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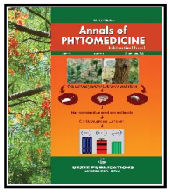

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Development and evaluation of thermoresponsive *in situ* nanoemulgel of myricetin for diabetic retinopathy

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Abstract

Diabetic retinopathy (DR) is a complication of diabetes, high blood sugar levels that damage the retina. If left untreated and undiagnosed, it can lead to blindness. DR progress has been reported to be reduced by flavonoids with antioxidant potential. Flavonols like myricetin, which occur naturally, have antioxidant properties. The present work summarises the development of thermoresponsive *in situ* gelling nanoemulsion containing myricetin providing sustained release and prolonged therapeutic effect for the treatment of diabetic retinopathy. Hydroxypropyl methylcellulose (HPMC) was used as a thermoresponsive gelling agent. Optimized nanoemulgel provided sustained release of myricetin with satisfactory rheological properties and pharmaceutical properties. In conclusion, the nanoemulsion formulations containing *in situ* gelling thermoresponsive polymer could serve as a promising drug delivery system providing superior therapeutic efficacy and better patient compliance for the treatment of DR.

1. Introduction

Diabetic retinopathy (DR) is an eye condition that occurs due to diabetes. It is the leading cause of new cases of blindness in adults, as well as the most common cause of vision loss for people with diabetes (Liu and Wu, 2021; Tiwari *et al.*, 2013; Tiwari and Rana, 2015). Oxidative stress has been demonstrated to play a key role in the pathophysiology of DR and has been postulated as a nexus with other biochemical pathways, which commonly brings about inflammation, neurodegeneration, and microvasculopathy (Rajeshwari *et al.*, 2013; Oshitari, 2022). Studies have confirmed that specific antioxidants and supplements could reduce the rate of DR progression by strengthening the antioxidant defenses (Sravanthi *et al.*, 2013; Alfonso-Muñoz *et al.*, 2021). Flavonoids having antioxidant potential have been proven to reduce DR progression (Yadav and Srivastava, 2014; Testa *et al.*, 2016). Myricetin (3, 5, 7, 32,42, 52 - hexahydroxyflavone) is a naturally occurring flavonoid in tea, berries, fruits, vegetables, and medicinal herbs. It inhibits the proliferation of human lens epithelial cells and protects retinal cells, retinal ganglion cells, and corneal epithelial cells. However, owing to main drawbacks such as low solubility, instability, poor bioavailability, the clinical application of myricetin is limited (Kim *et al.*, 2015; Liao *et al.*, 2017).

The unique characteristics of the ocular tissues and the anatomical and physicochemical barriers of the ocular globe make the penetration of ophthalmic drugs a challenging task. Less than 5% of the drug used for traditional formulations can penetrate the cornea (He *et al.*, 2021). The major complications associated with the administration of ocular dosage forms are precorneal loss due to the nasolacrimal drainage and high turnover of tear fluid. Hence, traditional formulations are not appropriate for administration due to their low bioavailability (Diebold and Calonge, 2010). To address these limitations and increase ocular drug bioavailability, several strategies include microparticles, colloidal carriers [*e.g.*, micelles, drug nanosuspensions, nanoemulsions, liposomes, nanostructured lipid carriers (NLC), and polymeric nanoparticles], have been developed and investigated (Li *et al.*, 2013). Among these new delivery systems, nanoemulsions appear to be the most promising candidate for ocular drug delivery (Liu *et al.*, 2016). Nanoemulsion is a pharmacokinetically stable liquid solution, usually with a droplet diameter within the range of 10-100 nm. Nanoemulsion has several advantages, including enhanced drug solubility, easy penetration to the ocular cavity, reduction of dose amount, and reduced side effects of dose as compared to conventional formulations (Liu *et al.*, 2016; Bhalerao *et al.*, 2020).

