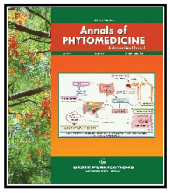
Annals of Phytomedicine 13(1): 1031-1045, 2024

DOI: http://dx.doi.org/10.54085/ap.2024.13.1.111

Annals of Phytomedicine: An International Journal

http://www.ukaazpublications.com/publications/index.php

Print ISSN : 2278-9839 Online ISSN : 2393-9885



Original Article : Open Access

Development, characterization and acute toxicity study of nanoemulsion containing thymoquinone, hesperetin along with vitamin C for improving oral bioavailability

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Article Info

Article history

Received 11 May 2024

Revised 22 June 2024

Accepted 21 June 2024

Published Online 30 June 2024

Keywords

Oral delivery

Nanoemulsion

Solubility

Bioavailability

In vitro release

Abstract

Thymoquinone, hesperetin and vitamin C are well known for their potential pharmacological properties such as antioxidant, anti-inflammatory, immunomodulatory and antidiabetic activity, *etc*. Poor oral bioavailability owning to limited aqueous solubility of thymoquinone and hesperetin limits its effective therapeutic delivery. Vitamin C is a water soluble drug but it is degraded by alteration of stomach pH leading to instability. The aim of this study is to develop a combined nanoformulation to enhance the oral bioavailability that can provide synergestic effect. The optimized nanoformulation (NE-17) was found to be clear and transparent with mean particle size 19.96 nm, 22.33 zeta potential, droplet size >60 nm, PDI 0.129, viscosity 9.62, refractive index 1.312 with 98.9% transmittance. *In vitro* release rate of NE-17 was found to be higher (86.97 ± 2.51%, 87.34 ± 2.29%, 89.24 ± 2.59) in simulated gastric fluid and (90.45 ± 4.009%, 89.81 ± 3.12%, 88.07 ± 2.23%) simulated intestinal fluid than conventional. Oral bioavailability of NE-17 was considered to be highly significant (***p*<0.001) with a C_{max} (15.23 ± 0.22 µg/ml, 14.83 ± 0.67 µg/ml, 14.33 ± 0.98 µg/ml), AUC_{0-24} (594.01 ± 0.23 µgh/ml, 598.32 ± 0.98 µgh/ml, 607.09 ± 0.23 µgh/ml,), AUC0-∞ (595.09 ± 0.87, 645.41 ± 0.91, 798.05 ± 0.43) and t½ (4.9 h, 4.7 h, 5.0 h), respectively. T_{max} was found to be (2.34 ± 0.34 h, 2.12 ± 0.12 h, 2.78 ± 0.12 h) that was highly significant (***p*<0.001) when compared with pure drug suspension. In acute toxicity, rats were subjected for a single oral dose (2000 mg/kg body weight) of NE-17 and monitored for 14 days. The results showed no sign of morbidity and mortality in any of the treated groups. Hematological, biochemical and histological assay was found to be normal when compared to control rats. This study result indicates that developed nanoemulsion was safe and improved the oral bioavailability as compared to conventional formulation by exhibited maximum *in vitro* and *in vivo* drug release.

1. Introduction

Oral drug delivery system is an expedient method as it increases conformance of a patient comparison to other route. However oral administration of poor aqueous soluble drugs has some disadvantages due to their low bioavailability, permeability, poor dissolution rate, and instability in gastrointestinal fluid (GIT) which obstacle their clinical application (Ye *et al.,* 2020). A drug substance must release drug particle to systemic circulation at an ideal place in order to produce the intended therapeutic response, mainly relies on the bioavailability of the drug. To overcome these constraints of hydrophobic drugs, nanotechnology based formulations have been invented such as nanocrystals, nanoparticles, phytosome, liposomes, nanoemulsion, and self emulsifying drug delivery system, *etc.* (Moksha, *et al.,* 2015; Alhakamy *et al.,* 2020; Singh *et al.,* 2018; Deka *et al.,* 2021; Jahan *et al.,* 2023; Aqeel *et al.,* 2022; Aqeel *et al.,* 2023). Nanoemulsion appeared to gained more attention across these techniques, due to their lower toxicity and potential to substantially increase the bioavailability. It is a thermodynamically stable isotropic system of a two immiscible liquid, size range is 10-100 nm. It forms two types of emulsion either water in oil (w/o) or oil in water (o/w) by using surfactant as well as cosurfactant. Nanoemulsion prepared by either low or high energy method. Low energy method is most preferable method as it required less energy and protect the active ingredient from physical destruction. Some drugs are thermolabile in nature; they can deteriorate by high energy method (Zhang *et al.,* 2014; Srivastava *et al.,* 2018). In this study, the nanoemulsion has been developed to prevent the drug from enzymatic degradations as well as enhanced bioavailability of poor water soluble drug, give better absorption, high permeability, dissolution rate and good stability. This unique attribute of nanoemulsion increase drugs therapeutic efficacy and render desired therapeutic effect (Alhamdany *et al.,* 2021). In this research article thymoquinone (TQ), hesperetin (HP) and vitamin C (vit. C) loaded combination nanoformulation was developed in order to implement a controlled released within therapeutic window and overcome the rest of limitations of hydrophobic and hydrophilic drug as well as reduces the side effect that could act as a functional food. Thymoquinone (Kamel *et al.,* 2011) and hesperetin both are hydrophobic phytochemicals used against wide variety of disease (Kyung et al., 2003). Thymoquinone obtained from plant *Nigella sativa*, belongs to the family Ranunculaceae (Jafri *et al.,* 2010). It has acquired substantial

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importance owning to its medicinal properties including immunomodulating, chemoprotective, anti-inflammatory, antioxidant, antidiabetic, *etc.* (Mahgoub *et al.,* 2003). Hesperetin (hesperetin 7-O-rutinoside) is the aglycon of hesperedin constitute from citrus fruits. It possess antioxidant, anti-inflammatory, anticancer, immunomodulating and hepatoprotective activity (Bai *et al.,* 2017). Vitamin C additionally referred to as ascorbic acid is an essential nutrient found in potatoes, citrus fruits, peppers, berries, *etc* (Qin *et al.,* 2019). Vitamin C showed several medicinal properties, *viz*: immunomodulating (Jeong *et al.,* 2011), antioxidant (Xiong *et al.,* 2017), anti-inflammatory (Raeeszadeh *et al.,* 2021), antidiabetic, *etc.* (Alper *et al.,* 2006). Therapeutic potential of both thymoquinone and hesperetin has impeded because of their poor bioavailability. Intestinal bacterial enzyme degraded C ring of hesperetin leads to fabricated degraded product by first pass metabolism and affect its bioavailability (Zeng *et al.,* 2021). Thymoquinone similar as hesperetin undergoes first pass metabolism and decreased bioavailability (Rathore *et al.,* 2022). Clinical use of vitamin C inspite of many health benefits is restricted due to its poor stability in the body (Raeeszadeh *et al.,* 2021) and physiological environment. Vitamin C consist of 4 hydroxyls and 1 lactone group which get oxidized and degraded when came into the contact of air, light, pH alteration and higher temperature produces dehydroascorbic acid product, which disrupted its bioavailability (Tian *et al.,* 2009; Gupta *et al.,* 2023). Several separate nanoformulations of thymoquinone (Harkaeh *et al.,* 2021; Rathore *et al.,* 2022), hesperetin (Maiti *et al.,* 2009; Zeng *et al.,* 2021) and vitamin C (Duarah *et al.,* 2017; Luo *et al.,* 2018; Raeeszadeh *et al.,* 2021) have been developed to increased their bioavailability, but no study have been found involving these phytochemicals together in one formulation (Shariq *et al.,* 2023; Kucuksayan *et al.,* 2021; Kumari *et al.,* 2023). For this reason thymoquinone, hesperetin and vitamin C in combination loaded in nanoemulsion by low energy method to offer an elegant approach for the delivery of water insoluble drug as well as preventing drug degradation of thymoquinone hesperetin and vitamin C. In addition formulation enhanced the oral bioavailability as well as improving the absorption of drug from intestine. Although, formulation may impart economically advantage and provide alternative to the current pharmacological treatment against disease due to presence of several activity.

