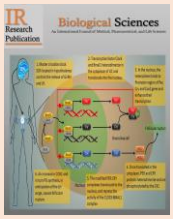
RESEARCH ARTICLE

IR Research Publication

Biological Sciences

(An International Journal of Medical, Pharmaceutical, and Life Sciences)

Journal home: https://irrespub.com/biolsciences/index.php/1/index



Myricetin loaded nanoemulgel: in vitro characterization and anti-inflammatory efficacy assessment in Sprague Dawley rats

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ARTICLE HISTORY

Received: 17-04-2024
Revised: 03-06-2024
Accepted: 04-06-2024
Online: 08-06-2024

KEYWORDS

Myricetin
Anti-inflammation activity
Pseudo-ternary phase diagram
Nanoemulgel

ABSTRACT

Myricetin, a commonly found natural flavonoid, exhibit a wide range of potential therapeutic activity including antidiabetic, anticarcinogenic, neuroprotective, and antiinflammatory activity. It belongs to BCS class II and has poor aqueous solubility, which limits its therapeutic application in the treatment therapy. This study was aimed to design and characterize the nanoemulgel formulation of Myricetin for topical delivery with the possibility of improved solubility, skin penetration and antiinflammatory activities. In this work Myricetin loaded nanoemulgel was prepared by water titration method and evaluated for particle size, zeta potential, pH, spreadability, rheology, drug content, skin permeation, skin irritation studies. In-vivo anti-inflammatory study of optimized Myricetin nanoemulgel was compared with marketed preparation (Voveran® emulgel®). The prepared Myricetin nanoemulgel containing Carbopol 934P (2%) as a gelling agent, Tween 20 (20%) as a surfactant, Ethanol (20%) as a co-surfactant and Sefsol 218 (20%) as an oil. The optimized Myricetin nanoemulgel was highly stable and it showed no sign of edema, skin irritation. It also showed significant anti-inflammatory activity. Transdermal permeation study demonstrated higher penetration of Myricetin from nanoemulgel compared to the marketed emulgel (Voveran® emulgel®). The Myricetin nanoemulgel showed promising results and can be further considered as a potent natural alternative to the synthetic drug for the treatment of inflammation.

Introduction

Myricetin is a common plant-derived antioxidant flavonoid and is found abundantly in our dietary supplies such as berries, beans, grapes, vegetables, red wine, and tea. It can be isolated from the root and bark of vine tea or Myrica rubra (1). It can be used as a functional nutraceutical as it possesses various beneficial biological activities including antidiabetic, anticarcinogen, neuroprotective, hepatoprotective, antibacterial, and anti-inflammatory activity (2). Unique multihydroxyl structure of Myricetin (figure 1) impart strong antioxidative property to it which enables the reduction of systolic blood pressure and changes vascular reactivity, making it a good candidate for the treatment of coronary heart disease (3). Myricetin also possesses several activities related to the central nervous system and may be beneficial in the treatment of Parkinson's and Alzheimer's diseases (2). Despite various health benefits, the

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DOI: https://doi.org/10.55006/biolsciences.2024.4202
Published by IR Research Publication;

Abbreviations: PDI: Polydispersity index; PCS: Photon correlation spectroscopy; NEG: Nanoemulgel; ANOVA: Analysis of variance

application of Myricetin in the treatment therapy is very limited due to low absorption efficacy, low bioavailability (less than 10%), and poor dissolution profile because of poor aqueous solubility (4,5). Therefore, it is essential to improve the bioavailability and solubility of the Myricetin to increase its therapeutic effectiveness.

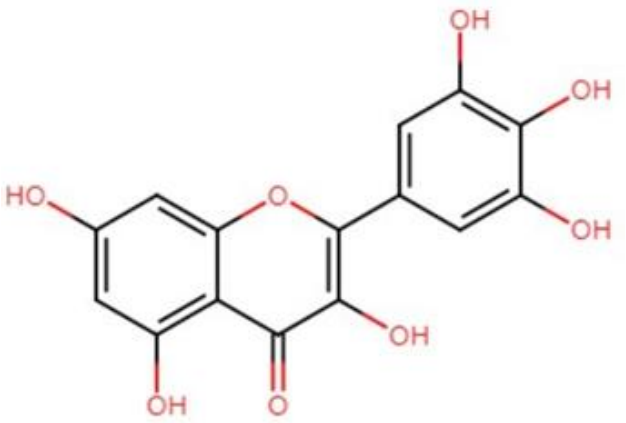

Figure 1. The chemical structure of Myricetin.

Recent studies have come up with certain strategies to overcome the limitation of poor bioavailability and solubility of drugs such as chemical and/or physical modification of drug moiety and/or changing the drug delivery systems approaches by using various carrier systems like liposome, microemulsion, nanoemulsion, niosomes, solid-lipid nanoparticles, etc. For an instant, nanococrystals of Furosemide-Caffeine have been synthesized to improve the solubility of the insoluble drug, Furosemide, from 17 µg/ml to 30 µg/ml and the intrinsic dissolution rate of Furosemide-Caffeine nanococrystals was found to be increased by approximately 6 times than that of the pure free drug solution (6). Similarly, the nanoparticle of poorly water-soluble drug Fenofibrate was prepared to enhance bioavailability. It was observed that the nanoparticle formulation showed greater solubility with a better dissolution profile and the bioavailability was found to be increased 2.5-fold than that of free drug solution (7). Overall, these studies have shown that the modification of the drug delivery system to nanoformulations gives feasible strategies to overcome the bioavailability issue of insoluble drugs. Though there are many drug delivery systems approaches emulsion-based drug delivery system has gained much interest in lipophilic drug delivery and can be considered industrially more feasible.