Recently, the use of responsive polysaccharides containing *in situ* gel formulation has increased due to its biocompatible nature, non-toxicity, and biodegradability (Liu *et al.*, 2016; Pathak *et al.*, 2013). Hydroxypropyl methylcellulose (HPMC) is a thermoresponsive gelling agent. The development of a thermoresponsive *in situ* nanoemulgel system combines the benefit as a drug carrier with the virtue of *in situ* gelling delivery systems to overcome the problem of low ocular bioavailability due to poor aqueous solubility of the drug and rapid drug loss caused by ocular protective mechanisms (Ribeiro *et al.*, 2011; Wu *et al.*, 2019). Thus, the present study aims to design a novel formulation of a myricetin-loaded *in situ* gelling nanoemulsion system for enhanced ocular delivery for the treatment of DR.

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2. Materials and Methods

2.1 Materials

Myricetin was purchased from Aktin Chemical Inc. (98%, Chengdu, China). Triacetin was supplied by Qualigens Fine Chem. Mumbai and Tween- 80 was supplied by SD Fine Chem. Mumbai. Sefsol was purchased by Nikko Japan. PEG 400 was generously provided by Fisher Scientific. Brij 35 and Kolliphor RH40 were purchased by BASF Chemical Company (Mumbai). HPMC 4KM was supplied by Colorcon Asia Pvt. Ltd. All other reagents were of analytical grade.

2.2 Screening of oil, surfactant, and co-surfactant

Solubility of myricetin was determined in various oils (Sunflower oil, Olive oil, Castor oil, Sefsol 218, Corn oil, Oleic acid, Triacetin), surfactants (Tween 80, Kolliphor RH40, Tween 20, Span 80, Span 20), and co-surfactants such as (Ethanol, PEG 400, 1,2-Propanediol, Glycerin, Isopropanol, Brij 35) by adding an excess amount of the drug in 2 ml of the selected vehicle in 5 ml capacity stoppered vials, and mixed using a vortex mixer (Pathak *et al.,* 2013; Abdel-Rashid *et al.,* 2019).

2.3 Construction of pseudoternary phase diagrams

Based on solubility studies, Kolliphor RH40 was used as the oil phase for the development of nanoemulsion. PEG 400, Brij 35, and double-distilled water were used as a surfactant, cosurfactant and aqueous phase, respectively. Surfactant and cosurfactant were mixed (S_{mix}) in different volume ratios (1:0, 1:1, 1:2, 2:1). These S_{mix} ratios were selected in increasing concentrations of surfactant concerning co-surfactant and increasing concentration of the co-surfactant concerning surfactant for detailed study of the phase diagrams. Phase diagrams were developed using an aqueous titration method. From each phase diagram constructed, different formulations were selected from the nanoemulsion region, so that a single dose of the drug could be easily incorporated into the oil phase (Bhalerao *et al*., 2020; Pathak *et al.,* 2013; Abdel-Rashid *et al.,* 2019; Morsi *et al*., 2017).

2.4 Thermodynamic stability testing of nanoemulsions

To find out the stable nanoemulsion and to discard the unstable nanoemulsions, the placebo nanoemulsions were subjected to a freeze-thaw cycle, centrifugation studies, and heating-cooling cycle (Bhalerao *et al.,* 2020; Pathak *et al.,* 2013; Ali *et al.,* 2014).

2.5 Preparation of myricetin-loaded *in situ* nanoemulgel

The *in situ* nanoemulgel was prepared by the cold method. For preparations of HPMC gel firstly 1% w/w HPMC 4KM was dissolved in distilled water using a mechanical stirrer. Following complete dissolution, the mixture was stored at 4°C for 12 h to ensure complete wetting. Then, optimized nanoemulsion with myricetin was added dropwise to the sol system with continuous magnetic stirring to form a homogeneous mixture. Finally, the mixture was neutralized with triethanolamine and stored at 4°C for 24 h (Almeida *et al.,* 2014; Sheshala *et al.,* 2015).

2.6 Determination of visual appearance and clarity

Appearance and clarity were determined visually against a white background at room temperature (20^0C) (Almeida *et al.,* 2014; Sheshala *et al.,* 2015).

2.7 Determination of pH and viscosity

The pH of the gel was measured in triplicate with a pH meter at 25°C. The viscosity of gel was determined using a Brookfield viscometer equipped with spindle number CC14 rotated at a speed of 5 rpm for a 10-s period at 37°C (Chowhan and Giri, 2020).

