
40
20
0
200
100
50
25
12.5
6.25
3.12
1.56
0
Concentration of TQ-SLNs (µg/ml)

Table I
Characterizations of formulated TQ-SLNs.

Characterizations	Formulated TQ-SLNs
Particle size (nm)	172.1 ± 7.41
Poly Dispersity Index	0.225
Zeta potential (mV)	-45.4 ± 2.68
Total drug content (%)	62.35 ± 2.94
Entrapment efficiency (%)	84.49 ± 3.36
Maximum drug release in 72h (%)	86.15 ± 2.76

RESULTS

Observation on Particle size, Zeta potential, TEM and Entrapment efficiency

The measurement of size of the developed TQ-SLN formulations was determined to be 172.1 ± 7.41 nm (Fig 1A) with a polydispersity index of 0.225 which denotes uniform dispersion of the particles. Similarly, the surface charge of the particles was found to be -45.4 ± 2.68 mV, higher the zetapotential greater the stability of the nanomicrons (Fig 1B). The TEM image showed a relatively smooth spherical morphology of the formulations (Fig 1C). TQ-SLNs showed an average drug content of 62.35 ± 2.94% which was entrapped efficiently at 84.49 ± 3.36% in a lipid core (Table I).

In vitro drug release kinetics

The in-vitro drug release data revealed a maximum of 86.15 ± 2.76% drug release for TQ-SLNs while 45.52 ± 3.97% for TQ–S in 72 hrs (Fig 2), which suggests initial burst release followed by controlled release of a drug from the TQ-SLNs formulations. The release characteristic was incorporated into various release kinetics models and linear regression analysis was carried out (Fig 2A-F).

XRD record

XRD patterns exhibited intense sharp peaks indicating a crystalline nature of TQ (Fig 3A). Similarly, SLNs showed diffused peaks and undefined sharp peaks (Fig 3B). Subsequently, TQ-SLNs exhibited relatively diffused peaks with low intensity, suggesting deformity of crystal lattice (Fig 3C) of TQ.

In vitro Cytotoxicity profile

The cytotoxicity study by MTT analysis of developed TQ-SLNs on vero cell culture was attempted (Fig 4). Cells were incubated with different drug formulations containing 1.56 µg/ml to 200 µg/ml of TQ-SLNs. There was a decline in the cell viability over time, with increase in the concentration of TQ-SLNs formulations. From the study, IC_{50} values of TQ-SLNs calculated after 24 hrs incubation was found to be 35.5 ± 10.5 µg/ml, denotes a sufficient amount of drug required for the cell survival.

DISCUSSION

Hot homogenization followed by micro emulsion method of formulated Solid lipid nanoparticles encapsulated Thymoquinone was found to contain highly solubilized and partitioned Thymoquinone in the solid core. The stirring rate and homogenization speed were highly responsible for a size of the nanoparticles. Accurate sized nanoparticles have an effective targeting efficiency[32]. The appropriate sized particles could retain in the blood stream for a longer period of time, which are effectively responsible for the targeted drug delivery. An ideal nanoparticle was large enough to pilot through the leaky blood vessels

and small enough to escape the macrophage attack and easily permeate into the targeted cells[33]. The formulated TQ-SLNs particle was likely to be small with a particle size ranging 172.5 ± 7.41 nm. Poly dispersity index of TQ-SLNs was also found to be quite nearer to zero, thereby signifies the uniform distribution of particles in the colloidal system. Further, the negative potential difference ensures physical stability of the nanomicrons, where the zetapotential implies upon the repulsion action of particles. Preferably, zetapotential of the nanoparticles higher than -30 mV were more stable[34], where the TQ-SLNs formulation showed a zetapotential of -45.4 ± 2.68 mV suggesting higher stability of the nanoparticles. Likewise, the TEM visualization of formulated TQ in the solid core (TQ-SLNs) appears to be spherical in shape as they emphasizes round colloidal basis. Our results are well in accordance with the previous study, reported by Sandhir R et al[35]. TQ-SLNs formulation also achieved a good entrapment efficiency which might be due to the ionic interaction between the functional groups of TQ with the stearic acid leading to gelation[36]. Consequently, the efficiently encapsulated drug in the solid core was determined to be applicable for the further studies.The results obtained from this study are in agreement with Rohit B and Pal KI[29], who reported solid lipid nanoparticles, encapsulated Isoniazid with increased entrapment efficiency. Pronounced ($P<0.05$) significant difference was observed on the in-vitro drug release kinetics between TQ-SLNs and TQ-S. A maximum drug release was obtained for TQ-SLNs when compared to that of TQ-S. Typically, a nanoparticulate system has more surface area which facilitates the controlled drug release than TQ suspension. Hence, the study of in-vitro release kinetics on different models revealed that TQ-SLNs and TQ-S found to fit best in Korsmeyer–Peppas model and Higuchi model respectively. Thus, the calculated drug release exponent (n) value for Korsmeyer–Peppas kinetics was 0.209, suggesting Fickian diffusional transport of drug, whereas Higuchi kinetics refers to dissolution controlled drug release. From the release profiles, we understood the outer shell of TQ-SLN was composed of lecithin, which contains fewer quantity of the drug that was released in the form of burst initially. The inner core of TQ-SLN mainly consisted of stearic acid and a portion of drug, that was released more gradually[37]. However, the characterization of the molecular arrangement of the drug and the lipid needs to be further investigated. Similar studies with in-vitro drug release potential were described by Singh A et al[38]. Further, XRD data verified the existence of crystalline drug into the solid matrix as molecularly dispersed state. The dispersed state of TQ in the solid lipid core was mainly due to the change in the arrangement of crystal lattice of TQ that provides maximum solubility and consequently enhances bioavailability of poorly water-soluble drug in lipid core, whereby viscosity of matrix was high enough to prevent recrystallization on storage. Similar results were also published previously demonstrating that rapid quenching of the nanoemulsion does not allow the drug to crystallize again[39]. Additionally, cytotoxicity study demonstrated that the lipid matrices showed lower cell toxicity compared to other nanoparticles. TQ-SLNs found to carry a negative surface charge (data shown in Fig 1B). Therefore, the electrical interaction force between the particles and vero cell membrane were repulsive, thereby reduced the particle-membrane interference, thus prevented an excess uptake of TQ-SLNs, and promoted the cell viability[40]. Moreover, positively charged nanoparticles induced a high risk of apoptosis, in general. C. Olbrich et al[41] observed marked toxicity when cells were incubated with SLNs consisting of stearic acid at concentrations of 0.01%, whereas SLNs consisting of triglycerides, cetylpalmitate or paraffin did not exert major cytotoxic effects at the same concentrations. In our study, we suggests that the cytotoxic effects were most likely caused by the products of enzymatic degradation of SLNs that produces free stearic acid which may preferably has toxic effect on cells at increased concentration.

CONCLUSION

The present study is concerned with the formulation, characteristics and evaluation of

cytotoxicity effect of TQ-SLNs. The prepared TQ-SLNs nanoformulation has obtained steric stability with spherical homogeneous distribution of particles, maximum drug entrapment, and sustained or controlled drug release potential. In addition, the reduced crystallinity of TQ-SLNs nanomicrons and its safer dosage determined by cytotoxicity analysis proved solid lipid nanoparticles (SLNs), a promising drug carrier of Thymoquinone. Hence, the further studies are planned to evaluate the pharmacodynamics activities of TQ-SLNs on suitable in vitro and vivo models to treat Huntington's disease

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