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Oral bioavailability enhancement and hepatoprotective effects of thymoquinone by self-nanoemulsifying drug delivery system

Mohd Abul Kalam [a], Mohammad Raish [a], Ajaz Ahmed [b], Khalid M. Alkharfy [b], Kazi Mohsin [a], Aws Alshamsan [a], Fahad I. Al-Jenoobi [a], Abdullah M. Al-Mohizea [a], Faiyaz Shakeel [a,*]

[a] Department of Pharmaceutics, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia
[b] Department of Clinical Pharmacy, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia

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ABSTRACT

Thymoquinone (TQ) is a poorly water soluble bioactive compound which shows poor oral bioavailability upon oral administration. Due to poor aqueous solubility and bioavailability of TQ, various self-nanoemulsifying drug delivery systems (SNEDDS) of TQ were developed and evaluated for enhancement of its hepatoprotective effects and oral bioavailability. Hepatoprotective and pharmacokinetic studies of TQ suspension and TQ-SNEDDS were carried out in rat models. Different SNEDDS formulations of TQ were developed and thermodynamically stable TQ-SNEDDS were characterized for physicochemical parameters and evaluated for drug release studies via dialysis membrane. Optimized SNEDDS formulation of TQ was selected for further evaluation of in vivo evaluation. In vivo hepatoprotective investigations showed significant hepatoprotective effects for optimized TQ-SNEDDS in comparison with TQ suspension. The oral administration of optimized SNEDDS showed significant improvement in in vivo absorption of TQ in comparison with TQ suspension. The relatively bioavailability of TQ was enhanced 3.87-fold by optimized SNEDDS in comparison with TQ suspension. The results of this research work indicated the potential of SNEDDS in enhancing relative bioavailability and therapeutic effects of natural bioactive compounds such as TQ.

1. Introduction

Thymoquinone (TQ) [chemical name: 2-isoprppyl-5-methyl-1,4-benzoquinone (Fig. 1)] is a poorly water soluble bioactive natural compound which is obtained from *Nigella sativa* [1,2]. The essential oil of *Nigella sativa* is traditionally used in folk medicine. TQ showed a broad range of therapeutic activities in literature which includes anti-oxidant, hepatoprotective, cardioprotective, neuroprotective, antidiabetic, anti-inflammatory, antimutagenic, anticarcinogenic and sepsis [1–5]. Although, TQ is potential candidate for treating hepatic disorders, high doses are required for this purpose due to poor water solubility and bioavailability of TQ. The solubility of TQ in water has been reported as <1.0 mg/ml at room temperature [2]. The poor aqueous solubility of TQ would results in poor in vitro dissolution rate which in turns will result in poor oral bioavailability. Most of the therapeutic effects of TQ are believed to be due to its strong antioxidant activity [4–6]. Recently, scientists have paid much attention in the development of nanotechnology-based drug delivery systems such as nanoemulsions, microemulsions, polymeric nanoparticles (PNP), solid-lipid nanoparticles (SLN), liposomes, niosomes, nanocrystals, nanostructured lipid carriers (NLC), self-microemulsifying drug delivery systems (SMEDDS) and self-nanoemulsifying drug delivery systems (SNEDDS) for bioactive natural compounds/nutraceuticals in order to enhance their bioavailability and therapeutic efficacy and thus minimizing adverse effects [7–10]. Oral nanoemulsions and SNEDDS are capable of encapsulating hydrophobic bioactive compounds/nutraceuticals from their plant sources into their internal oil phase which could effectively improve drug solubility, enhance therapeutic efficacy and bioavailability and reduce adverse effects [7,10]. SNEDDS fall under the category of lipid-based nanodrug delivery systems which composed of drug, an internal oil phase, surfactant and cosurfactant which can form very fine nanoemulsions (<100 nm in size) upon dilution with an aqueous media such as gastrointestinal (GI) fluids or water [11–13]. The potential of oral nanoemulsions and SNEDDS has been proved in enhancing the solubility, GI permeability, bioavailability and pharmacological effects of various poorly soluble bioactive natural compounds [8,9,14–18]. Various nanomedicine-based drug delivery system of TQ such as liposomes, nanoemulsions, PNP, SLN and NLC have been investigated recently to enhance its bioavailability and pharmacological effects [2,7,19–24]. SNEDDS offer some potential advantaged over these nanomedicine-based drug delivery systems such as thermodynamic stability, ease of preparation, low cost and

* Corresponding author.
E-mail address: faiyazs@fastmail.fm (F. Shakeel).

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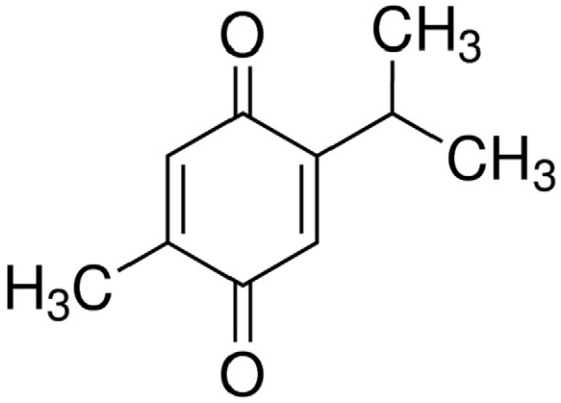

Fig. 1. Molecular structure of TQ; thymoquinone (TQ).

self-nanoemulsification efficiency [11–13,25,26]. As per our knowledge, hepatoprotective effects and bioavailability of TQ has not been investigated by incorporating it into SNEDDS. Therefore, the objective of this research work was to develop various SNEDDS formulations of TQ via construction of pseudo-ternary phase diagrams in order to enhance its hepatoprotective effects and oral bioavailability. Various TQ-SNEDDS were prepared using Capryol-90 (oil phase), Tween-20 (surfactant), isopropyl alcohol (IPA) [cosurfactant] and deionized water (aqueous phase).

2. Experimental

2.1. Materials

TQ (purity > 98.0%) was purchased from "Sigma-Aldrich (St. Louis, MO)". Capryol 90 (propylene glycol monocaprylate) was donated by "Gattefosse (Lyon, France)" and used as received. Tween 20 (polyoxyethylene sorbitan monolaurate) was purchased from "E-Merck (Schuchardh, Hokenbrunn, Germany)". IPA was purchased from "WINLAB (Leicestershire, UK)". Phosphate buffer (pH 6.8) was prepared as per the European Pharmacopoeia. Purified water was obtained by "Milli-Q® water purifier (Millipore, France)". All other chemicals used were of analytical grade and the solvents used were of HPLC grade.

2.2. Solubility studies

In order to select proper components for the preparation of SNEDDS, the solubility of drug molecule in different components is one of the most important measures. Hence, the solubility of crystalline TQ in different excipients/components such as oils, surfactants and co-surfactants was measured by adding an excess quantity of crystalline TQ in 1 ml of each component in stoppered glass. The glass vials were vortexed for 5 min and transferred to biological shaking (Julabo, PA) for continuous shaking at 25 ± 1 °C for 48 h to get the equilibrium. The methanolic extracts of the equilibrated samples were then subjected for centrifugation at 5000 rpm for 10 min (PRISM-R, Labnet International Inc. Edison, NJ), after centrifugation the TQ concentration in the obtained supernatant was determined using a validated HPLC at 254 nm [27].

2.3. Construction of pseudo-ternary phase diagrams

Based on the solubility data of crystalline TQ in different components, Capryol 90, Tween 20 and IPA were selected as oil, surfactant and co-surfactant for the development of SNEDDS of TQ. However, deionized water was selected as the aqueous phase due to its frequent use in SNEDDS/nanoemulsion preparation [11,12,25]. Tween 20 and IPA were mixed (S_{mix}) in mass ratios of 1:0, 1:1, 1:2, 2:1, 3:1 and 4:1. For each phase diagram, oil and specific S_{mix} ratio was mixed well in mass ratios of 1:9 to 9:1. Phase diagrams were constructed using aqueous titration method as reported in literature [25]. The software used for constructing phase diagrams was PCP Disso V2.08 software (Pune, India).

