BJPS

Brazilian Journal of
Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902022e20123

Article

New topical microemulsions of etofenamate as sufficient management of osteoarthritis

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In this study, microemulsions containing etofenamate were prepared and evaluated as dermal delivery carriers. The developed microemulsions consist of oleic acid, Span 80, Tween 20, Cremophor EL, Transcutol and ethanol. The percentage of etofenamate loading in the microemulsions was 5% (w/w). The characterization of formulations included droplet size, zeta potential, pH, conductivity, PDI, refractive index and viscosity. Moreover, e*x vivo* penetration study was carried out using mice abdominal skin. The developed formulations were analyzed for their cytotoxicity via MTT assay and tested for their anti-inflammatory properties opposed to LPS-stimulated nitrite production in RAW 264.7 cells. As ideal formulation, $M2_{ETF,}$ was chosen due to its greater permeation, lower penetration as well as higher anti-inflammatory activity compared to other microemulsions.

Keywords: Etofenamate. Nonsteroidal anti-inflammatory drug. Microemulsion. Ex vivo. Cytotoxicity. Anti-inflammatory activity.

INTRODUCTION

Osteoarthritis belongs to the most frequent chronic pain syndromes. Only in United States the osteoarthritis incidents have been estimated at 27 million (Plotnikoff *et al*., 2015). Clinicians as first-line medications suggest the use of acetaminophen. However, due to its ineffectiveness, nonsteroidal anti-inflammatory drugs (NSAIDs) are considered as the next step in therapy. Nonetheless, NSAIDs might present various side effects thus their topical administration instead of oral is preferred (Herndon, 2012). The topical administration of NSAIDs such as diclofenac and etofenamate, has been widely recommended for the localized pain and smaller, superficial joints. In fact, these formulations found to be efficacious in patients with osteoarthritis.

Etofenamate (ETF), 2-{[3-(trifluoromethyl) phenyl] amino}benzoic acid 2-(2- hydroxy ethoxy)ethyl ester is a non-steroidal anti-inflammatory drug (NSAID) with antipyretic, analgesic as well as antirheumatic properties which non-selectively inhibits cyclo-oxygenase (COX). ETF exists as a viscous, yellow liquid with high lipophilicity. ETF marketed formulations are either injectable or topical gels whereas they are used for the treatment of conditions as lumbago, arthritis, joint and muscular pain (Marto *et al*., 2015; Peraman *et al*., 2013).

Topical or transdermal administration of drugs can be achieved via skin (Siafaka, Barmbalexis, Bikiaris, 2016a; Langasco *et al*., 2017; Tuncay, Özer, 2013). Human skin is a physical, immunological, and sensory barrier given that it protects human body from external hazardous materials such as chemicals, microorganisms, UV radiation and prevents loss of water and biological constituents (Brown *et al*., 2006; Okur *et al*., 2020b; Siafaka *et al*., 2016b, 2016a;

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Telaprolu *et al*., 2016). The multilayered structure of skin, most particularly stratum corneum (SC)-the outer layer of the epidermis, plays an important role as main barrier (Prausnitz *et al*., 2012). Dermal drug administration could provide several benefits but also present some limitations. For instance, by applying dermal delivery carriers, the first pass metabolism can be avoided and side effects in association with systemic toxicity can be reduced. In further, a sustained and controlled release of the drug can be achieved via dermal delivery (Brown *et al*., 2006; Siafaka *et al*., 2020). Although dermal route is quite advantageous, it presents various limitations which should be regulated, in order to achieve a successful transportation of drug to the biological membranes. There are several dermal carriers, such as films, ointments, creams in situ gels or microemulsions. Microemulsions (ME) are among the most useful dermal carriers, since they present useful features as small droplet sizes as well as protection of the entrapped drug from degradation and hydrolysis. Moreover, some components of MEs could act as permeation enhancers.

MEs as promising vehicles for dermal and transdermal delivery, comprised from oil phase, aqueous phase, surfactant and co-surfactant. MEs present thermodynamically stability because of co-surfactant incorporation (Üstündağ Okur *et al*., 2011; Pillai *et al*., 2015). It has been proposed that low interfacial tension leads to MEs enhanced stability (Pillai *et al*., 2015). MEs can be easily prepared and they can load both hydrophilic and lipophilic substances. They also have long shelf-life due to their marvelous stability (Üstündağ Okur *et al*., 2011; Kajbafvala, Salabat, Salimi, 2018). These systems can be found in three main structures as swollen micellar (oil-in-water, O/W), reverse micelles (water-in-oil, W/O) and bicontinuous structures (Üstündağ Okur, Çağlar, Siafaka, 2020; Xavier-Junior *et al*., 2017).