2.8 Determination of flowability and the gelation temperature

The phase behavior and gelation temperature of the examined formulations were determined by the tube inversion method (Chowhan and Giri, 2020; Vengurlekar *et al.,* 2014). Briefly, test tubes were filled with 1 g *in situ* nanoemulgel sample and incubated at 5 ± 1°C (storage temperature), 25 ± 1°C (average room temperature), and 35 ± 1°C (precorneal temperature), respectively. The tubes were shaken at 50 rpm. Samples were investigated under two different conditions. One condition was with dilution by simulated tear fluid (STF) at a ratio of 40:7 (ISG: STF, v/v), the other condition was without dilution by STF (Ribeiro *et al.,* 2011; Gambhire *et al.,* 2013). The examinations were carried out in triplicates.

2.9 Determination of drug content

Myricetin content from pre-weighed nanoemulgel was determined by dissolving in 50 ml freshly prepared simulated tear fluid (STF; pH 7.4). An aliquot of 5 ml was withdrawn and suitably diluted with STF. Myricetin content was analyzed for drug quantification using a UV-visible spectrophotometer at 328 nm (Tian *et al.,* 2013).

2.10 *In vitro* release study

In vitro release studies on *in situ* gel solution and nanoemulsion were performed by using a Franz diffusion cell. The study was conducted using a dialysis membrane that was previously soaked overnight in the dissolution medium at room temperature. The membrane was then mounted carefully between the donor and receptor compartments of a diffusion cell. The receptor compartment was filled with 50 ml freshly prepared simulated tear fluid (STF; pH 7.4) while accurately weighed *in situ* gel solution was placed in the donor compartment. The cell assembly was kept on a magnetic stirrer and stirring was maintained at 100 rpm at 37°C with thermostatic control. At appropriate time intervals, 5 ml aliquots of the receptor medium were withdrawn and immediately replaced by an equal volume of fresh receptor solution up to 8 h. The samples were analyzed by a UV spectrophotometer at 328 nm (Bhalerao *et al.,* 2020; Jain *et al.,* 2016; Tian *et al.,* 2013).

2.11 Stability studies

Stability studies on optimized nanoemulgel system were performed by keeping the sample at 25°C ± 2°C/60 ± 5% RH and 40°C ± 2°C/ 65 ± 5% RH for 3 months. Samples were placed in ambient color vials sealed with aluminum foil. These studies were performed for 3 months. The clarity, pH, gelling capacity, rheological evaluation, drug content, and *in vitro* drug release were determined at 0, 1, 2, and 3 months. Stability studies were performed as per the international conference on harmonization (ICH) guidelines (Ali *et al.,* 2014; The Indian Pharmacopoeia, 2010; Nagesh *et al.,* 2012).

3. Results

3.1 Screening of oil, surfactant and cosurfactant

Based on the solubility study, Sefsol 218 was selected as the oil phase. Kolliphore RH40 and PEG 400 were selected as surfactant and co-surfactant, respectively based on solubility studies.

3.2 Construction of pseudoternary phase diagrams

As illustrated from Figure 1, it was found that as the ratio of surfactant in S_{mix} was increased, the area of the nanoemulsion isotropic region changed slightly. In the ternary phase diagrams, the existence of a small or large nanoemulsion region depends on the capability of the particular S_{mix} to solubilize the oil phase. Four S_{mix} ratios, *i.e.*, 1:1, 1:2, 1:3, and 2:1 were selected for thermodynamic stability studies.