Nanoemulsions has the ability to enhance the permeation and penetration of the drug through the skin due to their characteristic properties such as high interfacial area, small droplet size (20-200 nm), high solubilizing capacity, and high kinetic stability (8-10). Thus, improving the absorption of drug moiety into the skin without the addition of any penetration enhancer (11,12). Nanoemulsion is prepared spontaneously by oil, surfactant, co-surfactant, and aqueous solution. Drugs with poor water solubility can easily be loaded in the oil phase and transported across the skin membrane (9). However, due to low spreadability, poor retention on the skin surface, and low viscosity application of nanoemulsion to the skin is inconvenient (13). Limitation of nanoemulsion formulation as a topical drug delivery system can be overcome by increasing the viscosity of the formulation by the application of the suitable gelling agent. The nanoemulsion encapsulating the lipophilic drug in the oil phase can be then added to the hydrogel base to form nanoemulgel (14). Nanoemulgel is a nanoemulsion based hydrogel system that is formed by the incorporation of the nanoemulsion into a hydrogel matrix (8). Hydrogel system faces the limitation of inability to transport lipophilic drugs (15). Henceforth, solubilization of poorly aqueous soluble drug into the oil phase of nanoemulsion and then adding to the hydrogel base will eliminate the limitation of hydrogel along with promising improvement in stability and drug release kinetics (16). A combination of hydrogel with nanoemulsion gives a unique feature to the nanoemulgel, attracting the attention of many researchers for the development of the formulation of various lipophilic drugs with great potential of skin disorders treatment. Besides nanoemulgel formulation being non-sticky, non-greasy, and easily spreadable, it also improves the patient acceptability and compliance (16). So far, nanoemulgel have been used by various researcher to increase the utility of insoluble drugs such as Curcumin (14), Quercetin (17), Mangosteen extract (18), Celecoxib (19), Selegiline Hcl (20) and Aceclofenac (21), etc. However, no study has been reported till date reporting better solubility of Myricetin through topical nanoemulgel preparation.

Therefore, this study was performed to design the nanoemulgel preparation of Myricetin and improve its solubility and bioavailability for transdermal application. Briefly, the solubility of Myricetin in various excipients was investigated and the pseudo-ternary phase diagram was constructed to screen the stable nanoemulsions. Further, characterization of prepared nanoemulsions was conducted and optimized nanoemulsion was incorporated in the gel matrix to get final product, nanoemulgel, which was later evaluated. To validate the transdermal application, the interaction study of Myricetin

nanoemulgel with the skin membrane was studied. Later, skin irritation study, ex-vivo skin permeation study, and in-vivo anti-inflammatory activity were performed and compared with the marketed formulation (Voveran®emulgel®).

Material and methods

Chemical and reagents

Myricetin was gifted by Xi'an Lyphar Biotech Co., Ltd., (Mainland, China), Cremophor RH-40 and Captex 355, Carrageenan, Brij 35 were purchased from Sigma Aldrich (Mumbai, India). Sefsol 218 and Transcutol HP (diethylene glycol monoethyl ether) were kindly gifted by Gattefosse India Pvt. Ltd. (Mumbai, India). Polyethylene glycol (PEG) 400, propylene glycol, Tween 80 (polyoxyethylene sorbitan monooleate), Span 20, Triacetin (glycerol triacetate), and Span 80 were purchased from Merck India Ltd., (Mumbai, India). Methanol and Ethanol were purchased from S.D. Fine Chem Ltd. (Mumbai, India). Carbopol 934P and Triethanolamine (TEA) were bought from the Central Drug House Pvt. Ltd., New Delhi, India. All other solvents were of HPLC grade and chemicals were of analytical grade. Fresh double distilled water used throughout the study was filtered through a 0.22 µm membrane filter.

Animals and housing

Adult healthy male Sprague Dawley rats, weighing 150-180 g were used for carrageenaninduced paw edema study. All the animals were carefully lodged under standard laboratory conditions (25 ± 2 ℃ temperature and 40-60% RH) in polypropylene cages with free access to water and food. Before experiment, animals were acclimatization for seven days. The approval of the study protocol was taken from the Integral University, Institutional Animal Ethical Committee (Approval no.: IU/IAEC/18/29) and CPCSEA guideline was maintained throughout the study.

Screening of oils, surfactant, and co-surfactant

The solubility of Myricetin in various oils, co-surfactants, and surfactants was measured. Excess amount of Myricetin was mixed thoroughly using a vortex mixture with 2 mL of each of the selected oils (sefsol 218, isopropyl myristate, triacetin, captex 355), co-surfactants (PEG 400, Transcutol HP and ethanol), and surfactants (Tween 20, Span 80, Brij 35, Cremophor RH 40) in 5 mL vials separately. The vials were then kept in an isothermal shaker for about 72 hrs at 25 ± 2 ℃ to reach the state of equilibrium. Keeping vials for 72 hrs will ensure the maximum possible solubility of the compound as a saturated solution in the equilibrium. The equilibrated samples were centrifuged for 10 min at 3500 rpm and the supernatant obtained was filtered through 4.5 µm membrane filtrate before analysis. The concentration of Myricetin was determined in the selected oils, surfactants, co-surfactants using a UV-spectrophotometer at 376 nm. Based on the solubility data, ingredients were selected for the construction of the pseudo-ternary phase diagram (22).

Construction of pseudo-ternary phase diagram

Aqueous titration method was used to plot the pseudo-ternary phase diagram using XLSTAT software. Based on the solubility studies, Sefsol 218, Ethanol, and Tween 20 were selected as the oil, co-surfactant, and surfactant respectively, whereas double distilled water was used as the aqueous phase. Surfactant and co-surfactant were mixed in the different volume ratios of 1:0, 1:1, 1:2, and 2:1 namely Smix ratios, based on the increasing co-surfactant concentration in the Smix. To construct the pseudo-ternary phase diagram, oil was added to each Smix ratios in different volume ratios and mixed well. Total of sixteen different combination ratios of oil and Smix, (1:9, 1:8, 1:7, 1:6, 1:5 1:4, 1:3.5, 1:3, 3:7, 1:2, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1) were mixed, covering maximum possible ratios to delineate all the boundaries of the phases accurately in the phase diagrams. Each ratio of the oil and Smix was slowly titrated with the aqueous phase under the vortex condition at room temperature, and a visual examination was carried out for the transparent nanoemulsion (22).

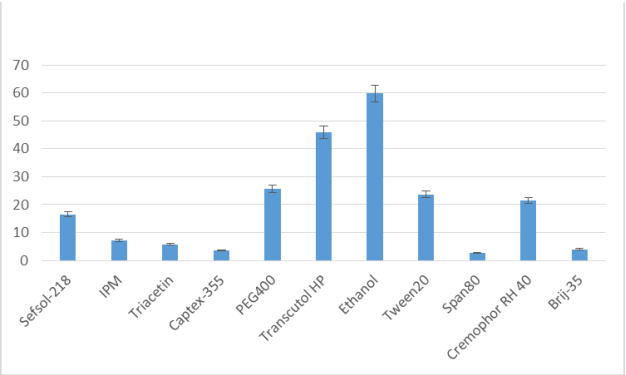

Figure 2. Solubility of Myricetin in different oils (sefsol-218, Isopropyl myristate (IPM), triacetin, and captex-355), surfactants (PEG 400, Trancutol HP and ethanol) and co-surfactants (tween 20, span 80, cremophore RH 40 and Brij-35).