In the past, the use of solid lipid nanocarriers for topical delivery of ETF has been reported in the literature. However, to the best of our knowledge this is the first time where microemulsions have been developed for the topical delivery of ETF. Thus, the aim of this study was the development and characterization of new ETF loaded microemulsions ($M1_{ETF}$, $M2_{ETF}$, $M3_{ETF}$, $M4_{ETF}$) as dermal delivery carriers. Therefore, in this study, the formulation, physicochemical characterization, *ex vivo* penetration studies as well as anti-inflammatory activities of the novel MEs carried out, to explore the suitability of the carriers.

MATERIAL AND METHODS

Material

Etofenamate was kindly gifted from Recordati, Turkey and Transcutol HP (diethylene glycol monoethyl ether) from Gattefosse, France. Span 80 and Tween 20 were purchased from Merck Company (Germany) whereas oleic acid, ethanol from Sigma-Aldrich (Germany) and Cremophor EL from Sigma (Germany). Acetonitrile and methanol were of High-pressure liquid chromatography (HPLC) grade and they were used for HPLC studies. All other chemicals and solvents were of analytical or HPLC grade. RAW 264.7 macrophage cell line (American Type Culture Collection-ATCC), DMEM (Gibco, UK), FBS (Gibco, USA), 1% streptomycin and penicillin (Gibco, USA), Griess reagent (1% sulfanilamide obtained by Sigma-Aldrich, USA, 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride were obtained by Sigma-Aldrich, USA in 5% phosphoric acid which was purchased by Mettler, Switzerland). In addition, LPS (lipopolysaccharide, from *E. coli* 0111:B4, Sigma, USA), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, AppliChem, Germany), isopropanol (Sigma-Aldrich, Germany), sodium nitrite (Fluka Chemika, Germany) and microplate reader (Microplate photometer, Multiskan Ascent, Finland) were used for cell culture studies.

Preparation of microemulsions

Pseudo-ternary phase diagrams were fabricated to determine microemulsion regions and ideal formulation ratios. Various potential microemulsion concentrations which can form a single phase could be selected by using the aforementioned diagrams. The titration method was chosen in order to construct a pseudo-ternary phase. Oil, surfactant and co-surfactant mixtures were prepared and titrated by adding dropwise water at 25±°C; the mixtures

were being stirred until mixture to become clear (Üstündağ Okur, Ege, Karasulu, 2014a; Üstündağ Okur *et al.*, 2014b).

It can be said that the ideal microemulsions were selected by the regions in the phase diagram (Tuncay, Özer, 2013), and ETF was loaded by dissolving it in the oil phase ($M1_{ETF}$, $M2_{ETF}$, $M3_{ETF}$, $M4_{ETF}$). The compositions of ideal formulations are given in Table I.

TABLE I - Composition of ideal microemulsion formulations (%, w/w)

Formulation code	Water	OA	Cre EL	SP80	TW20	EtOH	Transcutol HP	ETF
M1	11.32	21.45	-	4.43	5.17	57.63	-	-
$M1_{ETF}$	10.75	20.38	-	4.21	4.91	54.74	-	5
M2	28.42	13.24	29.17	-	-	29.17	-	-
$M2_{ETF}$	2.7	12.58	27.71	-	-	27.71	-	5
M3	28.51	12.37	29.56	-	-	-	29.56	-
$M3_{ETF}$	27.08	11.75	28.08	-	-	-	28.08	5
M4	19.16	12.25	-	15.83	18.47	-	34.3	-
$M4_{ETF}$	18.2	11.64	-	15.04	17.54	-	32.58	5

Solubility studies

The materials of formulations were chosen based on the solubilization capacity of ETF. The solubility of ETF was determined in several surfactants and solvents. Excess amount of ETF was added in 1 mL of selected surfactants, co-surfactants and oils and was shaken at 25 °C at 200 rpm for 24 h. Afterwards, the mixture was centrifuged at 15.000 rpm at 25 °C for 15 minutes. 10 µL of the supernatant parts were diluted to 1 mL in vials and analyzed by HPLC. Each experiment was performed in triplicate.

HLPC analysis

HP Agilent 1100 HPLC with pump and UV detector was used for the sample analysis. The used column was C18 (5µm, 150× 4.6 mm) whereas mobile phase consisted of methanol (MeOH)/acetonitrile (ACN)/purified water (H_2O) (45/35/20, v/v/v). Flow rate set at 1.0 ml/min and 10 µL sample injection volume was chosen. The UV detector was applied to monitor, at 286 nm and retention time was 6.5 minutes. The temperature adjusted at 25 °C in the auto sampler chamber (Marto *et al.*, 2015).