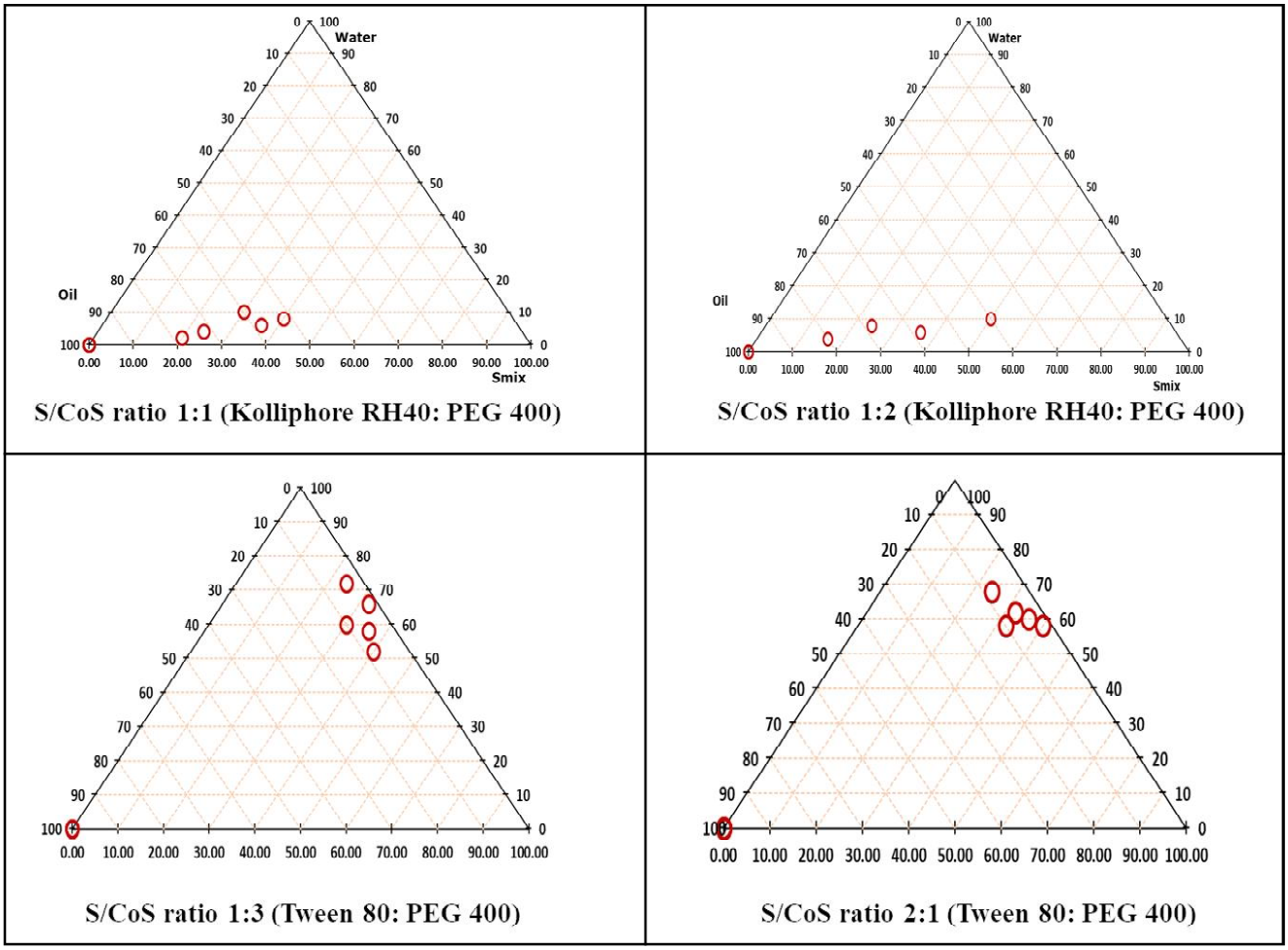

Figure 1: Pseudoternary phase diagrams system containing the following components: Sefsol 218 as oil, Kolliphor RH40 as a surfactant, PEG 400 as co-surfactant. The dotted area shows the nanoemulsion region in different ratios of surfactant to cosurfactant in 1:1, 1:2, 1:3, and 2:1.

3.3 Thermodynamic stability studies

Optimized nanoemulsions were subjected to different stress stability tests like heating-cooling cycles, centrifugation, and freeze-thaw cycle. Results of thermodynamically stable formulations were shown in Table 1. Thermodynamically stable formulations were selected for further studies.