Preparation of blank and Myricetin loaded nanoemulsion

Based on the nanoemulsion region of the pseudo-ternary phase diagram (figure 2), different formulation ratios were selected for the blank formulation. Specific quantities of oil (Sefsol 218),

surfactant (Tween 20), co-surfactant (Ethanol), and the aqueous phase (double distilled water) were mixed and vortexed for 2 min. The emulsion form was characterized for size and Myricetin was loaded into optimized nanoemulsion. Myricetin was dissolve in oil with vigorous mixing using a vortex. Further addition of surfactant, co-surfactant, and water was done the same as a blank formulation (22).

Characterization of optimized Nanoemulsion

Thermodynamic stability

Thermodynamic stability study was done to determine the physical stability of the prepared nanoemulsion and to detect any phase separation of the excipients as physical stability not only affect the visual appearance of the formulations but also the performance of the formulation (23).

Centrifugation study

The Nanoemulsions were centrifuged for 30 min at 5000 rpm and observed visually for phase separation, precipitation or creaming, if any, at every stage (23).

Heating and cooling cycle

The nanoemulsions with no creaming, cracking, or phase separation after centrifugation was subjected to six cycles of heat and cool to see the effect of temperature variation on its stability. Formulations were stored at the temperature between 4 to 40 0C each for at least 48 hours and stable formulations were further subjected to freeze-thaw cycle for a stress test (23).

Freeze-thaw cycle

In this test, the nanoemulsion formulations were stored at a temperature between -21 and +25 0C each for at least 48 hours to see the stability of formulations. The nanoemulsions that passed this stress test were selected as stable formulations for further studies (23).

Particle size, PDI and zeta potential

The average particle size and Polydispersity index (PDI) of the selected nanoemulsions were determined by Photon correlation spectroscopy (PCS) using zeta sizer 3000 (Malvern Instruments, Malvern, UK). The samples were subjected to sonication prior to dilution with ultra-pure water and light scattering was measured at the temperature of 25±2 ℃ (15). Zeta sizer nano ZS (Malvern Instruments, UK) was used to measure the zeta potential of the selected nanoemulsion for the analysis of surface charge. About 0.1 mL of nanoemulsion was diluted 100 times with ultra-pure water. The value was determined as a result of the electrophoretic mobility of oil droplets and the desired value of zeta potential is at least ±20 mv (24).

Viscosity and conductivity of Nanoemulsion

The viscosity of nanoemulsion was determined by Brookfield viscometer LV without diluting the formulation. The spindle size used was 64 and rotation speed was 100 rpm at 25±2 0C (25). The conductivity of nanoemulsion was determined by using conductivity meter at a temperature of 25 ±2 0C (25).

Preparation of Myricetin nanoemulsion loaded gel

To prepare nanoemulgel, the optimized nanoemulsion was mixed with Carbopol gel in the ratio of 1:1. The gel was prepared by the soaking the Carbopol 934P in water overnight with continuous stirring for the complete swelling of the gelling agent. Triethanolamine was added to neutralize the gel matrix whereas propylene glycol (5 % w/w) was added as a plasticizer. Optimized Myricetin nanoemulsion was slowly added to Carbopol 934P gel (1% w/w) under continuous stirring with three different drug concentrations (1%, 2.5%and 5% w/w). The nanoemulgels formed were coded as NEG1 (1% w/w Myricetin), NEG2 (2.5% w/w Myricetin), and NEG3 (5% w/w Myricetin) respectively. To prepare blank nanoemulgel (NEG), blank nanoemulsion was loaded into Carbopol-934P gel system (14).

Evaluation of Myricetin nanoemulgel

Viscosity measurement and Rheological behavior

Brookfield viscometer LV was used to determine the viscosity of nanoemulgel formulation. About 0.5 g of nanoemulgel was subjected to rotation of 20 rpm at 25°± 1°C using spindle size 7. The thickened system is considered to show good sensorial and biophysical benefits for topical drug delivery (22). The rheological behavior of the formulation was studied by Brookfield Viscometer at different rotational speeds (0.5, 1.0, 2.0, 2.5, 4, 5, 10, 20, 50, and 100 rpm) using size 2 spindle at 25±1 0C. Data observed was used to plot the graph of the rheogram and the position of the downward and upward curves was studied for the flow behavior (26).

Spreadability

To assess the spreadability of prepared nanoemulgel, the spreading diameter of formulation in between the two glass plates was measured. The formulation was kept for 48 hours before conducting the study. 500 mg weight nanoemulgel was placed within a circle of diameter 1 cm marked on the glass plate. A second glass plate was placed over it and a predefined weight was kept over the upper glass plate. The increase in diameter after 1 min due to the addition of weight was noted. The formula used to calculate the spreadability was:

S= m.l/t,

Where S is spreadability, m is weight added on the upper plate, l is the length of the upper plate, and t is the time taken in second (27).

pH determination

Nanoemulgel being topical formulation has to be applied on the skin, therefore it is essential to ensure the non-irritating nature of the formulation by determining the pH value. 2 g of nanoemulgel was dissolved in 100 mL solution of phosphate buffer and the pH of the resulting solution was measured at room temperature with digital pH meter (Microcup pH meter, Italy). If required, triethanolamine was added to neutralize the pH of the formulation (27).

Drug content determination

The amount of Myricetin content in the optimized nanoemulgel preparation was determined using UV spectrophotometer. A weighed amount of formulations equivalent to 20 mg of Myricetin was dissolved in 20 mL ethanol each and sonicated for 1 hour. The sonicated solution was filtered through Whatman filter paper and diluted 100 times with ethanol. The content of Myricetin in nanoemulgel formulations was determined at 376 nm (22).

Sustainability study

A sustainability study was conducted by performing a temperature stress test on the formulations. Each formulation was stored at different temperature conditions in sealed glass containers. The formulations were kept at accelerated temperature (40 0C), at room temperature (25 0C), and in a refrigerator (4 0C) for 60 days. The formulations were evaluated after 1, 7, 14, 21, 30, 45, and 60 days for any change in pH, drug content, or physical stability (27).