2. Materials and Methods

2.1 Materials

Bioactive thumoquinone (98%), hesperetin (99%), methanol, and dialysis bag all were purchased from Sigma-Aldrich (St. Louis, Missouri). Vitamin C procured from Yarrow Chem Products. Propylene glycol caprylate (Sefsol 218) was delivered from Nikko Chemicals Co. Ltd. Surfactant and cosurfactant were bought from M/s Qualikam fine Chemicals Ltd. Isopropyl myristate (IPM), triacetin, castor oil, olive oil and distilled water were procured from Merck Pvt. Ltd. India. Each drug and chemicals were of analytical grade and suitable for oral use.

2.2 Method

2.2.1 Solibility of TQ, HP and vit. C

The screening was done to obtain maximum solubility of drug in different oils. Separately three ml sefsol 218, IPM, triacetin, olive, and castor oil, were taken in 5 ml of glass vial. An excess amount of TQ and HP were added and vigorously mixed by vortex mixture. Similarly maximum amount of vit. C was added in 3 ml of water until solution became saturated. Each sample was kept on isothermal shaker to attained equilibrium at a controlled temperature (25 ± 1.0°C) for 72 h (Akhtar *et al.,* 2016; Ahmad *et al.,* 2017). Subsequently mixture was centrifuge for 15 min at 3000 rpm. The supernatant was filtered by 0.45µm membrane filter and concentration was determined using high performance liquid chromatography (HPLC) at wavelength 254, 288 and 243 nm, respectively (Ahmad *et al.,* 2018; Yousaf *et al.,* 2016). Miscibility of surfactant (Tween 20, 60, 80) and cosurfactant (PEG 200, 400 and 600) were also evaluated separately and visually observed turbidity, phase separation or cracking (Rathore *et al.,* 2022).

2.2.2 Preparation of phase ternary diagram

Nanoemulsion was formulated by water titration method. In a different volume ratio (1:0, 1:1, 1:2, 2:1) surfactant and cosurfactant (S_{mix}) was combined. Oil and S_{mix} (each) ratio was mixed in different ratio from 1:9, 1:8, 1:7, 1:6, 1:5, 2:8 (1:4), 1:3.5, 1:3, 3:7 (1:2.3), 1:2, 4:6 (1:1.5), 5:5 (1:1), 6:4 (1:0.7), 7:3 (1:0.43), 8:2 (1:0.25), 9:1 (1:0.1) and titrated with water (Akhtar *et al.,* 2014; Akhtar *et al.,* 2016). Each 5 % drop wise water added to the oil and S_{mix} mixture for observation of any sign of turbidity. The end point, at which nanoemulsion region appeared, plotted in ternary phase diagram with one axis water phase, second S_{mix} and third represent oil phase by using software (Prism Ternary Diagram 1.0) Pune India (Patel *et al.,* 2013).

2.2.3 Thermodynamic stability evaluation

After achieving the nanoemulsion region selected formulation was undergo stability test including centrifugation, heating cooling and freeze thaw cycle. In heating cooling parameter for each temperature cycle (45°C and 0°C) formulation was incubated for 48 h. Centrifugation was done at 5000 rpm for 30 min. Formulation was kept on -20°C and 20°C temperature in freeze thaw cycle (Khani *et al.,* 2016; Ahmad *et al.,* 2018).

2.2.4 Dispersibility test

To evaluate the efficacy of nanoemulsion 1 ml of each formulation (those passed the thermodynamic stability test) was mixed to 500 ml of 0.1Normal HCl and distilled water in a USP dissolution apparatus type 2 with rotating paddle at 75 rpm. Temperature was set at 37 ± 0.5°C. Visual observation was made by below mentioned grading system.

Grade A: Formulation showing clear appearance with in 1 min.

Grade B: Slight clear emulsion formed.

Grade C: Slight milky emulsion produced within 2 min.

Grade D: Faintly greasy look dull white emulsion formed after 2 min.

Grade E: Larger oil globule appears on the formulation surface, represent poor emulsification.

2.2.5 Loading of drug in to formulation

Formulation that passed the Grade A system was chosen for the drug loading. 20 mg drug was dissolved using smallest concentration of S_{mix} and oil (10%, 15%, 20% and 25%).

2.2.6 Dynamic light scattering assessment (DLS)

The DLS approach which uses a zetasizer ZS 90 (Malvern instrument) was used to examine the particle size, zeta potential and polydispersibility index (PDI). For light scattering evaluation formulation was diluted in 2 ml distilled water and observed at 25°C.

2.2.7 Viscosity measurement

The viscosity of the formulation was measured to know the formulation is either o/w or w/o emulsion. Brookefiled viscometer with spindle 40 was used to determine viscosity at room temperature (Alhamdany *et al.,* 2021; Ahmad *et al.,* 2018).

2.2.8 Electrical conductivity, refractive index and percentage transmittance determination

Conductometer, CDM 230 was used to measure the formulation conductivity with a frequency 94Hz and cell constant of $0.11cm^{-1}$ at 25 ± 1°C. Refractivity was determined by applying one drop of nanoemulation to the slide of Abbemat refractometer at 25°C. The percentage transmittance was assessed by high performance liquid chromatography (HPLC).

2.2.9 Transmittance of electron microscopy (TEM)

Using TEM, droplet structure was examined (Zeiss jenna germany). On a 200 mesh film grid, a single drop of 100 fold diluted sample was applied and allowed to dry at room temperature. Uranyl acetate was used to stain the sample, which was then exposed to the electron microscope at 200 KV (Khani *et al.,* 2016*;* Suyal *et al.,* 2022).

2.3 *In vitro* drug release

In vitro release study was carried out in simulated intestinal (pH 6.8) and gastric (pH 1.2) fluid, 900 ml of each using dissolution apparatus-II at 50 rpm at a room temperature. Dialysis bag was filled with one ml of drug loaded nanoemulsion. One ml of sample was taken out at regular interval 0, 0.5, 1, 2.4, 6, 8, 10, 12, 14, 16, 18 till up to 24 h and a little amount of each simulated fluid was substituted. Sample was determined by using HPLC at 254, 288 and 243 nm. Drug release of formulation was compared with conventional formulation.

2.3.1 Stability study

In glass bottle optimized nanoemulsion was kept at three different temperature with relative humidity (25°C/60°RH, 40°C/65°RH, and 60°C/75°RH) and observed for 0, 30,60 and 90 days under stability chamber Thermolab, Central Drug Research Institute (CDRI) Lucknow. The reason behind performing stability is to check that how the quality of the drug varies under the influence of temperature, humidity and shelf-life (Rathore *et al.,* 2022; Akhtar *et al.,* 2014; Hosny *et al.,* 2020).

2.3.2 *In vivo* oral drug release

Sparague Dawley (SD) rats (175-200 g) were purchased from CDRI Lucknow after taking endorsement from Integral University, Institutional Animal Ethics Committee (IAEC), Lucknow with approval number (IU/IAEC/22/12). Pharmacokinetic study was conducted to find out whether the developed formulation may increase the oral bioavailability as compare to conventional drug. Rats were divided into 2 groups, six rats in each. Group 1: received 22.5 mg/kg TQ, HP and vit. C containing nanoemulsion (NE-17) was given orally. While Group 2: given 22.5 mg/kg TQ, HP and vit. c containing conventional suspension. The animals were anaesthetized by diethyl ether and blood sample withdrawn at different interval (0, 0.5, 1, 2, 4, 6, 8 up to 24 h) from retro orbital in anticoagulant tube. Afterward blood sample was centrifuge at 10000 rpm for 10 min to accomplished plasma, stored at -20°C bioavailability of formulation in plasma was done by HPLC (Chavhan *et al.,* 2013).

2.3.3 Acute oral toxicity study

According to organisation for economic co-operation and development (OECD), guideline acute toxicity study was performed in SD rats. Animals were divided in 2 groups: (1) experimental group (animals receiving a single dose of NE-17, at 2000 mg/kg, orally), and (2) normal control (animals received 2 ml of normal saline, orally). Each group consisting of 3 animals in each. All animals were observed for clinical signs of toxicity such as mucus membrane, fur, skin, eyes and behavioral changes. Other parameters like tremors, salivation, diarrhea, sleep and coma were also observed. Normal body weight and food as well as water consumption were observed for 14 days and compared with control group (Alotaibi *et al.,* 2018).

2.3.4 Collection of blood sample

After 14 days of observation, all rats of both group were anesthetized with ketamine xylazine (10 mg/kg, body weight) by intraperitoneal injection (I.P), blood sample was collected by retro-orbital plexus for estimation of hematological and biochemicals. Animals were euthanized by cervical dislocation, and vital organs were dissected out for macroscopic/ microscopic evaluation.