Table 1: Thermostability of screened formulation ratios

Formulation with S_{mix} ratio	Heating cooling cycle	Centrifugation studies	Freeze-thaw cycle	Inference
1:1	√	√	√	Passed
1:2	√	√	√	Passed
1:3	×	×	×	Failed
2:1	×	×	×	Failed

3.4 Determination of visual appearance and clarity

The developed-*in situ* gel was light yellow and clear. The system was flowable liquid at a lower temperature (5ºC), while it turned into a viscous gel at physiological temperature (35ºC). This characteristic indicated the suitability of the developed nanoemulgel for ocular application.

3.5 Determination of pH, viscosity, and drug content of nanoemulgel

In the experimental observation, it was found that the drug content of both the selected formulations, *i.e.*, ISG 16 (S_{mix} ratio1:1) and ISG 17 (S_{mix} ratio1:2) was found in the range of 94.86 to 95.93% (Table 2). The result indicated that the incorporation of drug-loaded nanoemulsion to the nanoemulgel system accounted for a negligible loss of the drug. The pH values were found in the range of 6.9 - 7.1 which indicated the suitability of ocular drug delivery (Table 2).

The viscosity of *in situ* gel was estimated by a rheological instrument to predict retention behavior and physical integrity *in vivo*. As presented in Figure 2, when the temperature reached 34ºC, a sol to gel transition occurred which suggested that the system was more viscous. The increase in viscosity is expected to trigger prolonged retention of formulation on the corneal surface.

Table 2: Physicochemical evaluations of nanoemulgel

Formulation	Clarity	Visual appearance	pH	Drug content (%)	Gelling capacity test (Sec)	Gelation temperature
ISG16 (S_{mix} ratio 1:1)	√	Clear	7.1 ± 0.31	94.86 ± 0.68	60	35 ± 0.43°C
ISG17 (S_{mix} ratio 1:2)	√	Clear	6.9 ± 0.36	95.93 ± 0.73	60	35 ± 0.23°C

*All values are reported as Mean ± SD (n=3)

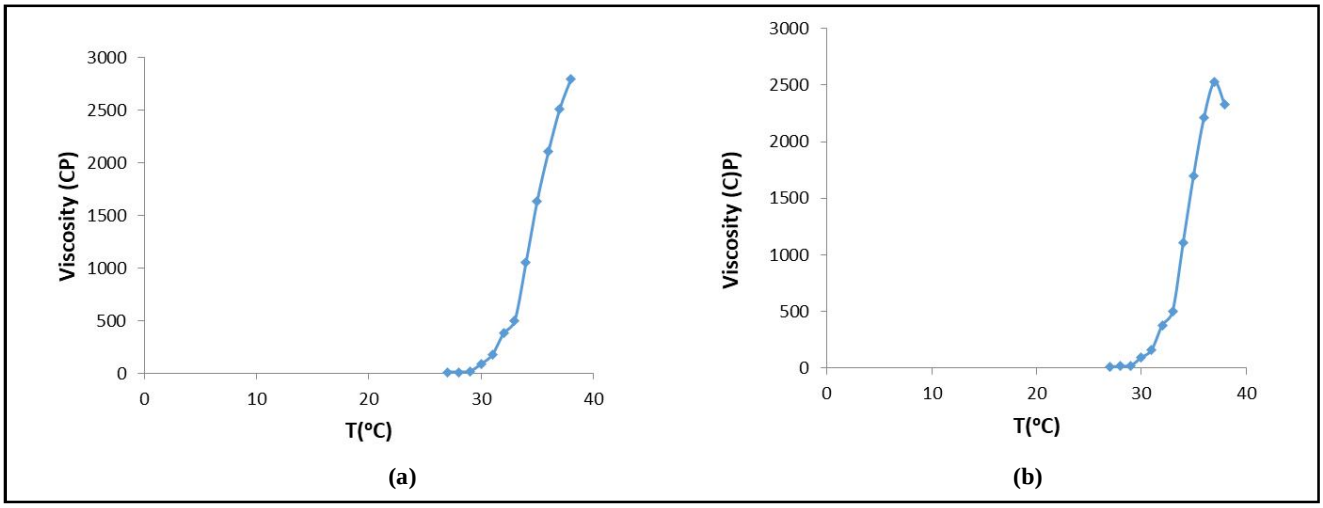

Figure 2: Rheological flow curves of optimized *in situ* gel formulations (a) ISG16 and (b) ISG 17 at different temperatures.