Skin Irritation study

Skin irritation test was carried out on SD rats weighing 150-180 g, according to the approval of the Animal Ethical Committee. Prior to the experiment, the animals were acclimatized for one week, in the standard laboratory conditions. The humidity of 45 ± 5 % RH and temperature of 25 ± 1 0C were maintained with free access to standard laboratory diet and water. SD rats were divided into two groups: Optimized nanoemulgel formulation and marketed formulation each containing 6 rats. A single dose of each marketed formulation and optimized nanoemulgel (NEG2) was applied to the right ear of the rats with the left ear considering as the control. The treated skin was monitored for 14 days for erythema and edema by visual observation (19).

Ex-vivo skin permeation study

The ex-vivo permeation of Myricetin through the freshly excised goatskin membrane from the optimized nanoemulgel formulation was studied using a Franz diffusion cell. The diffusion area was about 2.54 cm2 and media used was phosphate buffer pH 7.4 and ethanol in the ratio 3:1 at the temperature of 25 ± 2 0C. Goatskin was taken from the local slaughterhouse and was shaved without damaging the membrane. It was then washed with distilled water followed by saline and then placed carefully in between the donor and recipient compartment of Franz diffusion cell. The accurately weighed amount of optimized Myricetin nanoemulgel (NEG1) and marketed formulation (Voveran® emulgel®), containing an equivalent amount of standard drug as contained in the sample of nanoemulgel, was applied uniformly over the membrane surface. 1 mL aliquot sample was withdrawn from the receptor compartment at pre-determined time intervals, which was replaced by an equal volume of fresh solvent. The withdrawn samples were analyzed by U.V. spectrophotometry at 376 nm (28). The cumulative amount of drug permeated per unit surface area (µg/cm2) of the skin membrane was plotted against time to construct the permeation profile, and the slope of the linear part of the curve was taken as a steady-state flux (Jss). To calculate the permeability coefficient (Kp), the equation used was Kp=Jss/C, where C is the drug concentration of the sample (28).

Carrageenan induced paw edema model

In-vivo performance of the Myricetin nanoemulgel was assessed by the carrageenan-induced paw edema model for the anti-inflammatory activity. Adult healthy male Sprague Dawley rats of approximately 150-180 g weight were randomly divided into five groups of six rats each. Prior to the experiment, all the rats were kept for fasting overnight with free supply to water. The first group

was untreated and considered as control. NEG1 (1% Myricetin w/w), NEG2 (2.5% Myricetin w/w), NEG3 (5% Myricetin w/w), and marketed formulation (Voveran ® emulgel®) were applied on the subplantar region of the left hind paw of second, third, fourth, and fifth groups, respectively. 1 hrs. post topical application, 0.1 mL of freshly prepared carrageenan (1% w/v in saline) was injected into the subplantar region of the left hind paw of all the rats and paw volumes advancing till ankle joint were measured before and at predefined time intervals (1, 2, 3, 4, 5, 6 hrs.) by plethysmometer. The percentage reduction in paw edema of the treated groups was calculated by the following formula (26):

% edema (control) – % edema (treated group)

% reduction in edema =

% edema (control)

Statistical analysis

The results were expressed as mean values ± S.D. The analysis of variance (ANOVA) was applied to examine the significance of differences in the formulations. In all cases, $p < 0.05$ was considered to be significant.

Results and discussion

To screen components for nanoemulsion, the solubility of Myricetin in various oils, surfactants, and co-surfactants was analyzed as represented in figure 2. Among all oils tested (Sefsol 218, Triacetin, Captex 355, Isopropyl myristate), Sefsol 218 showed higher solubility and was selected as oil phase for the further studies in the pseudo-ternary phase diagram to identify its formulation capability. The solubility of Myricetin was found higher in Tween 20 and Cremophor RH 40 among the four surfactants studied (Tween 20, Span 80, Brij 35, Cremophor RH 40). Provided these two surfactants have been used commonly in foods, drugs, and nutraceuticals to form emulsion-based formulations, and tween 20 having higher HLB value, tween 20 was selected as the surfactant. Fluid interfacial film and transient negative interfacial tension are hardly achieved by the application of a single surfactant, therefore the addition of cosurfactant becomes essential in the emulsion-based formulation. Thus, based on the solubility data, ethanol was selected as co-surfactant among three co-surfactants (PEG 400, Transcutol HP, and ethanol).

Construction of pseudo ternary phase diagram

Based on the visual observation, the pseudo-ternary phase diagrams of blank nanoemulsion were constructed to optimize the concentration range of oil, surfactant and co-surfactant in the formulation and get a stable and transparent/transparent with bluish tint o/w nanoemulsion. The shaded areas of the pseudo-ternary phase-diagrams represent the stable and transparent o/w nanoemulsion. Whereas rest of the area represents the turbid and conventional emulsions. Figure 3(a) represents the pseudo-ternary phase diagram of sefsol 218 (oil phase) with surfactant only, i.e. Smix ratio (tween 20: ethanol) 1:0. It showed a significant area of nanoemulsion towards the higher concentration of surfactant. As reported earlier (29), a high concentration of surfactant can cause problems of skin irritation, so co-surfactant was added in the formulation. Equal concentration of surfactant and co-surfactant (Smix ratio 1:1) showed an increase in the area of nanoemulsion figure 3(b). Further rise in the concentration of co-surfactant from 1:1 to 1:2 resulted in a little reduction in the nanoemulsion area figure 3(c). However, as the surfactant ratio was increased from 1:1 to 2:1, a significant decrease in the area of nanoemulsion was observed figure 3(c). 1:2 ratio showed little reduction in the area nanoemulsion as compared to 1:1. Thus, the Smix ratio 1:1 and 1:2 was selected as an optimized surfactant and co-surfactant ratio for the preparation of nanoemulsion formulation.

Selection of formulation from the pseudo ternary phase diagram

Based on the criteria that the concentration of surfactant in the formulation should be optimized enough to be a non-irritant and good emulsifier, different concentrations of oil and optimized Smix ratio were mixed to form stable nanoemulsion. Sixteen blank formulations with different oil: Smix ratio from 1:9 to 9:1 and 1:2 to 1:8 were prepared for each selected Smix ratio (1:1 and 1:2). The stable transparent nanoemulsion formulation with no phase separation and optimum concentration of surfactant was considered as an optimized formulation for further drug loading and characterization studies (Table 1).