2.3.5 Hematological and biochemical analyses

Using an automated haematology analyzer (Sysmex-XT-1800, Norderstedt, Germany), haematological parameters were examined. All parameters that were assessed included: white blood cells (WBC), platelet count, mean corpuscular volume (MCV), haemoglobin concentration (Hb), hematocrit, and red blood cells (RBC), *etc*. Aspartate aminotransferase (AST), alkaline phosphatase (ALP), alanine transaminase (ALT) were assessed for liver function. Albumin, globulin, blood urea nitrogen (BUN) and serum creatinine levels were measured for renal function.

2.3.6 Histopathological analysis

All vital organs, *viz*: brain, liver, spleen, heart, and kidney of all animals were collected, and weighed. Relative organ weight of all rats were calculated and kept in 10% buffered formalin for histopathology evaluation (Zhao *et al.,* 2013).

2.3.7 Statistical analysis

All value were expressed mean ± SD for statistical significance by analysis of variance (ANNOVA) followed by Brown forsythe test using software graph pad prism.

3. Results

3.1 Solubility study

This study was performed to achieve pertinent oil, surfactant and cosurfactant as components for the development of nanoemulsion. The maximum solubility of both TQ and hesperetin were found in sefsol 218 (153.6 mg/ml, 144.9 mg/ml) as compared to sefsol 228

(84.51, 108.2 mg/ml), isopropyl myristate (77.43, 62.82 mg/ml), olive oil (21.88, 18.36 mg/ml), triacetin (48.87, 43.03 mg/ml), followed by castor oil (32.70, 25.98 mg/ml) (Figure 1). Vitamin C solubility in water was found to be 230.2 mg/ml. Surfactant (Tween 80) and co surfactant (PEG 400) was found to be more miscible than Tween 20, 60, PEG 200 and 600. Therefore, Sefsol 218 as oil phases tween 80 and PEG 400 preferred as surfactant and cosurfactant for formulating nanoemulsion.

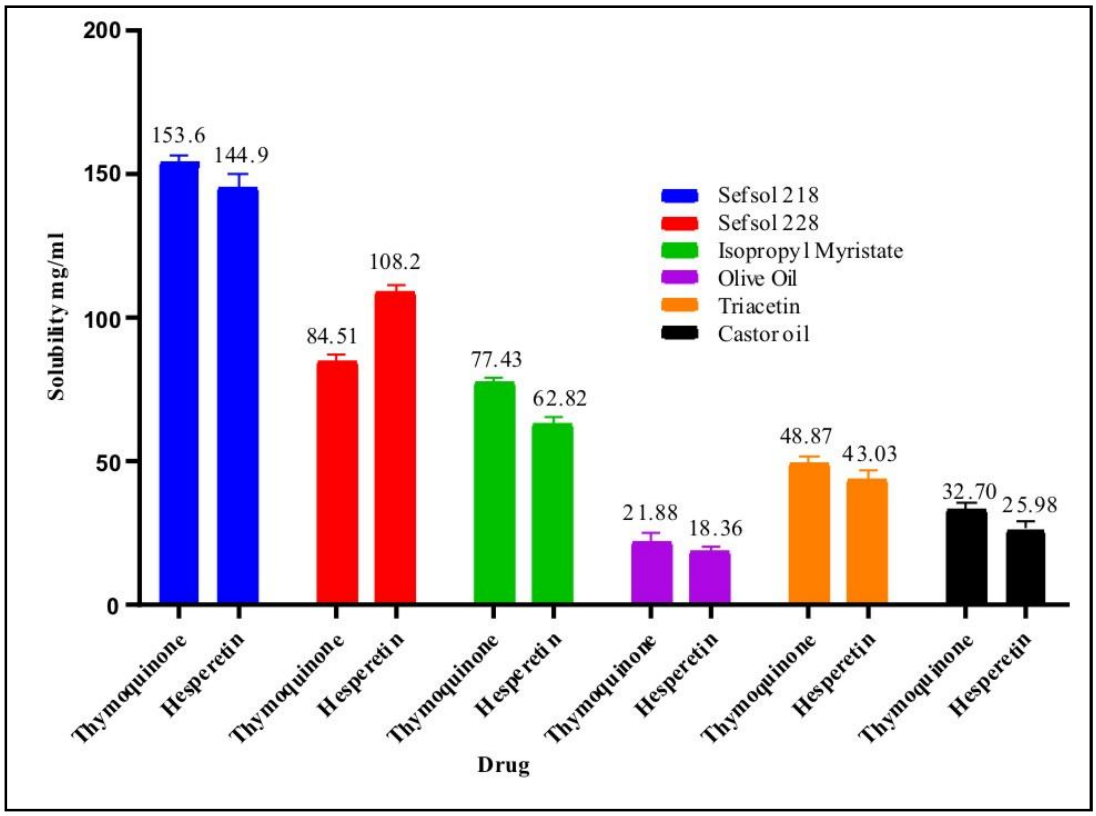


Figure 1: Solubility of TQ and HP.

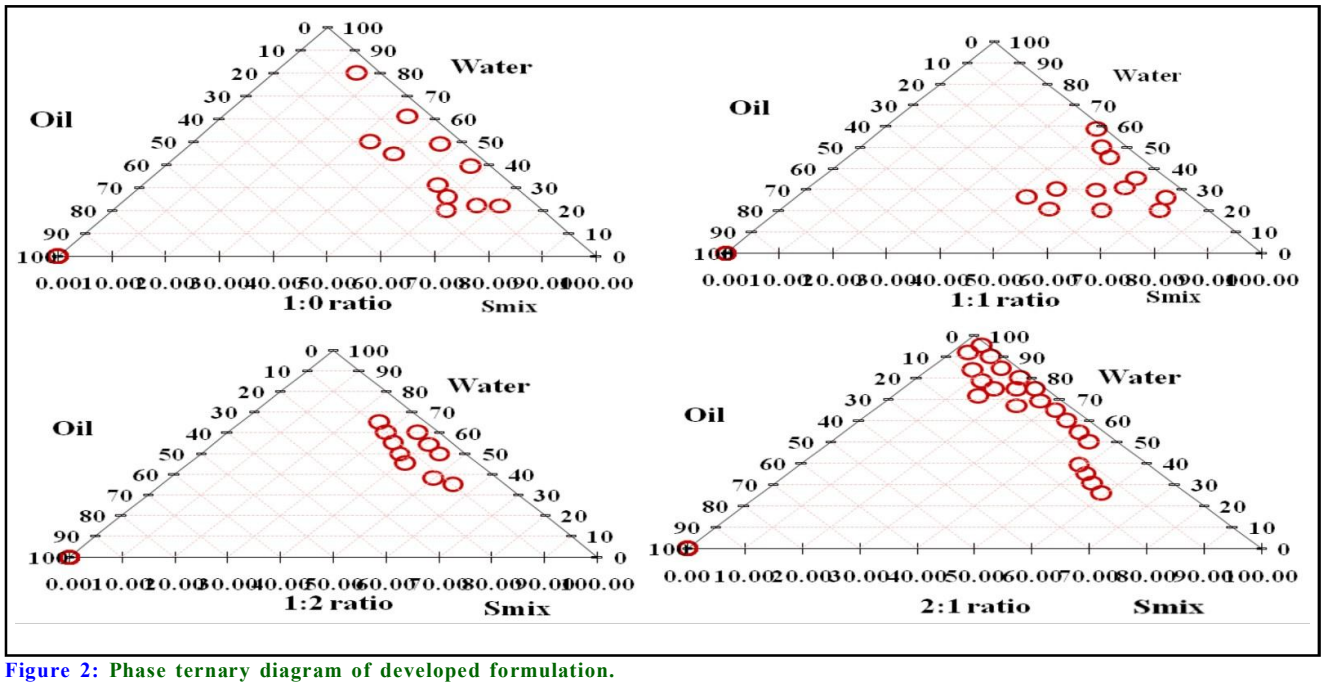


Figure 2: Phase ternary diagram of developed formulation.