Determination of n-octanol/water partition coefficient

The n-octanol phase was soaked with double distilled H_2O for 24h to establish the n-octanol/water partition coefficient of ETF. ETF was weighted directly in assay tubes due to its low solubility. Afterwards 2 mL of organic phase and 2 mL of double distilled water were added. The tubes were shaken for 24h at 25±2 °C at 200 rpm and then centrifuged for 15 min at 25±2 °C at 3500 rpm. Water phases were collected and diluted from 50 µL to 10 mL with mobile phase in order to determine the concentration of ETF by HPLC (Üstündağ Okur, Yavaşoğlu, Karasulu, 2014c). The partition coefficient was calculated with the equation given below:

$$Partition\ coefficient = \frac{ETF\ concentration\ in\ octanol\ phase}{ETF\ concentration\ in\ aqueous\ phase}$$

Characterization of the microemulsions

Determination of pH

The pH values of the microemulsions were measured at room temperature using calibrated pH meter (Mettler Toledo, Switzerland). Each sample tested in triplicate and the average of these results was taken as pH of the formulations (Okur *et al*., 2019).

Drug content

100 µL of the formulations ($M1_{ETF}$-$M4_{ETF}$) were dissolved in mobile phase up to 10 mL and analyzed on HPLC to determine drug concentrations (Üstündağ Okur *et al*., 2019a).

Determination of viscosity of microemulsions

AND Vibro Viscometer was used to measure viscosity. The microemulsions were placed in the sampler tube and analyzed at 25 °C. Each sample tested in triplicate.

Determination of zeta potential, droplet size and PDI

Dynamic Light Scattering method (Nano ZS, Malvern Instruments, U.K.) was applied to calculate the average droplet size and polydispersity index (PDI). Experiments to measure the particle size and PDI values were rerun five times at 25 °C. The results were acquired by obtaining the average of five measurements at an angle of 173° by utilizing disposable cells (Üstündağ Okur *et al*., 2014b).

Zeta potential

To measure the zeta potential of samples, disposable plain folded capillary zeta cells (Malvern Zetasizer Nano ZS) were used. The zeta potential was estimated by the electrophoretic mobility and via Helmholtz–Smoluchowski equation, under an electrical field of 40 V/cm. Software involved system was used for this process. The measurements were repeated five times at 25±2 °C (Üstündağ Okur *et al*., 2014b).

Conductivity measurement

Electrical conductivity measurement was performed in triplicate for each formulation at 25 ± 2 °C by using conductometer and conductometer prob (Milwaukee MW801, U.S.). The results are presented as mean ± SD (Üstündağ Okur *et al*., 2011; Üstündağ Okur *et al*., 2015).

Stability of ETF loaded microemulsions

$M1_{ETF}$, $M2_{ETF}$, $M3_{ETF}$ and $M4_{ETF}$ formulations were stored at 4±1 °C, 25±2 °C and 40±2 °C for 12 months, so as to examine their stability. ETF loaded microemulsions were examined in terms of their clarity, pH, viscosity, refractive index, phase separation, droplet size and electrical conductivity (Aksu *et al*., 2019). Centrifuge tests were performed, before their storing in the specified conditions, at 13.000 rpm for 30 min in order to determine the physical stability of microemulsions.

Besides, the heating-cooling cycle test was also carried out by storing formulations between 4±1°C and 40±1°C for 24h as one cycle. Six cycles were completed before investigating the formulations in terms of clarity, precipitation and phase separation (Üstündağ Okur *et al*., 2015).

Ex vivo studies (permeation and penetration studies)

Mice abdominal skin and diffusion cell of 0.785 cm^2 effective area was applied for *ex vivo* permeation evaluation of ETF loaded MEs. After mounting the skin samples on diffusion cells, receiver and donor compartments were prepared. 10 mL ethanol:PBS (ratio of 30:70) was used as a receptor compartment to ensure sink condition. 1 mL formulation was placed on a donor compartment and parafilm was used to prevent evaporation. Temperature was set at 37±1°C while magnetic stirring at 600 rpm was also performed during the experiment. 50 µL sample of the receiver compartment was withdrawn at a predetermined time and replaced with an equal volume of receptor solution. All samples were analyzed through HPLC (Üstündağ Okur *et al*., 2019b).

After 24h of the experiment, each skin was cut into pieces and placed in MeOH for penetration study. To determine the amount of ETF withheld in the skin, the tube containing methanol and skin was homogenized for 10 min, filtered through a membrane filter with 0.2 µm pore width. Samples from the solution were analyzed in HPLC.

Anti-Inflammatory activity and cytotoxicity of etofenamate formulations on RAW 264.7 macrophage cells

Cell cytotoxicity

RAW 264.7 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin, at 37 °C in 5% CO_2. RAW 264.7 cell line was plated using a density of 1×10^5 cells per well with 250 µL of growth medium. Cells were treated with $M1_{ETF}$, $M2_{ETF}$, $M3_{ETF}$ and $M4_{ETF}$ for 1 day in presence of LPS (1µg/mL). After the removal of cell medium, 100 µL of MTT solution (0.5 mg/ml) was added and incubated for 2 hours at 37°C. At the end of the incubation period, the medium was removed and 100 µL of isopropanol was added in each well and shaken gently to solubilize the dark blue crystals. Optical absorbance was assessed at 570 nm. The relative cell viability (%) was determined via the following formula, where OD depicts optical density (Aydin *et al*., 2018; Karadağ *et al*., 2019).