2.4. Formulation development

Different formulations were selected from SNEDDS zones of each phase diagram constructed, based on reason that the concentration of oil phase should be such that it is able to dissolve 10.1 mg of the TQ easily. From the developed pseudo-ternary phase diagrams, various amount of Capryol-90 that solubilized 10.1 mg of TQ was carefully chosen to prepare different SNEDDS of TQ. Moreover, the formulations were chosen from the ternary phase diagrams by giving an emphasis that has minimum concentration of surfactants. Suitable SNEDDS of TQ were developed by aqueous titration method. The composition of SNEDDS was selected on the basis of SNEDDS regions obtained by the pseudo-ternary phase diagrams. TQ was dissolved in Capryol 90 oil, the mixture of Tween 20 as surfactant and IPA as a co-surfactant was added in the oil phase in the preferred concentration, and deionized water was added drop by drop with continuous vortexing till the appearance clear transparent monophasic liquid. The composition of prepared SNEDDS of TQ is presented in Table 1.

2.5. Thermodynamic stability studies

Developed SNEDDS of TQ were subjected to different thermodynamic stability tests in order to eliminate metastable or unstable formulations for further studies. These tests were carried out via centrifugation, heating and cooling cycles and freeze-pump-thaw cycles by adopting the procedure reported in literature [11,25].

2.6. Self-nanoemulsification test

Self-nanoemulsification test was performed in order to evaluate phase separation or precipitation of TQ upon dilution with three diluents [water, acidic buffer (0.1 N HCl) and phosphate buffer (pH 6.8)]. This test was performed by diluting 1 ml of each TQ SNEDDS (NE1–NE9) with around 500 ml of water, 0.1 HCl and phosphate buffer. After dilution, the efficiency of TQ SNEDDS was investigated with the help of A–E grading systems reported in literature [11,12,25].

2.7. Physicochemical characterization of TQ SNEDDS

All the analysis for physicochemical characterization was performed in triplicates manner. The mean droplet size (mean Z value) and

Table 1
Composition of SNEDDS prepared using Capryol-90, Tween-20, IPA and deionized water.

Formulations	Formulation composition (% w/w)					S_{mix} ratio
	TQ (mg)	Capryol-90	Tween-20	IPA	Deionized water	
NE1	10.1	2.00	4.00	4.00	90.0	1:1
NE2	10.1	4.00	8.00	8.00	80.0	1:1
NE3	10.1	5.00	10.0	10.0	75.0	1:1
NE4	10.1	8.00	16.0	16.0	60.0	1:1
NE5	10.1	10.0	20.0	20.0	50.0	1:1
NE6	10.1	16.0	32.0	32.0	20.0	1:1
NE7	10.1	9.00	18.0	18.0	55.0	1:1
NE8	10.1	10.0	15.0	15.0	60.0	1:1
NE9	10.1	7.00	14.0	14.0	65.0	1:1
NE10	10.1	10.0	25.0	25.0	40.0	1:1

Self-nanoemulsifying drug delivery systems (SNEDDS); isopropyl alcohol (IPA); thymoquinone (TQ) and the mass ratio of the surfactant to cosurfactant (S_{mix}).

polydispersity index (PI) of each TQ SNEDDS (NE1-NE10) were determined at 25 °C by dynamic light scattering (DLS) method using a "Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK)". DLS analyzed fluctuations in the intensity of light scattering due to Brownian movement of the particles [11]. The scattering angle for measurement was set at 90°. The zeta potential (ZP) of TQ SNEDDS was also determined using the same instrument. For ZP measurement, sample was diluted with Milli-Q® water and by keeping the dispersant dielectric constant 78.5 for water, the electrophoretic mobility was determined at 25°. The ZP values were calculated through these electrophoretic mobility values using the software DTS Version 4.1 (Malvern, Worcestershire, UK) as described in literature [12].

The viscosity (η) of TQ SNEDDS (NE1–NE10) was measured using "Brookfield Viscometer (Brookfield Engineering Laboratories, Middleboro, MA)" at 25 °C as described by Shakeel et al. [11]. The refractive indices (RIs) of TQ SNEDDS (NE1–NE10) were measured using "Abbes' Refractometer (Precision Testing Instruments Laboratory, Germany)" at 25 °C and the percentage transmittance (%T) of TQ SNEDDS (NE1–NE10) was determined spectrophotometrically at 550 nm using methanol as blank as described in literature [11,25].

Transmission electron microscopic (TEM) studies were carried out in order to determine the shape of the internal oil droplets. A drop of optimized SNEDDS (NE3) was diluted suitably with purified water, applied to a copper grid and allowed to dry for 30 s. The grid was then kept inverted and a drop of phosphotungstic acid was applied to the grid for 10 s. Excess of phosphotungstic acid was removed by absorbing on a filter paper and the grid was analyzed using the "JEOL TEM technique (JEOL JEM-2100 F, USA)" at 100 kV which is capable of point-to-point resolution.

2.8. In vitro drug release studies

The in vitro drug release of TQ from prepared SNEDDS was determined using dialysis bag method. Phosphate buffer (pH 6.8) solution was used as medium for the in vitro release studies. 2.1 ml of formulation was placed in the treated and activated dialysis bag (single dose containing 10.1 mg of TQ), which was immersed in 50 ml of phosphate buffer (pH 6.8) in a beaker for 24 h, maintained at temperature 37 °C and kept on shaker at 50 rpm. Samples were withdrawn at predetermined time intervals (0, 0.5, 1, 2, 4, 6, 8, 12 and 24 h) for the analysis and the sink condition was maintained by replacing an equal volume of TQ free phosphate buffer (pH 6.8) solution. The TQ concentrations in the aspirated samples were determined by validated HPLC at 254 nm [27]. The release of TQ from different SNEDDS was compared with pure TQ suspension. TQ suspension was prepared by dispersing an appropriate amount of TQ in deionized water in order to obtain TQ suspension in the concentration of 5.0 mg/ml of TQ. No stabilizers or suspending agents were used for the preparation of TQ suspension because it was easily dispersed in water.

2.9. Physical stability on optimized SNEDDS (NE3)

The optimized SNEDDS NE3 was freshly prepared and subjected for physical stability in terms of rheological, optical, particle size distribution and drug content remained after three months of storage at 25 ± 2 °C. The samples were withdrawn at 0, 30, 60, and 90 days) and evaluated visually for any physical change in the formulation. Average Z value, PI, ZP, η and RI were measured as per the method described above as well as the amount of drug content remained was determined using HPLC [27] at 254 nm at the end of 1st month, 2nd month and 3rd month of storage.