3.2 Phase ternary diagram

Nanoemulsion region comprises sefsol 218 as oil phase tween 80 and PEG 400 as S_{mix}, titrated with water as aqueous phase was achieved by construction of ternary phase diagram (Figure 2).100 ml stock solution of Tween 80 and PEG 400 (S_{mix}) in each different volume ratio (1:0, 1:1, 1:2, 2:1 *v/v*) were prepared. Sefsol 218 and each S_{mix} was mixed in different volume ratio from 1:9 to 9:1 and titrated with water between 5-95% at every 5 min interval. Cosurfactant with hydrophilic and lipophillic nature is required to decrease the surface tension. Co surfactant break up liquid crystalline states, developed when the surfactant film is too hard, offer ability to flow easily by penetrating into the surfactant monolayer. S_{mix} in 2:1 ratio provided maximum nanoemulsion region (Bhosale *et al.,* 2016; Tubesha *et al.,* 2013; Ahmad *et al.,* 2018).

3.3 Physical stress and dispersibilty evaluation

Physical stress and dispersibility study was perfomed for optimized nanoformulation (Table 1). Selected nanoemulsion which remain stable in heating/cooling test, passed to the freeze thaw cycle than to centrifugation. In macroemulsion the interfacial energy exceeds entropy. A thermodynamically stable nanoemulsion forms as a result of free energy and dispersion entropy. Small droplet size thermally induced by temperature quenching, favour brownian movement, effectively disburses the particle to an optimum centrifugation with total free energy. Formulations that have passed these entire tests were further subjected to dispersibility test and rest of formulation discarded due to phase separation (Rocha *et al.,* 2022). Distilled water and 0.1N HCl (GI fluid) were used as a dispersion media in dispersibility test to determine stability of formulation after oral administration. Prerequisite for a robust nanoemulsion, is formulation containing droplets in nanosize and indefinite dilution. It is in view of the fact that GI fluid will cause the surfactant to desorb gradually at interface of the globule. Formulation that passed grade A was considered for further evaluation and grade B, C, D and E were discarded.

Table 1: Thermodynamic stability and dispersibility test of nanoemulsion

Formulation code	S_{mix} ratio	% Oil	% S_{mix}	% H_2O	Thermodynamic stability tests					Inference
					Heating and cooling cycle	Freeze thaw cycle	Centri-fugation	Dispersibility		
								0.1 N HCl	H_2O	
NE1	1:0	9.09	36.36	54.55	P	X	-	-	-	Failed
NE2		3.00	7.00	90.00	P	P	P	A	A	**Passed**
NE3		4.44	15.56	80.00	P	P	X	A	C	Failed
NE4		5.00	35.00	60.00	X	-	-	-	-	Failed
NE5		3.88	31.07	65.05	X	-	-	-	-	Failed
NE6	1:1	7.02	28.07	64.91	P	P	X	C	C	Failed
NE7		8.33	16.67	75.00	X	-	-	-	-	Failed
NE8		10.00	30.00	60.00	P	X	-	B	B	Failed
NE9		3.00	23.00	75.00	P	P	X	C	C	Failed
NE10	1:2	4.55	40.91	54.55	P	P	P	B	B	Failed
NE11		4.00	16.00	80.00	P	P	P	A	A	**Passed**
NE12		10.00	30.00	60.00	P	P	X	B	B	Failed
NE13	2:1	3.08	12.31	84.62	P	P	P	B	B	Failed
NE14		1.43	3.33	95.24	P	P	P	A	A	**Passed**
NE15		5.00	10.00	85.00	P	P	P	C	C	Failed
NE16		5.00	15.00	80.00	X	-	-	-	-	Failed
NE17		2.22	7.78	90.00	P	P	P	A	A	**Passed**
NE18		8.00	12.00	80.00	P	P	P	B	B	Failed
NE19		4.00	20.00	75.00	P	P	X	B	B	Failed
NE20		5.00	30.00	65.00	P	X	-	B	B	Failed

Nanoemulsion (NE), P alphabet denotes to pass while letter X denote to fail.

3.4 Fabrication of drug loaded formulation

All three drugs (TQ, HP and vit. C) were added in the formulation which passed the dispersibility test.

3.5 Charecterization of TQ, HP and vit. C loaded nanoformulation

3.6 Visual observation

The nanoemulsion was found clear liquid as compare to macro emulsion which was cloudy/white in color (Figure 3).

3.7 DLS and TEM evaluation

NE-17 has shown the best control of zeta potential, small particle size, and less than 60 nm droplet size compared to NE-02, NE-11 and NE-14 (Figure 4). Difference in droplet size may be attributing to variation in the penetration of sefsol oil into the hydrophobic region of S_{mix}. Low PDI value of NE-17 indicates uniformity of droplet size and homogenecity (Table 2). Therefore, NE-17 nanoformulation was selected for further study.

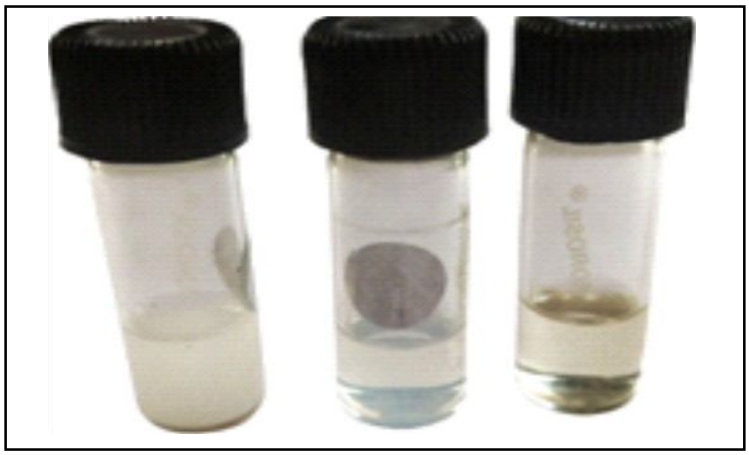


Figure 3: Observation of nanoemulsion vs macro emulsion.