Characterization of optimized nanoemulsion

Thermodynamic stability

Nanoemulsion system is regarded to be kinetically stable, form at a particular concentration of components (oil, surfactant, and water) and shows no sign of creaming, phase separation, and cracking (23). Therefore, optimized nanoemulsions were subjected to stress stability tests under different temperature conditions such as freeze-thaw cycle,

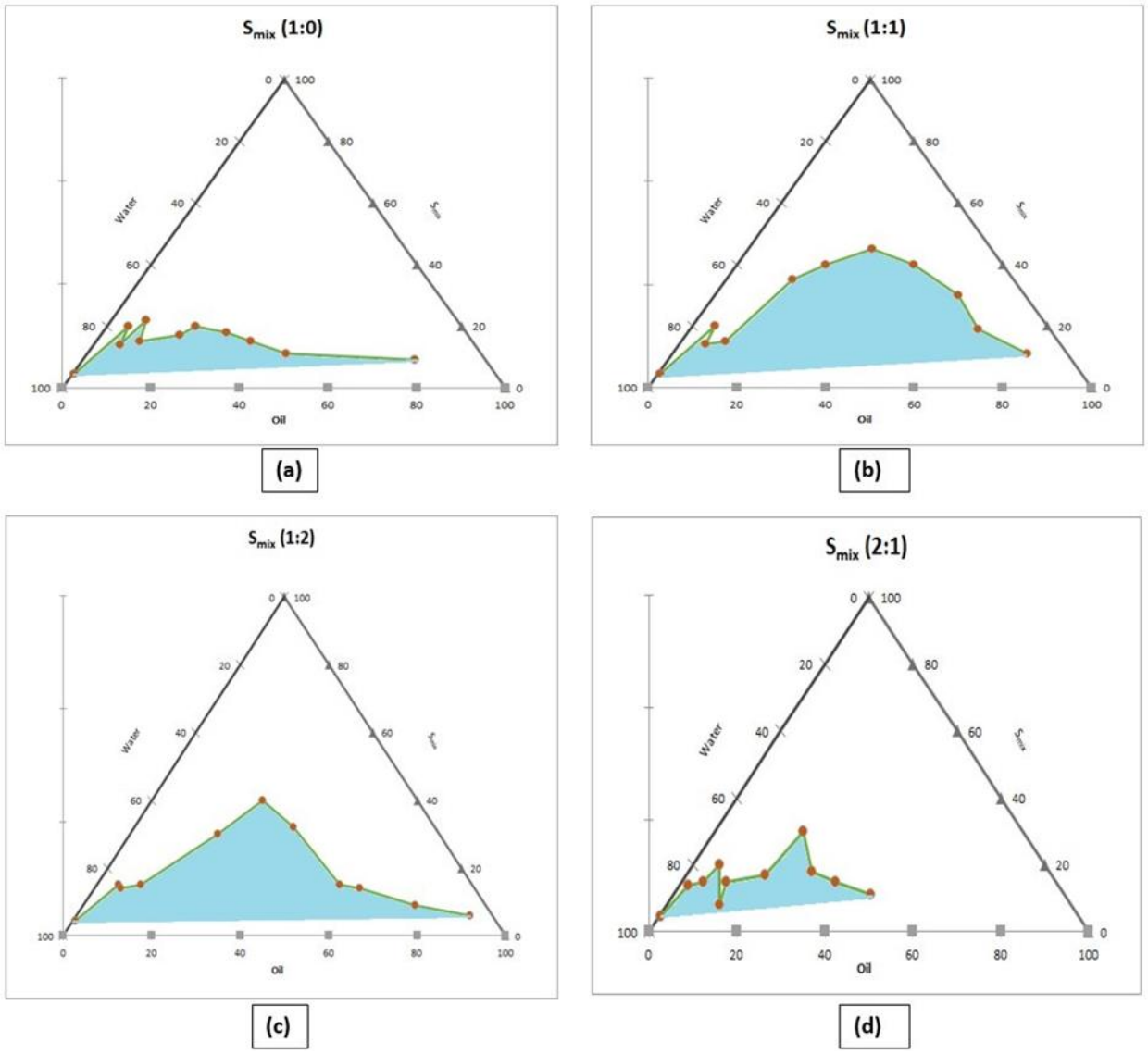

Figure 3. Pseudo-ternary phase diagram with surfactant (tween 20) and co-surfactant (ethanol) ratio (A) 1:0 and Oil: sefsol 218, (B) 1:1 and Oil: sefsol 218, (C) 1:2, and Oil: sefsol 218, (D) 2:1, and Oil: sefsol 218.

Table 1. Composition of nanoemulsions selected for optimization.

Formulation code	Oil: S_{mix}	S_{mix} ratio	Oil (%)	S_{mix} (%)	Water (%)
F1	3:7	1:2	15	35	50
F2	1:2	1:2	20	40	40
F3	3:7	1:1	15	35	50
F4	1:2	1:1	20	40	40
F5	1:3	1:1	15	45	40
F6	4:6	1:1	20	30	50

Table 2. Thermodynamics stability study data of selected formulation.

Formulation code	Centrifugation (5000 rpm)	Heating– cooling cycle (4^0C to 40^0C)	Freeze–thaw cycle. (-21^0C to $+25^0$C)	Inference
F1	✓	✓	✓	Pass
F2	✓	✓	✓	Pass
F3	✓	✓	X	Fail
F4	✓	✓	✓	Pass
F5	✓	✓	✓	Pass
F6	✓	✓	X	Fail