2.10. Hepatoprotective effects

Thirty male Wistar Albino rats weighing 220–250 mg/kg were taken from "Animal Care and Use Centre, King Saud University, Riyadh, Saudi Arabia". Before starting hepatoprotective experiment, all the rats were acclimatized and kept in plastic cages under standard laboratory conditions of animal care and storage and standard pellet diet was provided with water ad libitum. The studies were approved by the "animal care and use committee of King Saud University, Riyadh, Saudi Arabia". The guidelines of "animal care and use committee of King Saud University" were strictly followed throughout the study. Hepatotoxicity was induced using CCl_4 as a toxicant as it has been reported as a suitable toxicant for such experiments [28]. The animals were randomly distributed into five different groups and each group was having six rats. Group I animals were served as control which were provided a daily dose of 1 ml aqueous solution of 0.5% w/w carboxymethyl cellulose (CMC) (p.o.) for 5 days. Group II animals were serve as toxic control which were provided daily dose of aqueous solution of 0.5% w/w CMC (p.o.) along with a single dose of CCl4 (1 mg/kg, i.p.) on day 1. Group III animals were considered as the standard group which were treated with an oral suspension of standard silymarin (10 mg/kg) on all 5 days and CCl4 (1 mg/kg, i.p.) on days 2 and 3 after 1 h of the administration of silymarin (standard). Groups IV animals were served as test TQ group which were treated with TQ suspension (20 mg/kg) on all 5 days and CCl4 (1 mg/kg, i.p.) on days 2 and 3 after 1 h of the administration of TQ suspension. Groups V animals were served as test TQ SNEDDS NE3 group which were treated with optimized TQ SNEDDS NE3 (having 20 mg/kg of TQ) on all 5 days and CCl4 (1 mg/kg, i.p.) on days 2 and 3 after 1 h of the administration of TQ SNEDDS NE3. Around 1.5–2.0 ml of blood was collected in a sterile Eppendorf tubes from the tail vein of all animals fasted overnight on day 6 and kept at 37 °C for about 45 min. The serum was separated with the help of a sterile micropipette after centrifugation at 3000 rpm for 15 min. The serum samples were subjected to biochemical estimation to evaluate liver function.

Various biochemical parameters such as serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), γ-glutamyl transpeptidase (γ-GGT), serum bilirubin and alkaline phosphatase (ALP) were measured by adopting the procedures reported in literature [29–31]. At the end of experiment, the animals were sacrificed using ether euthanasia.

2.11. Preparation of liver homogenate for biochemical estimation of liver tissue

Fresh livers were collected from rats and weighed. 10% w/v of liver homogenates were prepared in 1.5 M KCl. The biochemical parameters such as catalase (CAT), glutathione peroxidase (GSH), superoxide dismutase (SOD) and melondialdehyde (MDA) were measured in liver homogenates as reported in literature [32,33].

2.12. Histopathological evaluation

The histopathology of liver was performed using a standard method. In order to perform this experiment, the autopsied livers were washed carefully using normal saline solution and fixed in 10% formalin solution for 2 days followed by a bovine solution for the period of 6 h. The samples were then paraffin-embedded and the sections of about 5 μm thickness were cut with the help of a microtome. These sections were stained with hematoxylin and eosin and processed in an alcohol-xylene series [34]. The slides were studied under a photomicroscope and photographs were taken for evaluation.

2.13. Pharmacokinetic studies

Twelve male Wistar Albino rats (220–250 mg/kg) were obtained from "Animal Care and Use Centre, King Saud University". Before starting the experiment, all the rats were acclimatized and kept in plastic cages under standard laboratory conditions animal care and storage and standard pellet diet was provided with water ad libitum. The studies were approved by the "animal care and use committee of King Saud

University, Riyadh, Saudi Arabia". The guidelines of "animal care and use committee of King Saud University" were strictly followed throughout the study. In a single dose parallel study designed, animals were randomly divided into two groups (6 in each) which served as TQ suspension (group I) and optimized TQ SNEDDS NE3 (group II) treatment groups, respectively. All the animals were fasted overnight before starting pharmacokinetic studies. Around 500 μl of blood samples were taken from the retro-orbital plexus into heparinized microfuge tubes at 0, 0.5 1, 1.5, 2, 4, 6, 12 and 24 h after oral administration of TQ suspension (10 mg/kg of TQ, p.o.) and TQ SNEDDS (10 mg/kg of TQ, p.o.). The rats were anaesthetized using ether before taking blood at each time interval. During blood sampling for 24 h, the animals were provided standard pellet diet with water ad libitum. The blood samples were centrifuged at 4500 g for 10 min for separation of plasma. The separated plasma samples were stored frozen at −80 till further use. At the end of experiment, the animals were sacrificed using ether euthanasia.

2.14. Determination of TQ content in plasma by validated HPLC-UV method

Shimadzu high performance liquid chromatographic (HPLC) unit coupled with ultraviolet (UV) detector "(Shimadzu, Kyoto, Japan)" was used for quantification of TQ in plasma samples using thymol as an internal standard (IS). The HPLC-UV system was equipped with a "LC 20AD binary solvent delivery pump, SIL 20A auto sampler, SPD 20A dual UV absorbance detector, and an inline vacuum degasser (Shimadzu, Kyoto, Japan)". The software used was "Shimadzu lab solutions software (version 3.05, Shimadzu, Kyoto, Japan)". The chromatographic separation of TQ and IS was performed on "Atlantis dC-18 bond pack column (Waters, St. Louis, MO, USA)" having a 5 μm packing as a stationary phase and maintained at room temperature. The mobile phase consisted of methanol: 10 mM KH_2PO_4 buffer (90:10% v/v, pH 4.6). The elution was carried out at a flow rate of 0.9 ml/min with UV detection at 254 nm for both TQ and IS. Samples were prepared by protein precipitation method using acetonitrile as a precipitation solvent. About 200 μl of rat plasma samples were transferred to 1.5 ml centrifuge tubes and 10 μl of IS (20 μg/ml) and 20 μl of TQ (20 μg/ml) were added. The samples were vortexed for 20 s and 1000 μl of acetonitrile was added for precipitation. The samples were shaken and centrifuged at 2500 g for another 10 min. The upper layer was taken and transferred to HPLC vials and subjected for the analysis [27]. The proposed HPLC method was linear in the concentration range of 0.5–50 μg/ml with coefficient of determination value of 0.997. The lower limit of quantification was recorded as 0.134 μg/ml. The accuracy of HPLC method was recorded in the range of 92–107.77%. The intra-day and inter-day precisions of HPLC method were recorded in the range of 3.92–12.36%.

2.15. Pharmacokinetic calculation and data analysis

The plasma concentrations of TQ against different time intervals were used to investigate pharmacokinetic profiles of TQ. TQ plasma concentration–time curves were plotted for this purpose. All pharmacokinetic parameters are expressed as mean ± standard error of mean (SEM). The software applied for calculation of these parameters was WinNonlin software (Pharsight Co., Mountain View, CA). The maximum plasma concentration (C_{max}) of TQ and time to reach maximum concentration (t_{max}) were estimated directly from plasma-concentration time profile curve. However, area under the curve from time 0 to t (AUC_{0-t}) and 0–∞ ($AUC_{0-\infty}$), area under moment curve from time 0 to t (AUMC0–t), area under moment curve from time 0-∞ ($AUMC_{0-\infty}$), elimination rate constant (K_e), half-life ($t_{1/2}$), volume of distribution (VD) and clearance (CL/F) were calculated using noncompartmental model as reported previously in literature [25,26].

2.16. Statistical analysis

Statistical data were compared using unpaired *t*-test using Graphpad Instat software. The value of $P < 0.05$ was considered as significant as compared to TQ suspension.

3. Results

The solubility data of TQ in various components are presented in Fig. 2. In this study, Capryol-90 was chosen as oil phase as it exhibited a highest solubility of TQ, Tween 20 was used as surfactant and it also presented a sufficient solubility of the TQ and IPA was selected as co-surfactant as it has shown a good solubility of the TQ, which are found sufficient to give a clear, transparent and stable SNEDDS of TQ.

Pseudo-ternary phase diagrams were constructed for each S_{mix} ratio for the development of suitable SNEDDS of TQ and results are presented in Fig. 3. The summary of results is presented in Table 2. From Fig. 3, it can be seen that S_{mix} ratio of 1:0 showed relatively low SNEDDS zones (Fig. 3A). However, in case of S_{mix} ratio of 1:1 (Fig. 3B), when IPA (cosurfactant) was used along with Tween-20 (surfactant), the SNEDDS zones were enhanced significantly as compared to Fig. 3A (S_{mix} of 1:0). In case of S_{mix} ratio of 1:2 (Fig. 3C), SNEDDS zones were observed to be enhanced as compared to 1:0 but reduced as compared to 1:1 ratio. When S_{mix} of 2:1 was investigated (Fig. 3D), SNEDDS zones were found to be decreased as compared to 1:1 ratio but increased in comparison with 1:2 ratio. Similarly, when S_{mix} ratios of 3:1 and 4:1 were studied (Fig. 3E and F, respectively), the SNEDDS zones were found to be decreased again as compared to S_{mix} ratios of 1:1 and 2:1 (Table 2).