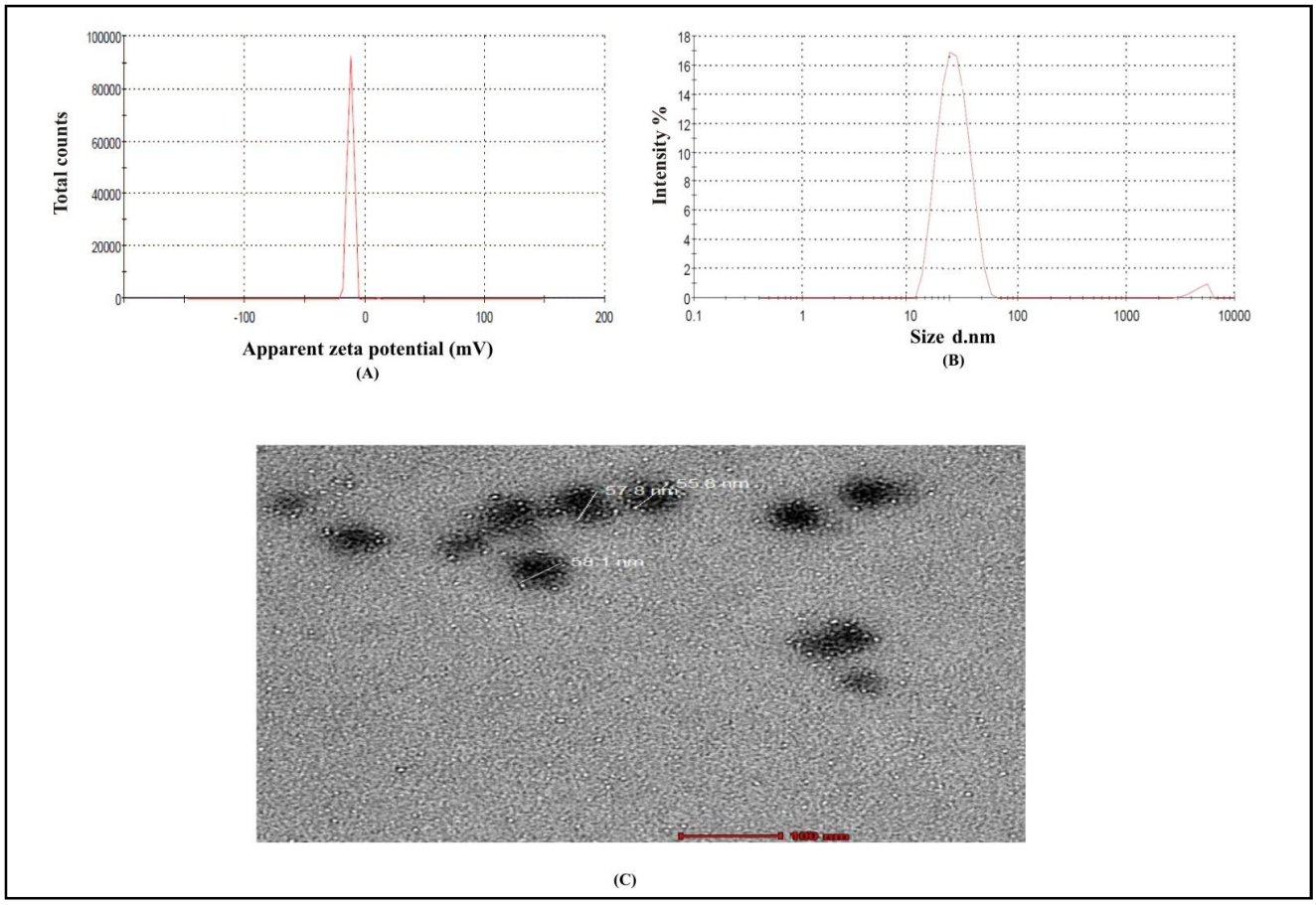


Figure 4: NE-17 loaded nanoemulsion (A) zeta potential (B) particle size (C) TEM (droplet size).

Table 2: Charecterization of optimized nanoformulation

F. code	Droplet size (nm)	Polydispersity index (PDI)	Zeta potential (mV)	Viscosity (cPs)	Conductivity µS/cm	Refractive index (RI)	% transmittace
NE-02	93.94 ± 3.185	0.298 ± 0.004	-10.867 ± 0.878	11.32 ± 0.031	470.99 ± 1.079	1.341 ± 0.004	96.01 ± 0.010
NE-11	82.78 ± 3.052	0.488 ± 0.382	-14.467 ± 4.843	11.89 ± 0.066	467.35 ± 1.068	1.376 ± 0.002	97.07 ± 0.543
NE-14	72.73 ± 2.650	0.173 ± 0.034	-17.01 ± 3.228	10.57 ± 0.522	475.37 ± 0.796	1.337 ± 0.006	97.12 ± 0.962
NE-17	57.23 ± 1.250	0.129 ± 0.105	-22.33 ± 1.060	9.62 ± 0.755	489.92 ± 0.456	1.312 ± 0.013	98.92 ± 0.612

Nanoemulsion (NE), Formulation code (F. code)

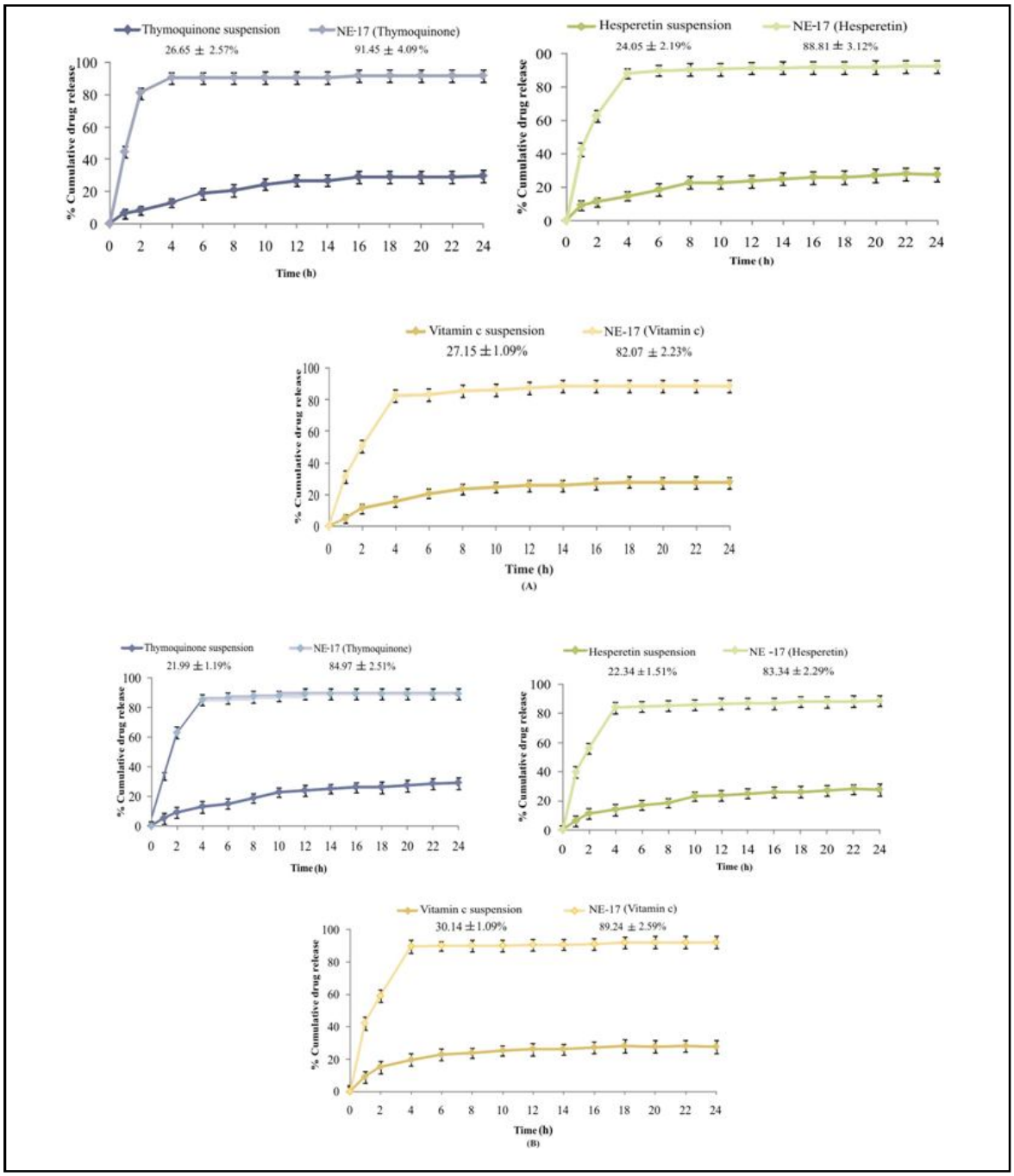


Figure 5: ***In vitro*** **drug release of developed nanoformulation in simulated gastric fluid (A) and intestinal fluid (B).**

3.8 *In vitro* drug release

The *in vitro* drug release of NE-17 nanoemulsion was evaluated and the concentration was calculated by plotting a graph between time and cumulative percentage release (Figure 5). NE-17 nanoemulsion showed maximum drug release with increased dissolution rate (86.97 ± 2.51%, 87.34 ± 2.29%, 89.24 ± 2.59) in simulated gastric fluid in 24 h and (90.45 ± 4.009%, 89.81 ± 3.12%, 88.07 ± 2.23%) in simulated intestinal fluid first 4 h as compared to conventional formulation (21.99 ± 1.19%, 22.34 ± 1.51%, 30.14 ± 1.09%) in GI fluid while (26.65 ± 2.57%, 24.05 ± 4.09%, 27.15 ± 1.09%) in intestine fluid. The conventional formulation exhibited very low drug release after 6 h is may be due to larger particle size of drug. The fastest release during first 4 h is because of larger surface area and small particle or globule size.

3.9 Accelerated stability evaluation

Accelerated stability parameter was performed for NE-17 nanoformulation by ICH guideline. As shown in Table 3, the average particle size distribution, droplet size, PDI, refractive index, viscosity and % transmittance did not change significantly at different temperature and humidity over a period of 3 months. At 90 days, a very slight changes in droplet size was found with decreasing transparency. The appearance of NE-17 loaded nanoemulsion was found to be clear and transparent, no precipitate was observed within 90 days. The smaller the droplet size having more stability thus does not cause any flocculation and aggregation.