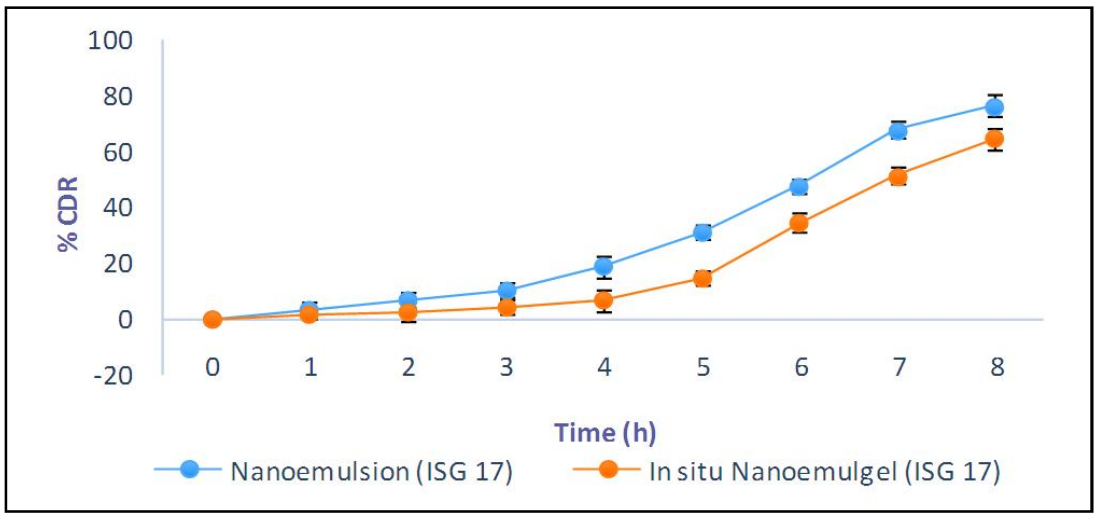

Figure 3: Cumulative *in vitro* drug release of myricetin loaded nanoemulgel. All values are expressed as Mean ± SD (n=3).

Table 3: Assessment of flowability

Formulation	Flowability at temperature (^{0}C)		
	5^{0}C	25^{0}C	35^{0}C
ISG 16	+++	++	++
ISG17	+++	+++	++

* Flowability: +++ Very good; ++ Good

3.6 Determination of flowability and gelation temperature

The flowability results for different samples are shown in Table 3. The ISG formulations diluted by STF are supposed to exhibit solution characteristics at 25ºC and change into gel at the body temperature. The STF diluting method was used to simulate the dilution after the formulation was administered. The sol-gel transition temperature was observed at 35°C. Gelling time was found 60 sec (Table 3). Samples flowing at 5ºC and 25ºC but not at 35°C within 30 s were accepted as optimal thermoresponsive *in situ* nanoemulgel.

3.7 *In vitro* release studies

The results of the *in vitro* drug release studies were displayed in Figure 3. As illustrated from the drug release pattern, a steady increase in the release of myricetin was observed with time. *In vitro* release of the drug from the *in situ* nanoemulgel follows the diffusion mechanism.

3.8 Stability studies

The stability study of optimized best formulation (ISG 17) was performed as per ICH guidelines. The developed thermoresponsive *in situ* nanoemulgel was found to be most stable at 25°C ± 2°C/60 ± 5% RH than at higher temperatures 40°C ± 2°C/65 ± 5% RH (Ali *et al.*, 2014). Results of stability studies are given in Table 4.