The qualitative results of thermodynamic stability tests are presented in Table 3. It was observed that most of the TQ SNEDDS (NE1–NE5 and NE7–NE9) were found to be thermodynamically stable at centrifugation, heating and cooling cycles and freeze-thaw-pump cycles. Only two formulations NE6 and NE10 were observed to fail these tests.

The qualitative results of self-nanoemulsification test are also presented in Table 3. It was observed that most of the TQ SNEDDS (NE1–NE5 and NE7–NE9) passed this test with grade A in the presence of all three diluents investigated. Grade A is considered as the best grade for this test. However, formulations NE6 and NE10 passed this test with grade B in the presence of all three diluents investigated.

The data of physicochemical investigation of TQ SNEDDS are presented in Table 4. The droplet size of TQ-SNEDDS NE1–NE5 and NE7–NE9 was observed as 54.25–87.62 nm. The PI of TQ-SNEDDS NE1–NE5 and NE7–NE9 was observed as 0.125–0.325.

The ZP values of TQ-SNEDDS NE1–NE5 and NE7–NE9 were obtained as −18.88 to −12.25 mV (Table 4). The lowest ZP value was obtained in formulation NE3 (−18.88 mV). However, the highest one was obtained in formulation NE1 (−12.25 mV).

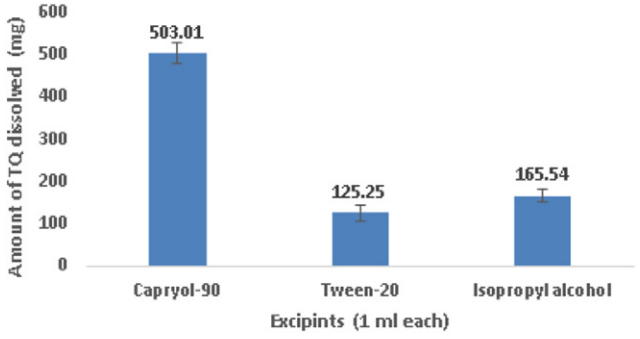

Fig. 2. Solubility profile of TQ in different components of SNEDDS; thymoquinone (TQ); self-nanoemulsifying drug delivery systems (SNEDDS) and the values are presented as mean ± SD, $n = 3$.

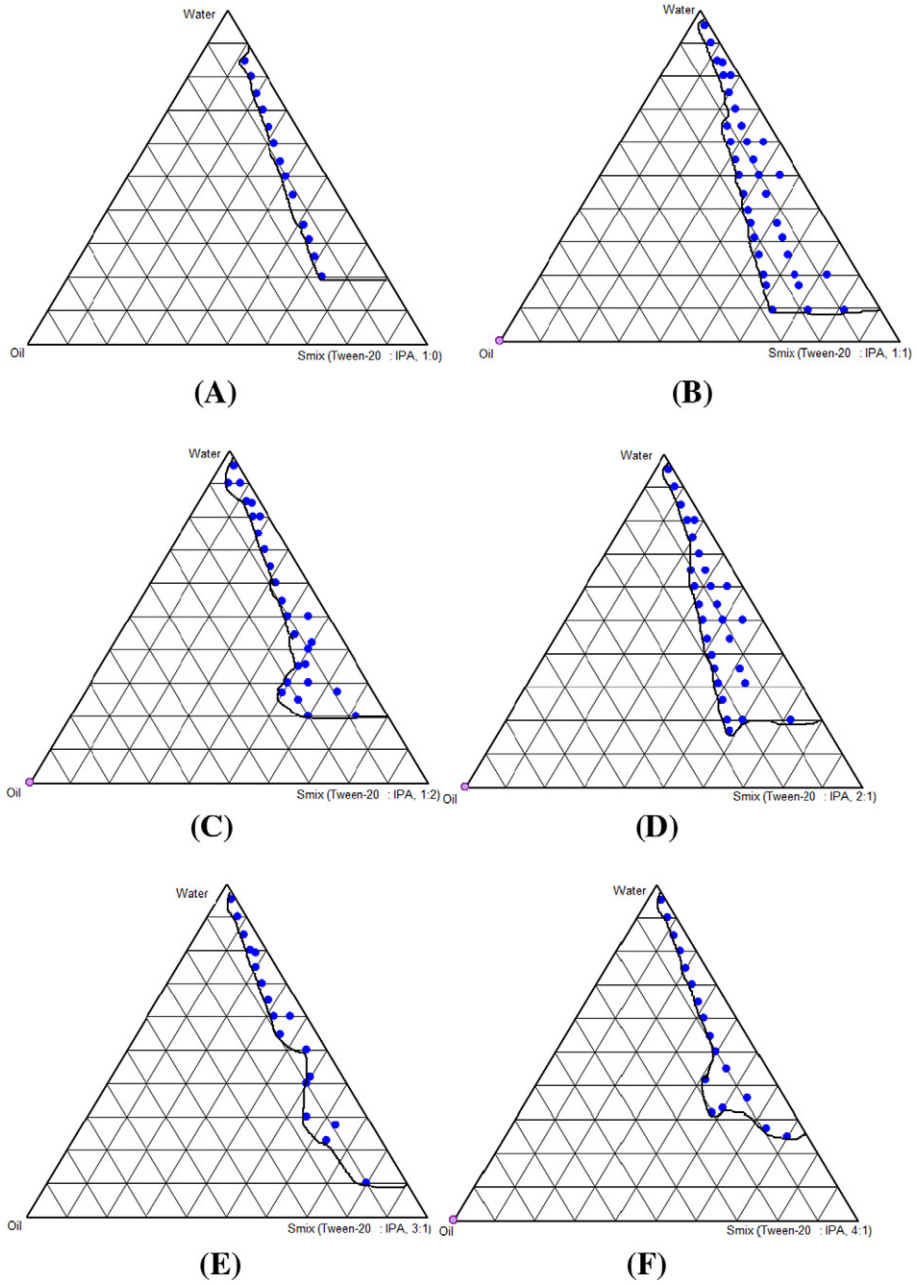

Fig. 3. Pseudo-ternary phase diagrams for SNEDDS zones of TQ (dotted area with outline) for oil phase (Capryol-90), aqueous phase (deionized water), surfactant (Tween-20) and co-surfactant (IPA) at S_{mix} ratios of 1:0 (A), 1:1 (B), 1:2 (C), 2:1 (D), 3:1 (E) and 4:1 (F); self-nanoemulsifying drug delivery systems (SNEDDS); isopropyl alcohol (IPA); thymoquinone (TQ) and the mass ratio of the surfactant to cosurfactant (S_{mix}).

The viscosity of TQ-SNEDDS NE1–NE5 and NE7–NE9 was observed as 18.25–55.75 cP.

The RIs of TQ-SNEDDS NE1–NE5 and NE7–NE9 were observed as 1.334–1.348. SNEDDS formulation NE8 showed the highest RI (1.448 ± 0.08). However, SNEDDS formulation NE3 showed the lowest RI (1.334 ± 0.09).

The %T of TQ-SNEDDS NE1-NE5 and NE7-NE9 was observed as 95.45–98.45% (Table 4). The highest %T was obtained in formulation

Table 2
Observations during the formulation of SNEDDS by aqueous phase titration method.