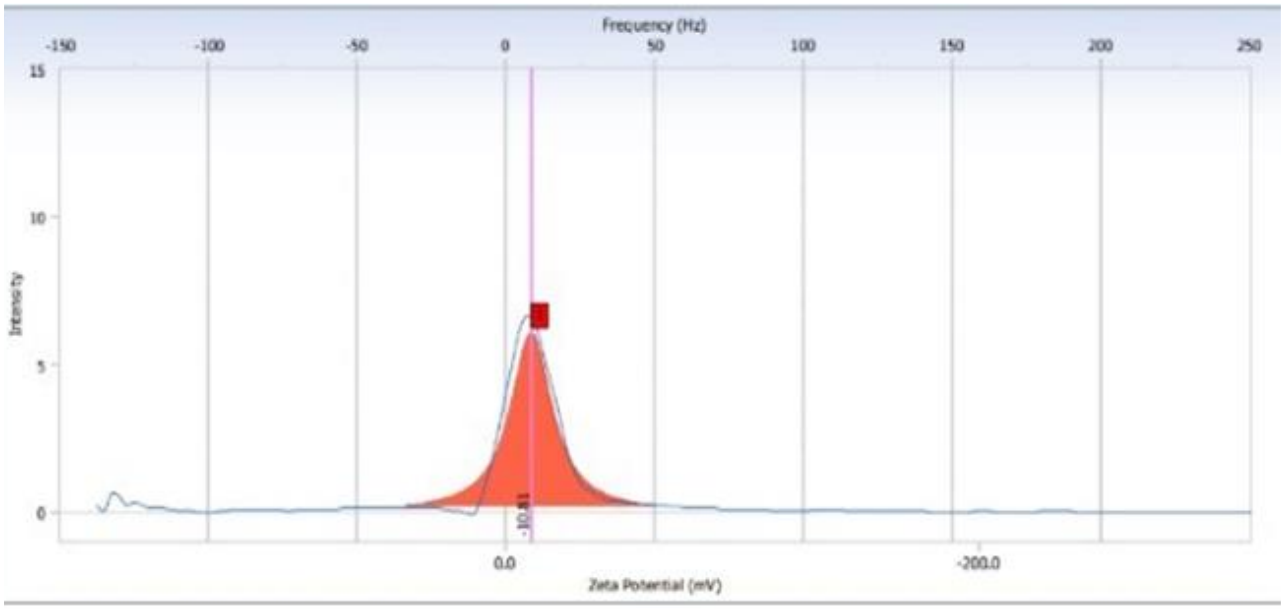

Figure 4. Zeta potential of nanoemulsion (F4).

Table 3. Characteristics of screened nanoemulsion.

Formulation code	F1	F2	F4	F5
Particle size (nm)	231.5±0.02	154.7±0.08	148.0±0.05	152.9±0.04
PDI	0.195±0.10	0.159±0.02	0.088±0.09	0.154±0.02
Zeta potential (mV)	-12.84±0.12	-19.95±0.06	-10.81±0.05	-13.13±0.30
Viscosity (cP)	33.0±0.27	32.0±0.02	33.5±0.12	32.0±0.02
Conductivity (μScm^{-1})	147.2±0.45	154.6±0.22	132.7±0.58	110.3±0.34
Appearance	Transparent	Transparent	Bluish	Transparent

Table 4. Comparative evaluation of blank nanoemulgel, nanoemulgel with three different concentration of myricetin and marketed emulgel.

Formulation code	Drug content (%)	pH value	Spreadibility ($gcmS^{-1}$)	Viscosity (cP)
NEG1 (1%)	99.1	7.5	5.8±0.36	15042
NEG2 (2.5%)	96.8	7.1	5.5±0.28	15095
NEG3 (5%)	95.7	7.4	5.8±0.81	15109
NEG (blank)	0	6.8	5.6±0.73	15545
Marketed preparation (Voveran® emulgel®)	98.3	7.2	6.0±0.59	15087

Table 5. Skin irritation score of myricetin nanoemulgel (NEG2) and marketed emulgel.

S. No	Group	Score after (Days)					Mean score	Standard deviation
		1	2	3	5	7		
1	Myricetin Nanoemulgel (NEG2)	1	3	3	2	2	2.2	0.83
2	Marketed emulgel (Voveran® emulgel®)	3	2	3	3	2	2.6	0.54

The nanoemulsions with no sign of Ostwald ripening during all the three stress tests were considered to pass the thermodynamic stability test for further characterization

Particle size, PDI and zeta potential

Particle size characterization of nanoemulsion is necessary to assure the safety and efficient dosing of the formulation (30). The droplet size was found to be in the nanosized range varying from 148.0 ± 0.05 nm to 231.5 ± 0.02 nm. Polydispersity index of the formulation indicates the uniform distribution of the droplet size within the formulation and was determine by calculating the ratio of the standard deviation to the mean size of droplets (30). The polydispersity of nanoemulsions was observed low (0.088±0.09 to 0.195±0.10), indicating that the droplet size was narrowly distributed within the formulation. Zeta potential values indicating the surface charge were recorded to be between -10.18±0.05 to -19.95 ±0.06 (Figure 4).

Viscosity and conductivity of Nanoemulsion

The viscosity of nanoemulsions was observed low, ranging from 32.0 ± 0.02 to 33.5 ± 0.12 mPaS. The low viscosity makes the nanoemulsion unsuitable for topical application, thus the addition of nanoemulsion to the hydrogel matrix to get the high viscosity nanoemulgel was justified.

The conductivity of nanoemulsion was observed to be high (Table 3) measuring from 110.3 ± 0.34 to 147.2 ± 0.45 µS/cm which confirms the o/w type of emulsion.

Formulation of Nanoemulgel

All the optimized stable formulations were in the range of nanosized particles with low viscosity, which hinders the transdermal use, and therefore, to impart applicability the viscosity of nanoemulsion was needed to be increased. Based on the observation of characterization profile formulation F4 was selected as optimized nanoemulsion Figure 5. Myricetin was loaded into nanoemulsion (F4), which was then incorporated into the hydrogel matrix of Carbopol 934P to prepare nanoemulgel NEG1, NEG2, NEG3 with different concentration of the drug, 1%, 2.5% and 5% w/w respectively.

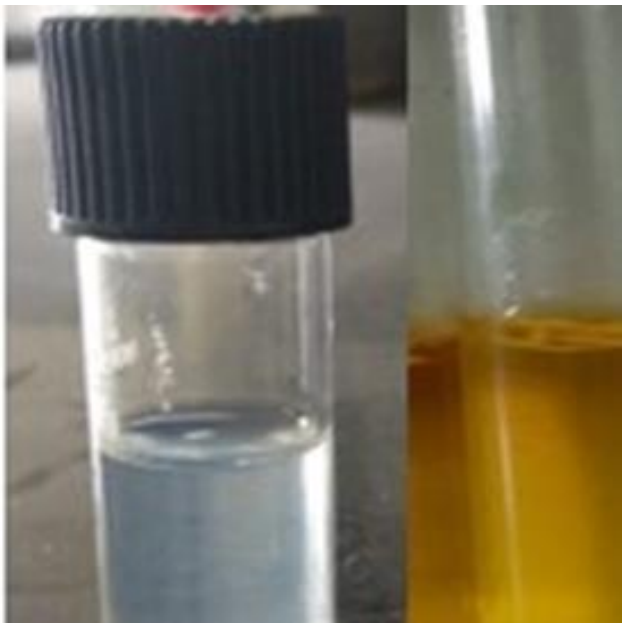

Figure 5. Optimized blank nanoemulsion F4 (left) and myricetin loaded nanoemulsion (right).