Table 4: Stability study of *in situ* nanoemulgel

Duration (Months)	Clarity	Visual appearance	pH	Isotonicity	Drug content (%)	Gelling capacity (sec)	*In vitro* drug release (%)
Stability at 25°C ± 2°C/60 ± 5%RH							
0	√	Clear	6.9 ± 0.36	√	95.93 ± 0.73	60	64.98 ± 0.53
1	√	Clear	6.8 ± 0.31	√	95.76 ± 0.43	60	64.97 ± 0.87
2	√	Clear	6.9 ± 0.42	√	95.63 ± 0.54	60	65.34 ± 0.21
3	√	Clear	7.1 ± 0.51	√	95.46 ± 0.61	60	65.98 ± 0.36
Stability at 40°C ± 2°C/65 ± 5%RH							
0	√	Clear	6.9 ± 0.36	√	95.93 ± 0.73	60	64.98 ± 0.53
1	√	Clear	7.0 ± 0.48	√	95.21 ± 0.43	60	65.12 ± 0.69
2	√	Clear	7.1 ± 0.53	√	94.56 ± 0.61	60	65.78 ± 0.31
3	√	Clear	6.9 ± 0.39	√	94.12 ± 0.57	60	66.08 ± 0.71

4. Discussion

The most important criterion for the screening of components is the solubility of the poorly soluble drugs in oil. The high solubility of the drug in the oil phase is important for the nanoemulsion to maintain the drug in the solubilized form. Since the drug exhibited the highest solubility in Sefsol 218, it was selected as the oil phase for the development of nanoemulsion. Similarly, Kolliphore RH40 and PEG 400 were selected as surfactant and co-surfactant, respectively based on solubility studies. The pseudoternary phase consisting of oil, S_{mix} (surfactant and co-surfactant mixture), and distilled water was developed using the aqueous titration method. Surfactant and co-surfactant were mixed in different volume ratios on the basis of increasing concentration of co-surfactant with respect to surfactant and vice-versa. Pseudoternary phase diagrams were constructed separately for each S_{mix} ratio.

Nanoemulsions are considered to be kinetically stable systems that are formed at a particular concentration of oil, surfactant, and water, with no phase separation, creaming, or cracking. Thermodynamic stability confers long shelf life to the nanoemulsion as compared to ordinary emulsions. It differentiates them from emulsions that have kinetic stability and will eventually lead to phase separation. Optimized nanoemulsions were subjected to different stress stability tests like heating-cooling cycles, centrifugation, and freeze-thaw cycle. Thermodynamically stable formulations were selected for further studies. Optimized ratios were converted into the gel and evaluated for acceptable gel characteristics. The system was flowable liquid at a lower temperature (5ºC), while it turned into a viscous gel at physiological temperature (35ºC). This characteristic indicated the suitability of the developed nanoemulgel for ocular application. The pH values were found in a nearly neutral pH range which indicated the suitability of ocular drug delivery. The viscosity of *in situ* gel was estimated to determine retention behavior and *in vivo* physical integrity. Formulations showed sol to gel transition in 60 sec. The increase in viscosity is expected to trigger prolonged retention of formulation on the corneal surface. The study results are in the agreement with the findings reported by Rui *et al.* (2016) and Zahraa *et al.* (2021).

Through the objectives covered best formulation was screened on the basis of clarity, pH, gelling capacity, rheology, and drug content

for further study. ISG 17 was selected as the best formulation and subjected for *in vitro* release study. Drug release studies were performed in simulated tear fluid (STF; pH 7.4) as a dissolution medium. Nanoemulgel system exhibited a better-sustained effect in comparison to nanoemulsion. The results clearly show that the gels can retain the drug for a prolonged period (> 8 hours) and that premature drug release will not occur. The developed thermoresponsive *in situ* nanoemulgel was found to be most stable at 25°C ± 2°C/60 ± 5% RH than at higher temperatures 40°C ± 2°C/65 ± 5% RH.

5. Conclusion

In the present work, myricetin-loaded thermoresponsive *in situ* nanoemulgel was successfully developed which combines the advantage as a drug carrier with the virtue of *in situ* gelling delivery systems to address the problem of low ocular bioavailability caused by poor aqueous solubility of the drug and rapid drug loss caused by ocular protective mechanisms. The developed optimized *in situ* nanoemulgel was satisfactory in terms of rheological properties and pharmaceutical characterization that offered the sustained release of myricetin. Therefore, study results suggested that the thermoresponsive *in situ* nanoemulgel containing myricetin has a great potential to be an ocular delivery system for the treatment of diabetic retinopathy.

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Conflict of interest

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

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