Figure	S_{mix} ratio	Surfactant	Cosurfactant	Nanoemulsion zones	Oil phase solubilized (% w/w)[a]	S_{mix} solubilized (% w/w)[b]
3A	1:0	Tween-20	IPA	Low	15.00	55.00
3B	1:1	Tween-20	IPA	Highest	41.67	81.81
3C	1:2	Tween-20	IPA	Higher than 3A, 3E & 3F but lower than 3B & 3D	25.93	63.63
3D	2:1	Tween-20	IPA	Higher than 3A, 3C, 3E & 3F but lower than 3B	27.27	72.73
3E	3:1	Tween-20	IPA	Higher than 3A & 3F but lower than 3B, 3C & 3D	25.00	58.33
3F	4:1	Tween-20	IPA	Higher than 3A but lower than 3B, 3C, 3D & 3E	16.00	64.00

Self-nanoemulsifying drug delivery systems (SNEDDS); isopropyl alcohol (IPA) and the mass ratio of the surfactant to cosurfactant (S_{mix}).
[a] The maximum amount of Capryol-90 (oil phase) that was solubilized.
[b] The maximum amount of S_{mix} (Tween-20: IPA) phase that was solubilized with respect to maximum amount of oil phase.

Table 3
Results of thermodynamic stability and self-nanoemulsification testing of SNEDDS in the presence of deionized water, phosphate buffer saline (pH 7.4) and 0.1 N HCl.

Formulations	Nanoemulsion test grade[a]	Thermodynamic stability tests		
		Centrifugation	Heating and cooling cycles	Freeze-thaw cycles
NE1	A	Passed	Passed	Passed
NE2	A	Passed	Passed	Passed
NE3	A	Passed	Passed	Passed
NE4	A	Passed	Passed	Passed
NE5	A	Passed	Passed	Passed
NE6	B	Failed	Passed	Failed
NE7	A	Passed	Passed	Passed
NE8	A	Passed	Passed	Passed
NE9	A	Passed	Passed	Passed
NE10	B	Failed	Failed	Passed

Self-nanoemulsifying drug delivery systems (SNEDDS).
[a] All the formulations (except NE6 and NE10) passed this test with Grade-A in the presence of deionized water, 0.1 N HCl and phosphate buffer saline (pH 7.4).

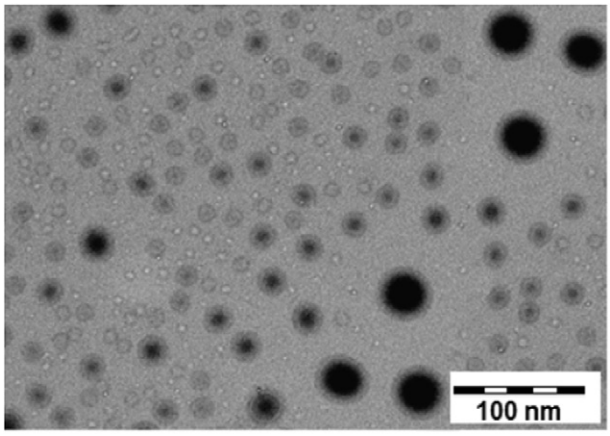

Fig. 4. TEM images of optimized SNEDDS (NE3) showing spherical shaped droplets within nanometer (around 25 nm) range; transmission electron microscopy (TEM) and self-nanoemulsifying drug delivery systems (SNEDDS).

NE3 (98.45 ± 0.25%). However, the lowest one was obtained in formulation NE9 (95.45 ± 3.25%).

TEM Photomicrographs of formulation NE3 were taken and interpreted for shape and size distribution and results are presented in Fig. 4.

TQ released from the different SNEDDS was found to be satisfactory and significant ($P < 0.001$) as compared to the TQ-suspension as shown in Fig. 5. The formulation coded as NE3 delivered the highest cumulative percent of TQ released (73.01%) as compared to the other formulations. Approximately 62% of TQ (NE3) was released in the initial 4 h of the release study while TQ-suspension showed a drug release of only 34%. Similarly, other SNEDDS have shown a satisfactory release of TQ in the initial 4 h of the study (NE4 = 51%, NE5 = 45%, NE7 = 58% and NE8 = 48%), and overall a sufficient quantity of TQ was released from the remaining four selected and optimized nanoemulsions (NE4 = 64%, NE5 = 57%, NE7 = 68% and NE8 = 61%) as compared to the TQ-suspension throughout the 24 h study period.

The results of physicochemical stability are presented in Table 5.

The results of various biochemical parameters of rat serum are presented in Table 6. It was observed that control group animals (group I animals) showed normal levels of serum biochemical parameters such as AST, ALT, ALP, γ-GGT and bilirubin. However, the administration of CCl_4 (group II animals) showed a marked increase in AST, ALT, ALP, γ-GGT and bilirubin. The oral administration of standard silymarin, TQ suspension and optimized TQ SNEDDS NE3 showed significant reduction in the levels of AST, ALT, ALP, γ-GGT and bilirubin in different proportions.

The mean levels of AST, ALT and ALP were enhanced extremely significantly in toxic control (group II animals) in comparison with group I (control) rats ($P < 0.01$). The administration of standard silymarin, TQ suspension and optimized SNEDDS NE3 along with CCl_4 administration showed significant reduction in AST, ALT and ALP levels in comparison with toxic control group ($P < 0.01$). There was around 2.82, 2.65 and 2.29 fold enhancement in AST, ALT and ALP levels in group II animals, respectively in comparison with group I animals. The levels of serum γ-GGT and bilirubin in control animals (group I) were observed as 1.48 ± 0.05 and 0.73 ± 0.01 U/L, respectively.

The results of various biochemical parameters of liver tissue are presented in Table 7. It was observed that control group animals (group I animals) showed normal levels of tissue biochemical parameters such as CAT, GSH, MDA and SOD. The GSH level in group I animals was recorded as 1.17 ± 0.02 nmol/mg. However, CCl_4 administration reduced GSH level to 0.47 ± 0.02 nmol/mg in group II animals. Moreover, the administration of standard silymarin suspension, TQ suspension and optimized SNEDDS NE3 also reduced the GSH level as compared to group I animals (Table 7). The tissue levels of CAT, MDA and SOD were recorded as 46.62 ± 1.09 U/mg, 3.26 ± 0.08 nmol/mg and 23.91 ± 1.13 U/mg, respectively in group I animals. The administration of CCl_4 in group II animals caused significant reduction in tissue levels of CAT, MDA and SOD ($P < 0.01$). The administration of standard silymarin suspension, TQ suspension and optimized SNEDDS NE3 also reduced CAT, MDA and SOD levels as compared to group I animals (Table 7).

The results of histopathology of normal, toxic control, TQ suspension and optimized SNEDDS NE3 are presented in Fig. 6. Normal group animals showed normal liver with signs of inflammatory cells and necrosis (Fig. 6A). The toxic group animals presented large hepatocytes with inflammatory cells and necrosis (Fig. 6B). However, the animals which

Table 4
Physicochemical characterization (mean ± SD, $n = 3$) of TQ-SNEDDS (NE1-NE10).

Formulations	Characterization parameters					
	Z-average ± SD (nm)	PI	ZP ± SD (mV)	η ± SD (cP)	RI ± SD	%T ± SD
NE1	58.15 ± 2.98	0.187	−12.25 ± 1.84	25.14 ± 1.85	1.343 ± 0.01	97.89 ± 0.53
NE2	55.58 ± 4.52	0.245	−13.23 ± 1.56	21.25 ± 2.45	1.347 ± 0.04	97.76 ± 0.28
NE3	54.25 ± 3.54	0.125	−18.88 ± 1.12	18.25 ± 1.28	1.334 ± 0.09	98.45 ± 0.25
NE4	61.28 ± 4.95	0.275	−16.23 ± 2.15	29.78 ± 2.85	1.346 ± 0.02	97.65 ± 1.24
NE5	75.25 ± 5.68	0.278	−17.28 ± 1.26	55.25 ± 4.75	1.343 ± 0.07	97.41 ± 1.08
NE6[a]	–	–	–	–	–	–
NE7	58.35 ± 5.45	0.242	−15.64 ± 1.56	51.25 ± 4.85	1.347 ± 0.01	96.12 ± 1.29
NE8	87.62 ± 5.87	0.325	−14.57 ± 2.32	55.75 ± 5.65	1.348 ± 0.08	95.85 ± 2.58
NE9	65.28 ± 3.98	0.247	−14.26 ± 1.85	48.25 ± 3.51	1.345 ± 0.05	95.45 ± 3.25
NE10[a]	–	–	–	–	–	–

Thymoquinone (TQ); self-nanoemulsifying drug delivery systems (SNEDDS); Z-average (average droplet size); PI (polydispersity index); ZP (zeta potential); η (viscosity); RI (refractive index); %T (percent of transmittance); SD (standard deviations) and the values are presented as mean ± SD, $n = 3$.
[a] NE6 and NE10 failed the thermodynamic stability testing, so these two were not subjected for further physicochemical characterization.