Table 3: Accelerated stability of NE-17

Condition T (ºC)/RH%	Duration (days)	Droplet size (nm)	PDI	Zeta potential (mV)	Viscosity (cPs)	Refractive index (RI)	% transmittance
25 ± 2/60 ± 5	30	57.23 ± 1.250	0.169 ± 0.012	−22.33 ± 1.060	9.62 ± 0.755	1.312 ± 0.013	98.62 ± 0.612
	60	59.01 ± 0.111	0.173 ± 0.143	−21.06 ± 0.516	10.02 ± 0.587	1.320 ± 0.190	98.12 ± 1.120
	90	61.21 ± 1.321	0.179 ± 0.341	−21.01 ± 1.078	10.14 ± 1.102	1.329 ± 1.016	98.02 ± 0.980
40 ± 2/65 ± 5	30	57.10 ± 0.365	0.170 ± 1.024	−21.12 ± 1.008	9.89 ± 0.074	1.318 ± 0.260	97.72 ± 0.732
	60	58.22 ± 0.005	0.177 ± 0.074	−20.41 ± 0.896	10.10 ± 1.365	1.326 ± 0.073	97.32 ± 0.412
	90	61.56 ± 1.710	0.185 ± 0.014	−19.35 ± 2.054	10.19 ± 0.222	1.349 ± 1.780	97.22 ± 0.962
60 ± 2/75 ± 5	30	58.35 ± 1.240	0.173 ± 1.056	−21.62 ± 0.142	9.87 ± 1.840	1.332 ± 0.086	97.09 ± 1.750
	60	59.88 ± 2.065	0.182 ± 0.168	−20.22 ± 1.490	10.27 ± 0.072	1.338 ± 1.206	96.62 ± 0.092
	90	61.47 ± 0.555	0.187 ± 0.019	−19.99 ± 2.150	10.39 ± 1.406	1.370 ± 0.006	96.59 ± 1.012

Temperature (T), Relative humidity (RH).

3.10 Pharmacokinetic study

In-vivo pharmacokinetic parameters of all three drugs loaded (NE-17) nanoemulsion was performed in order to determine whether the NE-17 nanoemulsion could improve the oral bioavailability. NE-17 exhibited maximum drug concentration (15.23 ± 0.22 µg/ml, 14.83 ± 0.67 µg/ml, 14.33 ± 0.98 µg/ml), *i.e.* (**p<0.001) as compared to conventional suspension. The $AUC_{0\text{-}24}$ (594.01 ± 0.23 µgh/ml, 598.32 ± 0.98 µgh/ml, 607.09 ± 0.23 µgh/ml,) $AUC_{0\text{-}\infty}$ (595.09 ± 0.87, 645.41 ± 0.91, 798.05 ± 0.43) and t½ (4.9 h, 4.7 h, 5.0 h) was found to be highly significant than suspension (*p<0.05) Figure 6. It is believed that a low T_{max} value contributes to the drug's fast action in the body. NE-17 nanoformulation showed lowest T_{max} (2.34 ± 0.34 h, 2.12 ± 0.12 h, 2.78 ± 0.12 h) than conventional. The improvement in bioavailability may be because of oil that increases the drug's solubility (Table 4). In addition, the membrane permeability could have changed as a consequence of the surfactant and cosurfactant present in the nanoemulsion system, allowing the concentration to reach its maximum in the shortest period of time. As a result, the current study demonstrated improved absorption and a faster onset of action for the oral bioavailability of drug.

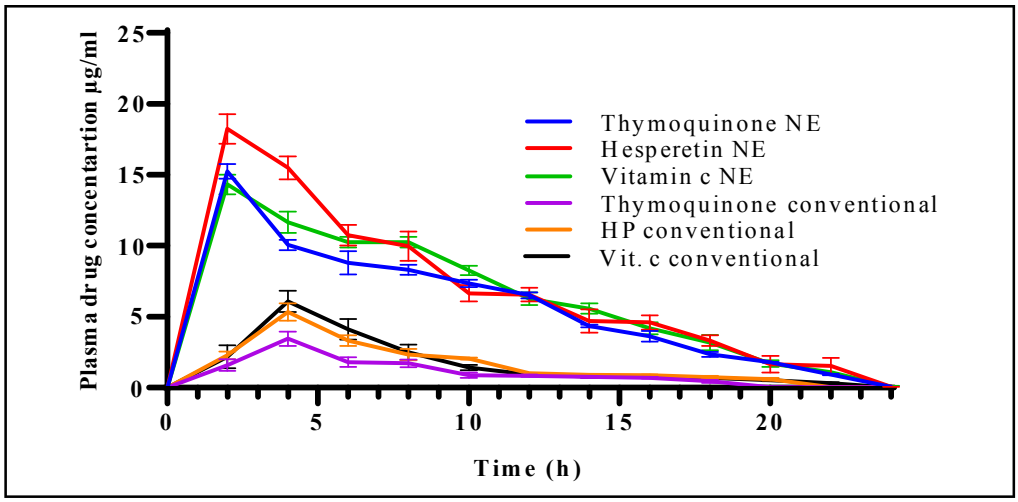


Figure 6: Plasma concentration curve after oral administration of NE-17 and conventional formulation in SD rats.

Table 4: Pharmacokinetic comparison between NE-17 and conventional

Formulation (NE-17)	T_{max} (h)	C_{max} (µg/ml)	AUC_{0-24} (µgh/ml)	$AUC_{0-\infty}$	t ½ (h)
TQ conventional	4.91 ± 0.16*	2.47 ± 0.49*	95.6 ± 0.16*	99.3 ± 0.32*	2 .6*
TQ NE	2.34 ± 0.34**	15.23 ± 0.22**	594.01 ± 0.23**	595.09 ± 0.87**	4.9**
Hesperetin conventional	4.52 ± 0.75*	5.31 ± 0.60*	98.7 ± 0.62*	105.7 ± 0.49*	1.5 *
Hesperetin NE	2.12 ± 0.12**	14.83 ± 0.67**	598.32 ± 0.98**	645.41 ± 0.91**	4.7 **
Vitamin C conventional	4.78 ± 0.36*	6.08 ± 0.75*	103.9 ± 0.42*	114.3 ± 0.84*	3.0 *
Vitamin C NE	2.78 ± 0.12**	14.33 ± 0.98**	607.09 ± 0.23**	798.05 ± 0.43**	5.0**

Nanoemulsion (NE). All values are expressed as Mean ± SD. **p<0.001= highly significant, * p<0.05= significant.

3.11 Acute toxicity study

3.12 Clinical observations

No abnormal behavior and mortality was found in experimental group during 14 days of period. Physical observations of the treated rats revealed that not one of them had any toxicological symptoms, including changes in their skin, fur, eyes, mucous membranes, tremors, salivation, diarrhoea, sleeplessness, or coma. In comparison to the control group, increase in body weight was seen (Table 5).

Table 5: Behavioral parameters

Parameters	30 min		4 h		24 h		48 h		7 days		14 days	
	NC	NE	NC	NE	NC	NE	NC	NE	NC	NE	NC	NE
Skin and Fur	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Mucous membrane	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Eyes	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Salivation	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Sleep	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
Lethargy	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Convulsion	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Coma	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Tremors	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Diarrhea	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Morbidity	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Mortality	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF

NC-Normal control, NE-Nanoemulsion, NR-Normal, NF-Not Found.

3.13 Body weight, food and water consumption

NE-17 treated and control rats body weights results are shown in (Table 6). After the first and 2 weeks of the study, the body weight was not increased significantly and found to be 0.91 and 0.74 of normal control and NE-17, respectively. There were slight increases in body weight of NE-17 and control rats. The food and water consumption of the treated rats were also not found significant when compared to the control rats (Table 7). Therefore slight increase in body weights during the study period may indicate the improvement in the nutritional state of the animals.

Table 6: Body Weight of treated group during 14 days

Groups	Body weight (g)			Weight gain (%)
	(day-0)	(day-7)	(day-14)	
NC	174.1 ± 0.9	174.3 ± 0.72	175.7 ± 0.37	0.91
NE	175.1 ± 1.05	175.9 ± 0.95	176.4 ± 0.17	0.74

Table 7: Food and water consumption of treated group during 14 days

Groups	Water intake (ml/day)	Food intake (g/day)
NC	27.09 ± 1.73	22.48 ± 1.37
NE	26.99 ± 1.37^{ns}	23.07 ± 1.58^{ns}

Results were expressed as mean ± standard deviation, ns = not significant

3.14 Relative organ weight

Figure 7 represent the organ weight (liver, brain, heart, kidneys, and spleen) in relation to body weight of the rat used in the acute toxicity study. The organ weight-to-body weight ratio did not alter following a single dose NE-17 (2000 mg/kg) compared to the control group.

3.15 Hematology parameter

Haematological parameters were assessed in this study to evaluate NE-17's toxicity in SD rats. Table 8 illustrates the hematological parameters of the experimental and control rats. None of the experimental group's rats showed changes in hematological profiles.