Spreadability

Topical formulations such as nanoemulgel which are applied on inflamed skin should exhibit maximum slip and drag and can be easily spread without any friction (26). Thus, to ensure better patient compliance the spreadability of nanoemulgel formulations was measured and was observed to be in a range of 5.5 ±0.28 to 5.8 ± 0.81 gcmS-1 (Table 4).

Rheological Behavior and Viscosity Measurement

Rheological behavior was studied to govern the performance of the topical preparation such as spreadability, drug release, and transdermal adherence of the formulation on the surface of the skin. The optimized nanoemulgel preparation was kept at 25±1 °C for 5 days prior to the rheological study. The rheogram of plain nanoemulgel and Myricetin loaded nanoemulgel (NEG2) was plotted with shear stress against shear rate. The superimposed flow curves of the rheogram (Figure 6) displayed that both the formulation has shear thickening (dilatant) flow behavior indicating that the presence of Myricetin in the nanoemulgel did not affect ($p>0.5$) the rheological behavior.

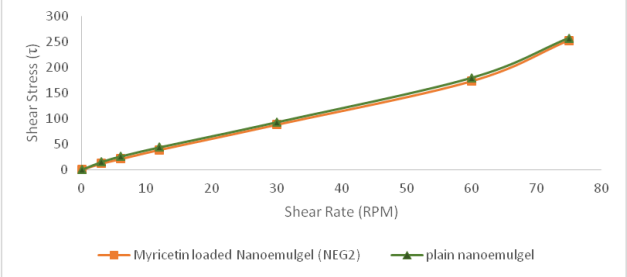

Figure 6. Rheogram depicting no effect of myricetin on nanoemulgel rheological behavior.

Drug Content and pH Determination of Nanoemulgel

In the study, the amount of Myricetin loaded in the nanoemulgel was found to be nearly the same in all the formulation and drug content was in the range between 95.7% and 99.1% (Table 4). The results indicated the uniform distribution of Myricetin throughout the formulation with minimum drug loss during formulation. The pH values of nanoemulgels were approximately neutral, in a range of 6.8-7.5 pH, signifying the suitability of the formulations for transdermal application without irritation.

Sustainability study

The nanoemulgel formulations kept at accelerated condition (at temperatures 25 ± 20C, 40 ±20C, and 40C) were found to be clear throughout 60 days. All the formulations were consistent with no significant change in their drug content, pH values, transparency, and phase separation during the sustainability study. Carbopol 934P in formulation enhanced the viscosity leading to the distribution of oil droplets in the gel matrix might have resulted in better stability of droplets (31).

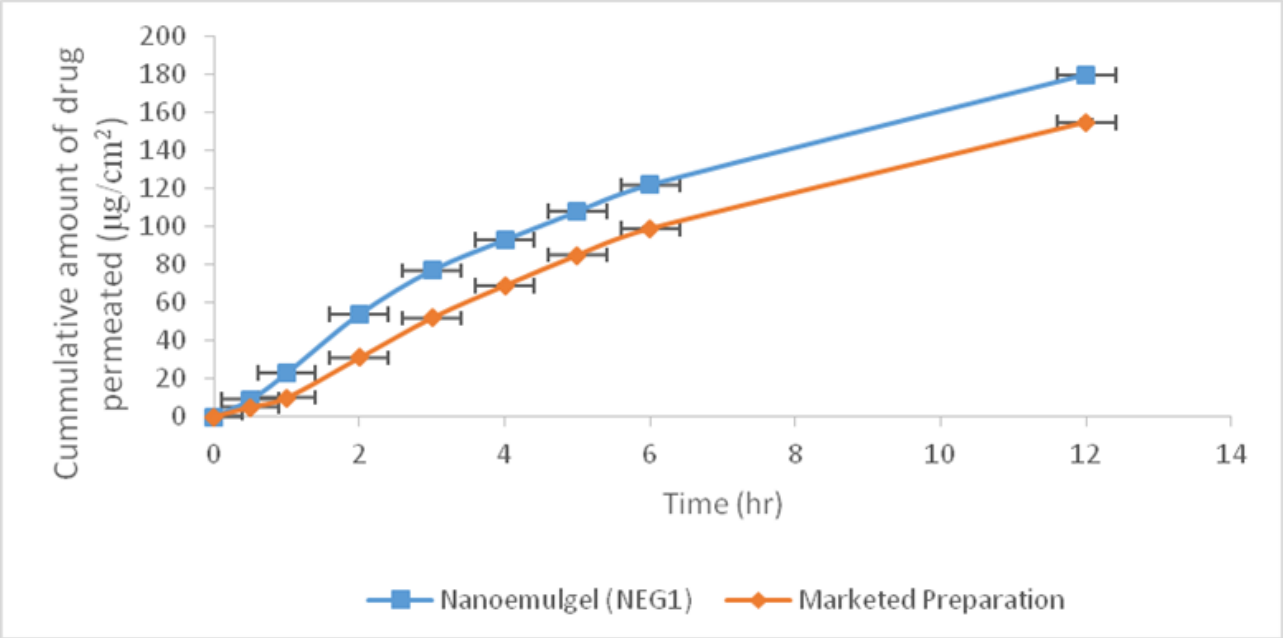

Figure 7. Comparative in-vitro permeation profile of optimized nanoemulgel (NEG1) and marketed emulgel.