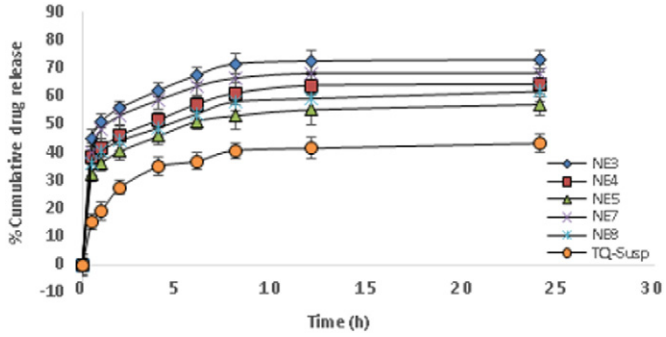

Fig. 5. In vitro drug release profile of TQ from SNEDDS (NE3-NE8) and TQ suspension; thymoquinone (TQ); self-nanoemulsifying drug delivery systems (SNEDDS) and the values are presented as mean ± SD, $n = 3$.

were treated with TQ suspension presented sinusoidal dilatation around the central vein with no sign of inflammation and necrosis (Fig. 6C). On the other hand, the animals which were treated with optimized SNEDDS NE3 showed almost normal appearance of liver parenchyma with no signs of inflammation and necrosis in hepatocytes (Fig. 6D).

The plasma concentration-time profile plots of TQ for optimized SNEDDS NE3 showed significantly greater enhancement in TQ in vivo absorption in comparison with TQ suspension (Fig. 7). The PK data of SNEDDS NE3 and TQ suspension are listed in Table 8. The t_{max} value was recorded as 2.0 h for NE3 and 4.0 h for TQ suspension. However, the C_{max} value was found to be 186.61 μg/ml for NE3 and 68.49 μg/ml for TQ suspension. AUC_{0-t} and $AUC_{0-\infty}$ of NE3 were found to be 1657.70 and 1995.60 μg·h/ml, respectively, which were highly significant ($P < 0.05$) in comparison with AUC_{0-t} (511.04 μg·h/ml) and $AUC_{0-\infty}$ (514.64 μg·h/ml) of TQ suspension. The relative bioavailability of NE3 with respect to TQ suspension was recorded as 387.76% as compared to TQ suspension.

4. Discussion

The important criteria to select the various components were based on the solubility of TQ in various components. The solubility of TQ in oil phase should be higher in order to maintain the drug in molecular state in developed SNEDDS. In this research work, various long chain and medium chain triglycerides were used for this purpose [35].

Overall, the maximum SNEDDS zones were observed in case of S_{mix} ratio of 1:1 (Fig. 3B). Therefore, different SNEDDS of TQ were selected from Fig. 3B. The entire SNEDDS zones in Fig. 3B were taken into an account and different oil compositions (2, 4, 5, 6, 7, 8, 9, 10 and 16% w/w) with different S_{mix} concentration (8, 16, 20, 28, 30, 32, 36, 40, 50 and 64% w/w) were precisely selected from Fig. 3B. Accurately weighed 10.1 mg of TQ was solubilized in required amount of oil phase and different concentrations of S_{mix} were added with vortexing. The required amount of deionized water was added drop by drop till clear and transparent SNEDDS of TQ obtained.

TQ SNEDDS (NE1-NE10) were evaluated for different thermodynamic stability tests to observe thermodynamic stability of formulations at different stress conditions.

Different TQ SNEDDS (NE1-NE10) were also subjected to self-nanoemulsification test in order to evaluate TQ precipitation/phase separation in the presence of water, 0.1 N HCl and phosphate buffer (pH 6.8) [36].

Formulations NE6 and NE10 were not investigated for physicochemical characterization because these formulations did not pass thermodynamic stability tests. SNEDDS formulation NE8 showed the largest droplet size (87.62 ± 5.87 nm) which was possible due to the presence of lower concentration of S_{mix} in formulation NE8. However, SNEDDS formulation NE3 showed the lowest droplet size (54.25 ± 3.54 nm) which was possible due to the presence of optimal concentrations of Capryol-90 (oil phase) and S_{mix} in formulation NE3. The PI of most of the SNEDDS (except NE8) was <0.3, indicating good uniformity of size distribution. SNEDDS NE3 had least PI value (0.125), which indicated the highest uniformity of droplets in formulation NE3. However, the highest PI value was observed in formulation NE8 (0.325).

The negative net charge on ZP for all TQ-SNEDDS was possibly due to the presence of fatty acid esters in Capryol-90 [11,12].

SNEDDS formulation NE8 showed the largest viscosity (55.75 ± 5.65 cP) which was possible due to the lowest droplet size and the presence of lower concentration of S_{mix} in formulation NE8. However, SNEDDS formulation NE3 showed the lowest viscosity (18.25 ± 1.28 cP) which was possible due to the lowest droplet size and the presence of optimal concentrations of Capryol-90 (oil phase) and S_{mix} in formulation NE3. The viscosity results were in accordance with droplet size analysis.

Table 5
Effect of storage time on physicochemical parameters (physical stability) and drug content remaining (mean ± SD, $n = 3$) of NE3.

Time (days)	Z-average ± SD (nm)	PI	ZP ± SD (mV)	RI ± SD	η ± SD (cP)	Drug content (%)
0	54.25 ± 3.54	0.125	−18.88 ± 1.12	1.334 ± 0.09	25.14 ± 1.85	100
30	55.41 ± 2.98	0.126	−17.08 ± 1.31	1.335 ± 0.07	25.57 ± 1.32	99.75
60	57.05 ± 3.15	0.125	−18.12 ± 1.08	1.342 ± 0.09	26.05 ± 1.52	99.12
90	58.26 ± 4.08	0.127	−16.58 ± 1.35	1.343 ± 0.08	26.09 ± 1.64	98.99

Z-average (average droplet size); PI (polydispersity index); ZP (zeta potential); η (viscosity); RI (refractive index); SD (standard deviations) and the values are presented as mean ± SD, $n = 3$.

Table 6
Effects of optimized TQ SNEDDS (NE3) and TQ treatments on various biochemical parameters of serum in CCl_4-induced hepatotoxicity in rats.

Groups	AST (U/L)	ALT (U/L)	ALP (U/L)	γ-GGT (U/L)	Bilirubin (U/L)
I	78.47 ± 1.74	35.94 ± 0.65	97.75 ± 1.59	1.48 ± 0.05	0.73 ± 0.01
II	221.52 ± 4.54	95.29 ± 1.73	224.71 ± 5.54	3.70 ± 0.10	1.06 ± 0.02
III	93.95 ± 0.86	48.39 ± 1.55	112.02 ± 1.70	2.00 ± 0.03	0.71 ± 0.01
IV	184.95 ± 3.32	76.60 ± 1.19	172.53 ± 2.74	2.64 ± 0.03	0.92 ± 0.01
V	117.92 ± 2.35	57.63 ± 1.67	119.79 ± 1.51	1.99 ± 0.04	0.69 ± 0.02
Normal levels*	75.80 ± 1.04	33.94 ± 0.98	81.09 ± 1.80	1.26 ± 0.06	0.72 ± 0.01

Thymoquinone (TQ); self-nanoemulsifying drug delivery systems (SNEDDS); aspartate aminotransferase (AST); alanine aminotransferase (ALT); alkaline phosphatase (ALP); γ-glutamyl transpeptidase (γ-GGT); the values are presented as mean ± SEM, $n = 6$ and the values were taken from reference [37] (*).