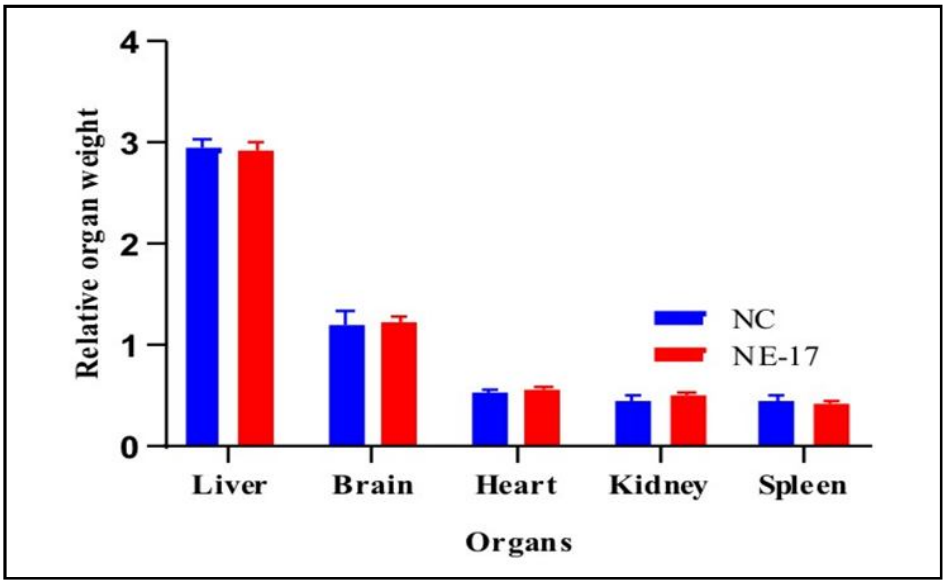


Figure 7: Effects of NE-17 and normal control treated rats on relative organ weights during acute toxicity study.

Table 8: Hematology parameter of normal control and NE-17

Parameters	NC	NE-17
TWBC (10^9/l)	2.1 ± 0.9	2.01 ± 0.05
Neutrophils (%)	39 ± 0.15	38 ± 0.04
Basophils (%)	00 ± 00	00 ± 00
Eosinophils (%)	02 ± 0.5	03 ± 0.9
Lymphocytes (%)	52 ± 0.11	53 ± 0.07
Monocytes (%)	07 ± 0.75	06 ± 0.32
Platelet (×109 /l)	432 ± 0.25	436 ± 0.12
RBC (×1012/l)	4.81 ± 0.21	5.12 ± 0.71
Hb (g/dl)	14.1 ± 1.01	15.3 ± 0.25
PCV%	30.2 ± 0.01	30.6 ± 0.04
M.C.V. (Fl)	81.4 ± 0.3	82.9 ± 0.1
M.C.H (Pg)	20.4 ± 0.4	19.5 ± 0.3
M.C.H.C (g/dl)	30.7 ± 1.01	31.5 ± 0.08
RDW-CV%	13.4 ± 0.5	14 ± 0.2

3.16 Liver and kidney functions tests

Liver and kidney function tests are important parameters in determining the safety of drug (Meenakshi *et al.,* 2012). Liver enzymes: AST, ALP, ALT, and kidney function test: BUN, creatinine, albumin and globulin level did not increase in both groups. Increases levels of these biomarkers are reliable indices of liver and kidney toxicity that altered integrity of cellular membrane and cell death. AST and ALT are the biomarkers of liver function tests. However, AST is not a highly specific indicator for liver injury because it is found in other tissues like the heart, muscles, kidney, and brain, unlike ALT which is found largely in the liver (Mariappan *et al.,* 2012). Hence, the acute oral toxicity study of NE-17 indicating that it does not have significant toxic effects on the liver and kidneys (Figure 8).

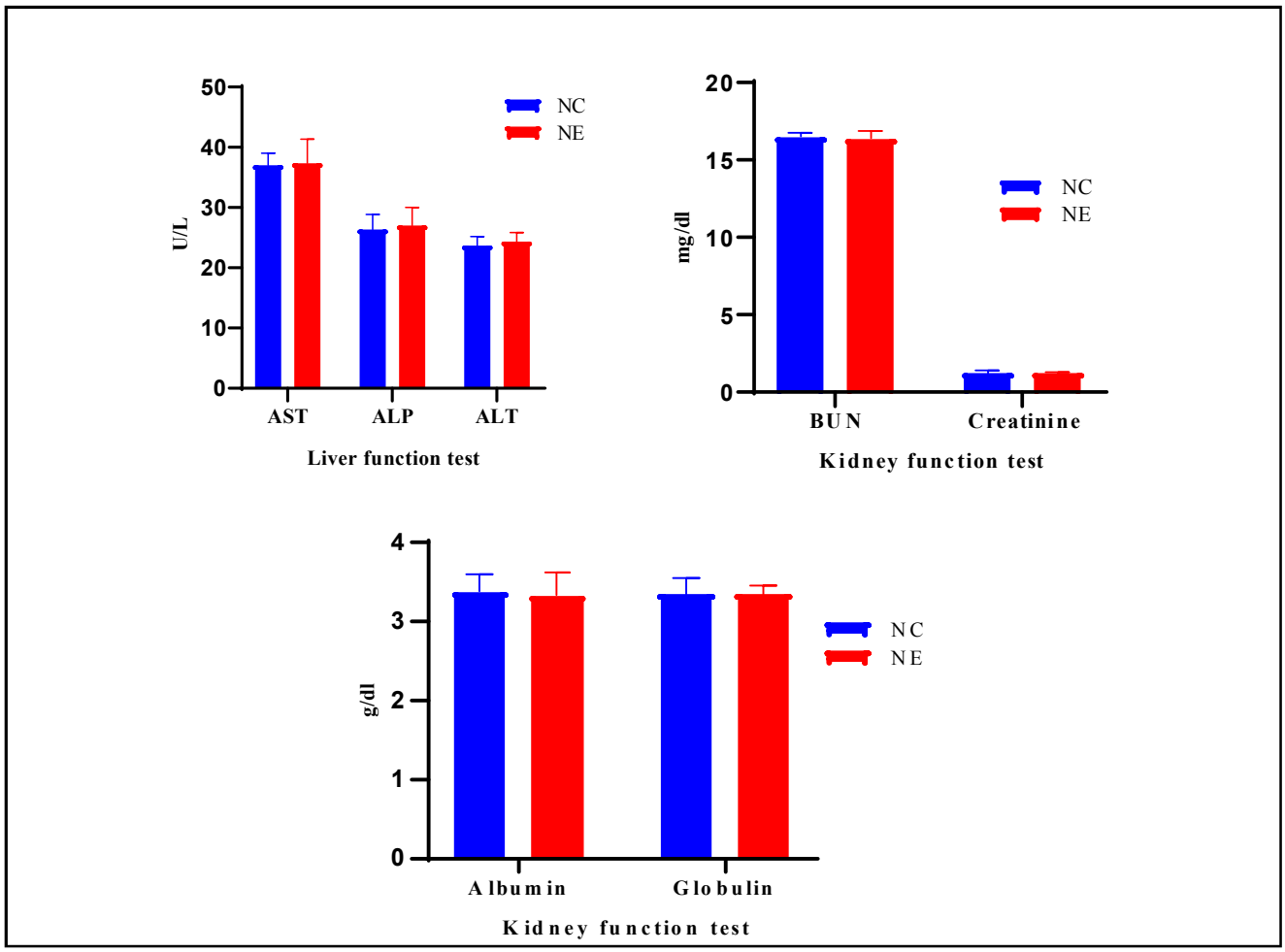
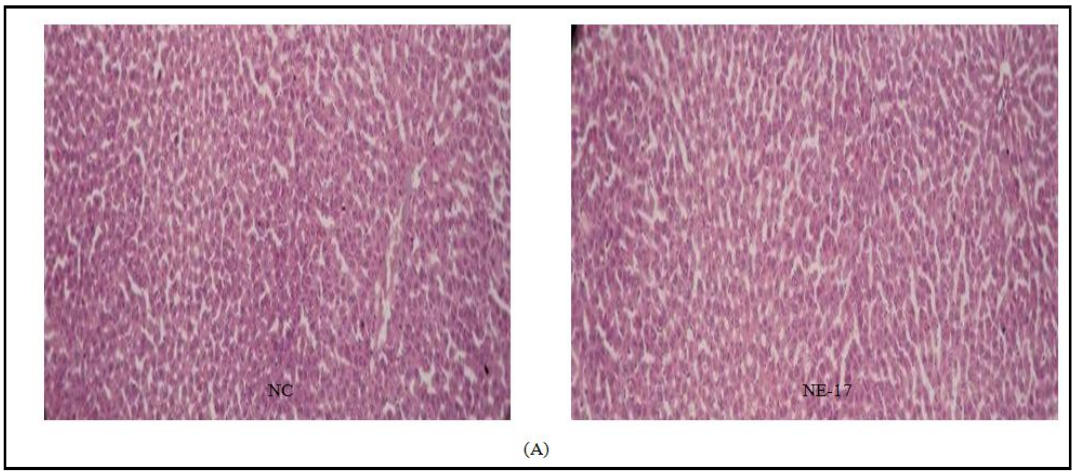


Figure 8: Effects of oral administration of NE-17 treated group and normal control on liver and kidney function tests. Values are expressed as mean ± SD of 3 rats per group.