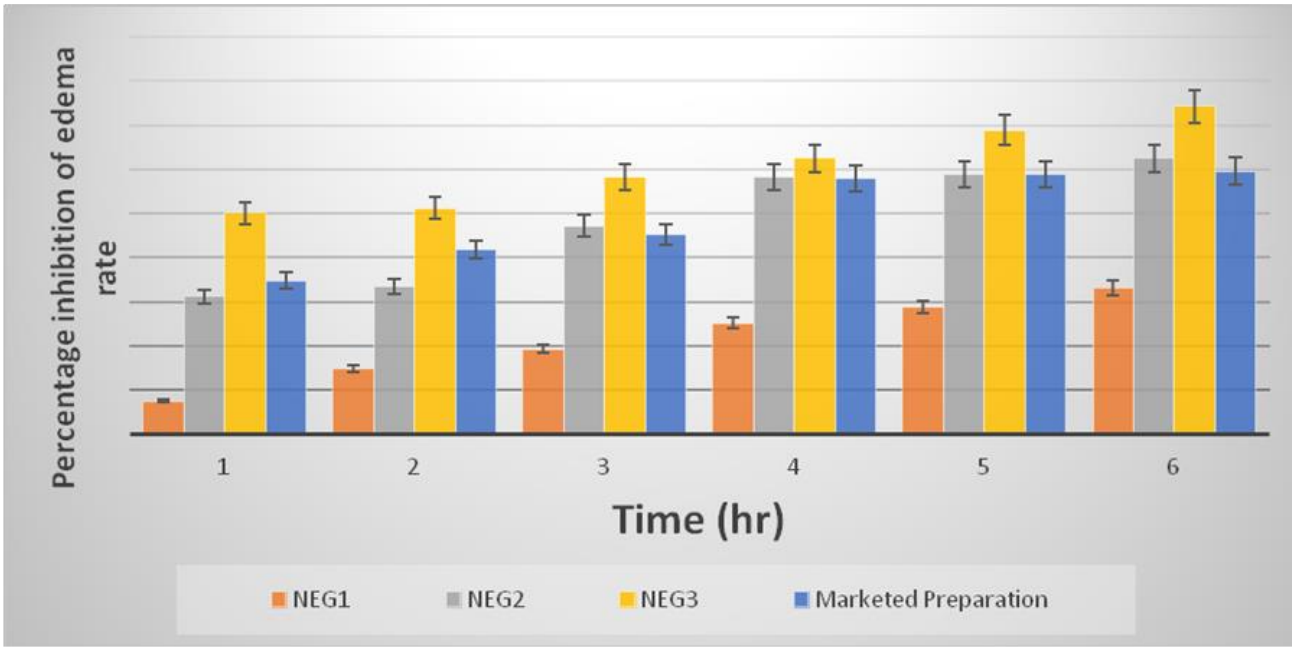

Figure 8. Comparative anti-inflammatory effect of nanoemulgel with different concentration of Myricetin (NEG1 has 1%, NEG2 has 2.5% and NEG3 has 5% w/w myricetin) and marketed emulgel.

Skin Irritation study

A skin irritation study was done to affirm the safety of the optimized nanoemulgel. The value 0 to 9 was given which indicated the irritation score of the formulation on the skin (32). The mean irritation score of skin for optimized nanoemulgel formulation (NEG2) and marketed gel (Voveran® Emulgel®) was found to be 2.2 ± 0.83 and 2.6 ± 0.54 respectively (Table.5). Lower irritation profile was shown by optimized nanoemulgel (NEG2) as compared to marketed product. It may be due to the herbal feature of Myricetin. No sign of erythema was recorded with a minimal skin irritation profile. From the result, it was concluded that the prepared nanoemulgel was significantly tolerated by rat skin and was safe for transdermal application.

Ex vivo skin permeation study

The permeation profile of the optimized Myricetin nanoemulgel (NEG1) and marketed formulation (Voveran ® emulgel®) is shown in Figure 7. The order of permeability was Myricetin nanoemulgel > marketed emulgel; and the amount of drug permeated was found to be 180 ±2.0 and 155 ±36 respectively, at the end of the 12 hours of the study. The significant difference between the permeation profiles of both formulations was observed may be

due the nanosized droplet and better permeability power of nanoemulgel (33).

The steady-state flux per unit area calculated from the slope of the linear portion of the graph was found to be 15.31 µg/cm2/h for Myricetin nanoemulgel and 14.69 µg/cm2/h for the marketed formulation, and permeability coefficient (Kp) was calculated to be 1.53 for Myricetin nanoemulgel and 0.59 for marketed formulation.

Carrageenan induced paw edema model

Prevention of paw edema induced by carrageenan is the most common and reliable model for screening of activity of the anti-inflammation drug in inflammatory diseases. Carrageenaninduced paw edema progress in two phases. The early phase start immediately after the injection and continuous for an hour. In this phase, cytoplasmic enzymes and serotonin are release from mast cells and prostaglandin level increases in the inflamed area. The next phase start after 3 to 5 hours of injection. Interleukin-1 was release inducing more polymorphic nuclear cells to accumulate in the inflamed area followed by release of lysosomal enzymes. The lysosomal enzymes destroy connective tissues and induce swelling of paw (34).

The mean percentage inhibition of nanoemulgel containing different concentration of Myricetin (1%, 2.5% and 5%) and marketed formulation (Voveran® emulgel®) containing 1.16% of diclofenac diethylamine were determined. All the formulation showed significant antiinflammatory activity when compared to control (Figure 8). From the percentage inhibition data, it was observed that the 5% w/w Myricetin nanoemulgel (NEG3) showed better inhibition profile than all other formulations with a maximum inhibition of 74.3 % after 6 hrs. Nanoemulgel with 2.5% w/w Myricetin (NEG2) showed almost similar result when compared with marketed preparation. The percentage inhibition of NEG2 and marketed preparation was observed 62.5% and 59.6% respectively after 6 hrs. Whereas nanoemulgel with 1% concentration of Myricetin showed significantly lower inhibition activity (33.1%) after 6 hrs, than all other formulations. It was also noticed that the NEG3 showed a drastic rise in inhibition followed by gradual increase whereas NEG2 and marketed formulation showed a consistent rise in the inhibition. Inhibition by NEG1 was not promising.

Conclusion

The optimized nanoemulgel of Myricetin could act as a promising surrogate carrier for transdermal delivery of Myricetin. Further it will encourage the application and commercial availability of Myricetin in pharmaceuticals as well as in supplementary food.

Contribution of authors

Nazneen Sultana: Designing, Investigation, Project administration, Writing-original draft, Writing-review & editing. Usama Ahmad: Project administration Methodology project and Formal analysis. Badruddeen: Supervision and Formal analysis. Juber Akhtar: Supervision, Conceptualization, Project administration, Formal analysis.

Acknowledgments

The authors are thankful to Xi'an Lyphar Biotech Co., Ltd., (Mainland, China) and Gattefosse India Pvt. Ltd. (Mumbai, India) for gifting the sample of drug and excipient respectively. They are also grateful to Integral University for providing technical support and assigning Communication reference no: IU/R&D/2020-MCN00212, for further communication.

Conflict of Interest

No conflicts of interest declared by the authors.

Funding

The authors are thankful to the Director of Integral University for a financial assessment.

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