Table 7
Effects of optimized TQ SNEDDS (NE3) and TQ treatments on various biochemical parameters of liver CCl_4-induced hepatotoxicity in rats.

Groups	CAT (U/mg)	GSH (nmol/mg)	MDA (nmol/mg)	SOD (U/mg)
I	46.62 ± 1.09	1.17 ± 0.02	3.26 ± 0.08	23.91 ± 1.13
II	19.56 ± 2.84	0.47 ± 0.02	10.13 ± 0.32	10.69 ± 0.35
III	43.71 ± 1.38	0.96 ± 0.01	4.11 ± 0.22	21.15 ± 0.73
IV	26.67 ± 0.94	0.73 ± 0.02	7.38 ± 0.18	16.35 ± 0.75
V	32.19 ± 0.82	0.88 ± 0.01	6.24 ± 0.15	19.57 ± 0.54
Normal levels*	45.09 ± 1.07	1.17 ± 0.02	3.20 ± 0.10	22.24 ± 0.41

Thymoquinone (TQ); self-nanoemulsifying drug delivery systems (SNEDDS); catalase (CAT); glutathione peroxidase (GSH); malondialdehyde (MDA); superoxide dismutase (SOD); the values are presented as mean ± SEM, $n = 6$ and the values were taken from reference [37] (*).

The RIs of all formulations were very close to RI of water (1.33), indicating o/w behavior of developed SNEDDS of TQ.

The higher values of %T indicated transparent behavior of all formulations investigated.

TEM analysis was performed in order to evaluate the surface morphology of optimized formulation NE3. The droplets of optimized formulation NE3 recorded by TEM were observed as spherical with nanometer range.

The higher release of TQ from the nanoemulsion (NE3) might be due to the small droplet which offered an overall large surface area in contact with the release medium for the delivery of TQ and hence causing immediate release of TQ. Overall, the percent cumulative amount of TQ released from the SNEDDS other than NE3 was almost comparable except NE5 and NE8 and variations was statistically significant ($P < 0.05$). This might be due to the fact that the NE5 and NE8 were having larger droplet size as compared to NE3.

On the basis of in vitro release, NE3 was found to delivered the highest amount of TQ, as well as it was found to have good physicochemical characteristics, like minimum droplet size (54.25 nm), smaller PI (0.125), and an optimum viscosity (25.14 cP), better RI (1.3349) and excellent transmittance (98.45%). Therefore, this optimized formulation was selected for further physicochemical stability and in vivo studies.

On physicochemical stability studies, the drug content remained and other physicochemical parameters were found slightly changed with the respect to the storage condition and time, but these changes were not significant statistically ($P < 0.05$).

The hepatoprotective effects of silymarin, TQ suspension and optimized SNEDDS NE3 were significant ($P < 0.05$). The normal values of serum AST, ALT, ALP, γ-GGT and bilirubin reported in rats are presented in Table 6 [37]. Standard silymarin suspension, TQ suspension and optimized SNEDDS NE3 groups were efficacious sufficiently to reduce AST, ALT and ALP levels in groups III, IV and V, respectively. However,

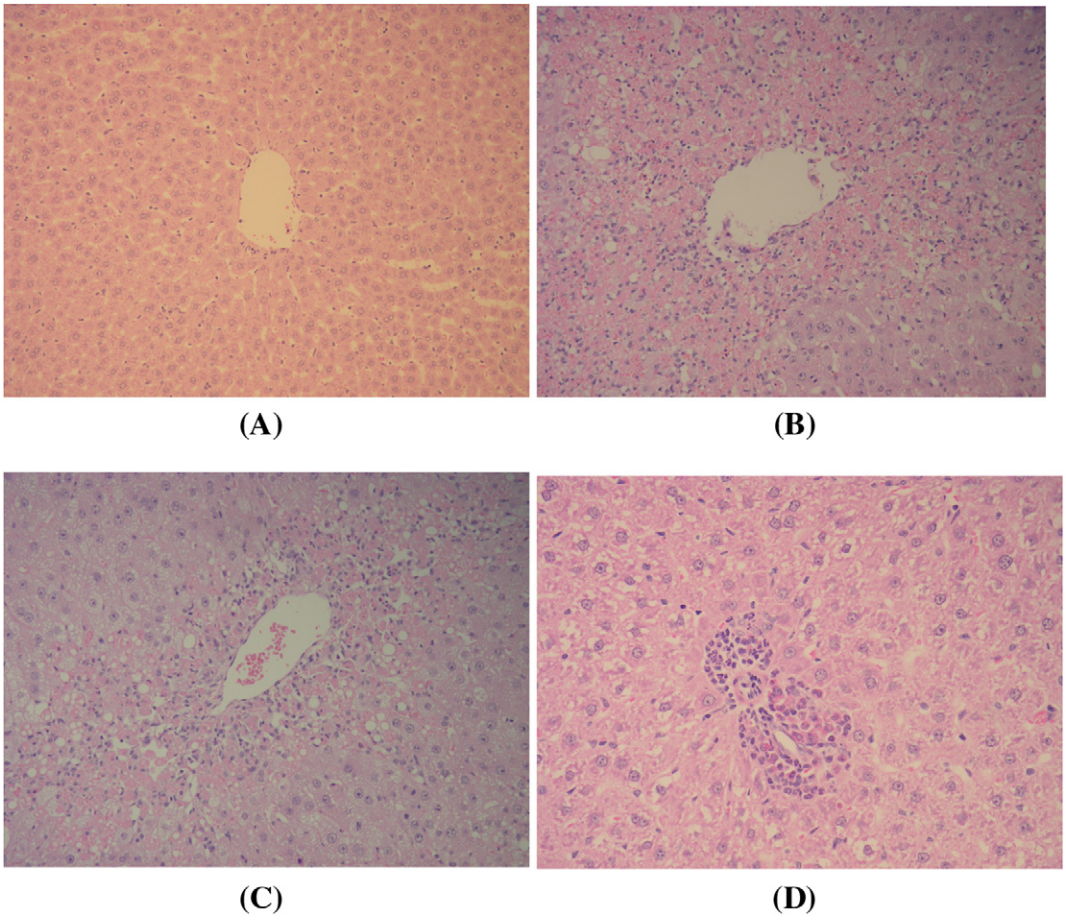

Fig. 6. Histopathological images of control (A), toxic control (B), TQ suspension (C) and optimized SNEDDS (NE3) (D); thymoquinone (TQ) and self-nanoemulsifying drug delivery systems (SNEDDS).