3.17 Histopathology

NE-17 treated rats showed normal liver structure with portal triad surrounded by the hepatocytes and narrow sinusoids open freely into the portal vein when compared to control group. Heart section showed individual cardiac muscle cells arranged in bundle normally and kidney showed normal glomeruli and tubular epithelial cell in in both group. No alterations were observed in the brain in both groups. Spleen section showed normal histomorphology of red and white pulp of spleen in NC and NE-17 treated group. Hence rats of both groups did not cause any evidence of tissue damage of organ as compared to control groups (Figure 9). Therefore, the results suggest that the administration of 2000 mg/kg NE-17 was not toxic after an acute exposure.



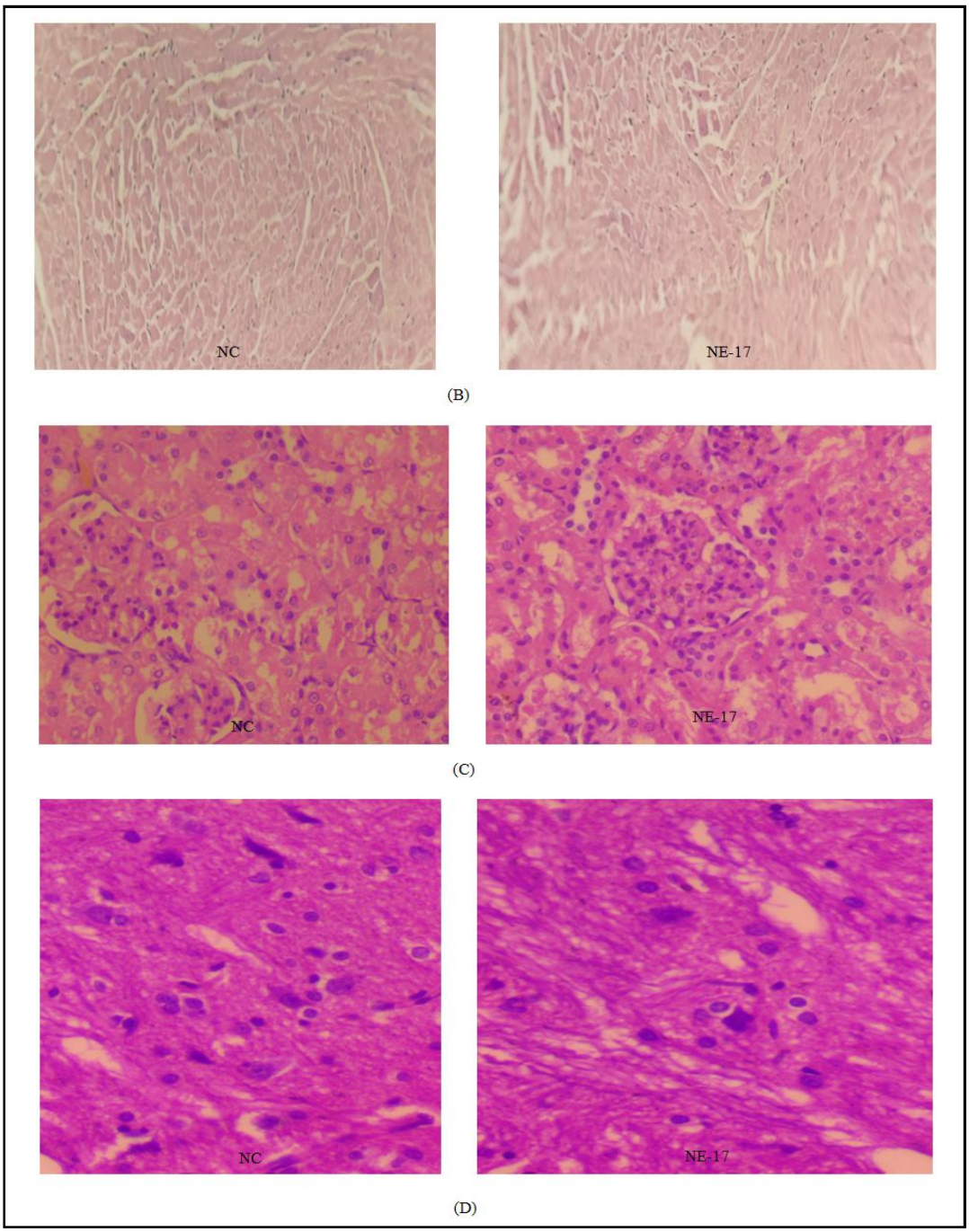


Figure 9: Histopathological evaluation, of liver (A), heart (B), kidney (C), brain (D), spleen (E) after NE-17 treatment.

4. Discussion

Increasing bioavailability and solubility of the poorly soluble drug is the main reason for the designing of new delivery system that enhances the effectiveness of already existing drug. Lipid based drug delivery system design in such a manner that gives an advanced approach to increase drug solubilization in gastrointestinal tract, consequently improve the oral bioavailability of poorly water soluble drug. Moreover, it also provides a protective carrier for preventing drug degradation. Drug containing nanoformulation was prepared by low energy (water titration) method which demonstrated good stability over 90 days and higher solubility. The cumulative % drug release of NE-17 nanoformulation was significantly increased in gastric as well as intestinal fluid when compare to suspension. According to *in vivo* pharmacokinetic study NE-17 (TQ 6.21, HP 6.06, vit. C) 5.84 fold higher than conventional nanoemulsion enhanced the drug absorption and improving oral bioavailability. In summary, the drug loaded nanoemulsion might enhance the bioavailability of drug by raising drug solubility and drug intestinal permeability. Although,

improved bioavailability would allow reducing the dose as well as side effects. The results of the acute toxicity study revealed that the experimental animals did not exhibit any behavioural changes, toxicological signs, or other negative effects when treating with NE-17 formulation orally for a period of 14 days. This finding suggests that NE-17 formulation has significant potential as a novel sustainable release medication against the disease that does not have acute toxic side effects. Therefore, the combination of thymoquinone, hesperetin and vitamin C in nanoformulation is of great importance owning to the potential of generating a new option as an effective adjuvant therapy in various diseases as two or more drug together can act synergistically.

5. Conclusion

NE-17 nanoformulation results indicate sustained release property for a longer duration and a higher relative bioavailability. These properties may be useful in preventing drug degradation and resolving issues with poorly water soluble drugs. 2000 mg/kg dose of NE-17 was found to be safe in rats and does not showed any toxic effect. The results provide safety information on combination nanoformulation of NE-17, which would further help researchers on its therapeutic use as a functional food.

Acknowledgements

The authors express their deep sense of gratitude to Faculty of Pharmacy, Integral University, for their encouragement and support. The author also thanks Dean, Faculty of Pharmacy, and Integral University for providing valuable suggestions while preparing this manuscript. The university has provided a manuscript communication number for further communication (IU/R&D/2024-MCN0002782).

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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Citation **Gazala Noor, Badruddeen, Juber Akhtar, Mohammad Ahmad and Mohammad Irfan Khan (2024). Development, characterization and acute toxicity study of nanoemulsion containing thymoquinone, hesperetin along with vitamin c for improving oral bioavailability. Ann. Phytomed., 13(1):1031-1045. http://dx.doi.org/10.54085/ap.2024.13.1.111.**