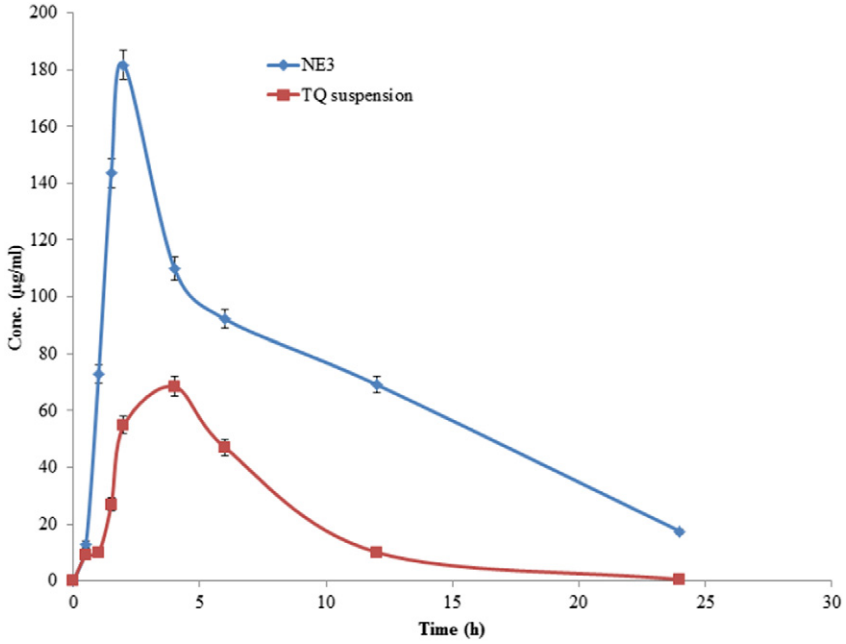

Fig. 7. Plasma concentration-time profile curve of TQ after oral administration of optimized SNEDDS NE3 and TQ suspension; thymoquinone (TQ); self-nanoemulsifying drug delivery systems (SNEDDS) and the values are presented as mean ± SEM, $n = 6$.

standard silymarin administration was the most efficacious in lowering AST, ALT and ALP levels. The administration of CCl_4 in group II animals resulted in marked increase in γ-GGT and bilirubin levels in comparison with control group animals. However, the treatment with standard silymarin suspension, TQ suspension and optimized SNEDDS NE3 caused marked reduction in γ-GGT and bilirubin levels in comparison with group II animals.

Various transaminases are present in the liver and their serum levels are increased further in patients suffering from liver diseases [15]. Serum AST, ALT and ALP are specific markers which are helpful in evaluation of hepatocellular damage. Measurement of serum bilirubin and ALP levels is one of the most widely investigated biochemical parameters in evaluation of hepatocellular injury [38,39]. In the present research work, a significant enhancement in the levels of bilirubin, AST, ALT and ALP were recorded. Significant hepatoprotective effects were recorded after oral administration of standard silymarin, TQ suspension and optimized SNEDSS NE3.

The normal values of liver CAT, GSH, MDA and SOD reported in rats are presented in Table 7 [37]. GSH shows an important role in cellular function of liver [15]. GSH is also known to detoxify to regulate gene expression, apoptosis and cellular transport of organic molecules. GSH scavenges free radicals and other reactive oxygen species effectively by enzymatic reactions. GSH is essential in order to maintain the reduced status of cells and tissues. However, its severe depletion could result in hepatic injury [15,40–42]. In the present work, the depletion of GSH level in the liver was recorded after administration of CCl_4 in comparison with control group rats. However, the oral administration of standard silymarin, TQ suspension and optimized SNEDDS NE3 reversed the hepatotoxic status by enhancing the GSH level in the liver.

The free radical of CCl_4 such as trichloromethyl is known to bind covalently with macromolecules and induces peroxidative degradation. This will ultimately results in production of MDA which damages membranes. The enhancement in MDA level in liver is responsible for enhanced lipid peroxidation which causes tissue damage and failure of antioxidant defense mechanisms [43–45]. In the present work, the level of MDA was significantly reduced after oral administration of standard silymarin, TQ suspension and optimized SNEDDS NE3. The levels of CAT and SOD were enhanced significantly after oral administration of standard silymarin, TQ suspension and optimized SNEDDS NE3 in comparison with toxic CCl4 group animals. The enhanced levels of SOD and CAT are beneficial for hepatoprotective effects.

Overall, these results indicated that optimized SNEDDS NE3 produced greater hepatoprotective effects against CCl_4-induced liver damage.

The results of biochemical examinations were supported by the histopathological investigations in rats.

Based on the best physicochemical parameters, in vitro drug release profile and better hepatoprotective effects, SNEDDS formulation NE3 was further evaluated for in vivo bioavailability and PK studies in rats. The bioavailability and PK studies were performed to quantify TQ contents in rat plasma after oral administration of optimized SNEDDS NE3 and TQ suspension. TQ is a poorly water soluble drug which exhibits poor oral bioavailability. High oral dose of TQ is required to achieve its therapeutic effects due to its poor bioavailability and poor aqueous solubility. Several nanotechnology-based approaches such as PNP, SLN, liposomes and NLC have been investigated in order to enhance the bioavailability of TQ [2,20–22]. Oral nanoemulsions/microemulsions and SNEDDS techniques have been investigated for the enhancement

Table 8
Pharmacokinetic parameters after oral administration of optimized TQ SNEDDS (NE3) and TQ suspension in rats.

Parameters	NE3	TQ suspension
C_{max} (µg/ml)	186.61 ± 1.24	68.49 ± 1.02
t_{max} (h)	2.00 ± 0.00	4.00 ± 0.00
K_e (h^{-1})	0.0519 ± 0.0006	0.1501 ± 0.0013
$AUMC_{0-t}$ (µg·h/ml)	13,683.00 ± 58.62	3012.20 ± 35.05
$AUMC_{0-\omega}$ (µg·h/ml)	28,318.00 ± 454.66	3122.60 ± 40.18
CL/F (ml/h)	5.00 ± 0.000	19.00 ± 0.000
VD (L)	71.00 ± 0.000	118.00 ± 0.001
$t_{1/2}$ (h)	13.58 ± 0.86	4.61 ± 0.55
AUC_{0-t} (µg·h/ml)	1657.70 ± 7.96	511.04 ± 5.31
$AUC_{0-\omega}$ (µg·h/ml)	1995.60 ± 12.69	514.64 ± 5.47
Relative bioavailability (%)	387.76	100

Thymoquinone (TQ); self-nanoemulsifying drug delivery systems (SNEDDS); maximum plasma concentration (C_{max}); time to reach maximum plasma concentration catalase (t_{max}); elimination rate constant (K_e); area under first moment curve from time 0 to t ($AUMC_{0-t}$); area under first moment curve from time 0 to ω ($AUMC_{0-\omega}$); area under curve from time 0 to t (AUC_{0-t}); area under curve from time 0 to ω ($AUC_{0-\omega}$); clearance (CL/F); half-life ($t_{1/2}$) and the values are presented as mean ± SEM, $n = 6$.

of bioavailability and therapeutic effects of drug molecules such as paclitaxel [46], ramirpril [25], saquinavir [47], curcumin [48], silymarin [14, 15] and carbamazepine [49]. Hence, in this work TQ was formulated into SNEDDS due to its poor aqueous solubility and bioavailability. By incorporating TQ in an internal oil phase of SNEDDS, the solubility was enhanced which results in enhanced oral bioavailability and hepatoprotective effects. TQ in SNEDDS showed significantly improvement in oral bioavailability as compared to TQ suspension. The enhanced oral bioavailability of TQ from SNEDDS was probably due to nanosized droplets of SNEDDS and the presence of solubilizers such as Capryol-90, Tween-20 and IPA in comparison with TQ suspension.

5. Conclusions

In order to enhance the hepatoprotective effects and bioavailability of a poorly water soluble bioactive compound TQ, various SNEDDS formulations were developed. Based on the best physicochemical parameters and in vitro drug release profile via dialysis membrane, SNEDDS formulation NE3 was further evaluated for in vivo hepatoprotective studies, bioavailability and PK studies in rats. Optimized SNEDDS NE3 showed significant hepatoprotective effects in comparison with TQ suspension. The oral administration of optimized SNEDDS NE3 showed significant improvement in absorption of TQ in comparison with TQ suspension. The relatively bioavailability of TQ was enhanced 3.87-fold by optimized SNEDDS NE3 in comparison with TQ suspension. The results of this research work indicated the potential of SNEDDS in enhancing bioavailability and therapeutic effects of natural bioactive compounds such as TQ.

Conflict of interest

"The authors report no conflict of interest associated with this manuscript".

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