Cell viability (%) = OD570 (sample)/OD570 (medium control) × 100

Nitrite assay

Plated RAW264.7 cell line (10^6 cells/ mL) was incubated at 37°C for 24 h. The cells were pretreated with formulations $M1_{ETF}$, $M2_{ETF}$, $M3_{ETF}$, $M4_{ETF}$ at different concentrations by serial dilutions with DMEM. Indomethacin (100 µM) was used as reference whereas non-cytotoxic concentrations were selected for the nitrite assay. The studied concentration ranges were as follows: Formulation $M1_{ETF}$: 0.16-2.50 (‰ v/v), Formulation $M2_{ETF}$: 0.04-0.63 (‰ v/v), Formulation $M3_{ETF}$: 0.04-0.63 (‰ v/v) and Formulation $M4_{ETF}$: 0.08-1.25 (‰ v/v). After 2 hours of pretreatment, cells were aroused alongside 1µg/mL LPS for additional 22 hours. The nitrite concentration in the medium was measured by using a colorimetric method based on the Griess reaction. Samples were subjected to absorbance reading by a microplate reader at 540 nm. A sodium nitrite standard curve was applied for the calculation of nitrite amount in the tested MEs (Aydin *et al*., 2018; Karadağ *et al*., 2019; Koksal *et al*., 2017) and % inhibition was determined via the following formula:

% Inhibition = $100-[(N_1*100)/N_0]$,
N_o: Nitrite amount (µM) of LPS-treated medium control,
N_1: Nitrite amount (µM) of MEs

Statistical analysis

The outcomes are shown as the mean ± SD of experiments. Statistical significance was determined via one-way ANOVA followed by Tukey's test, using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA)}. $p < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Determination of ETF solubility

It has been reported that the drug has to be dissolved in order to permeate the skin. Hence, solubility studies using different solvents and surfactants were carried out to identify the ideal ingredients of ETF microemulsion (Abd-Allah, Dawaba, Ahmed, 2010). It was revealed that ETF maximum solubility was obtained using EtOH and Transcutol HP at 253.108±13.590 mg/mL and 243.117±2.996 mg/mL, respectively. Thus, Transcutol HP was also selected as surfactant and EtOH as co-surfactant. Moreover, solubility on oleic acid was established at 134.547±2.520 mg/mL. This value was the greatest among the studied oils, so oleic acid was selected as oil phase for microemulsions. The results of solubility study are given in Table II.

TABLE II - Solubility of ETF in different surfactants, co-surfactants, and oils

Component	Solubility (mg/mL)
Distilled water	0.029 ± 08.347
EtOH	253.108 ± 13.590
Span 80	125.351 ± 18.750
Tween 20	176.047 ± 5.612
Transcutol HP	243.117 ± 2.996
Oleic acid	134.547 ± 2.520
Cremophor EL	177.991 ± 8.852
Tween 80	122.624 ± 11.345
Span 20	2.445 ± 0.497
Propylen glycol	116.127 ± 1.709
Labrafil	154.499 ± 3.703
Labrasol	200.493 ± 1.879
Capryol 90	215.483 ± 0.695
Labrafac PG	217.588 ± 8.723
Tween 60	173.631 ± 0.980
Cremophor RH 40	46.553 ± 8.151
Almond oil	111.340 ± 10.758
Castor oil	134.022 ± 13.732

Determination of n-octanol/water partition coefficient

The stratum corneum is the main lipophilic barrier of the skin which generally controls the molecules penetration according to their lipophilicity. Thus, drug lipophilicity is determined by logP of n-octanol/water. Herein, logP of ETF was calculated 4.21. This result shows that ETF is lipophilic compound and can permeate through the skin (Üstündağ Okur *et al*., 2011).

Preparation of ETF loaded microemulsions

Pseudo-ternary phase diagrams were applied to calculate the combination of phases. The domains of the existing transparent, isotropic systems are similar to microemulsion phases. The ternary phase diagrams of all the ratios are shown in Figure 1. It can be said, that more than one stable microemulsions were obtained and their component ratios are shown in Table I. Accordingly, the ideal formulations were selected and 5% ETF (w/w) was dissolved in their oil phase. It has been reported that hydrophilic surfactants promote oil-in-water (O/W) microemulsion formation. In our study, Cre EL (HLB 12-14), TW20 (HLB 16,7) and SP80 (HLB 15) were used, so an O/W microemulsion was prepared (Lu, Gao, 2010).

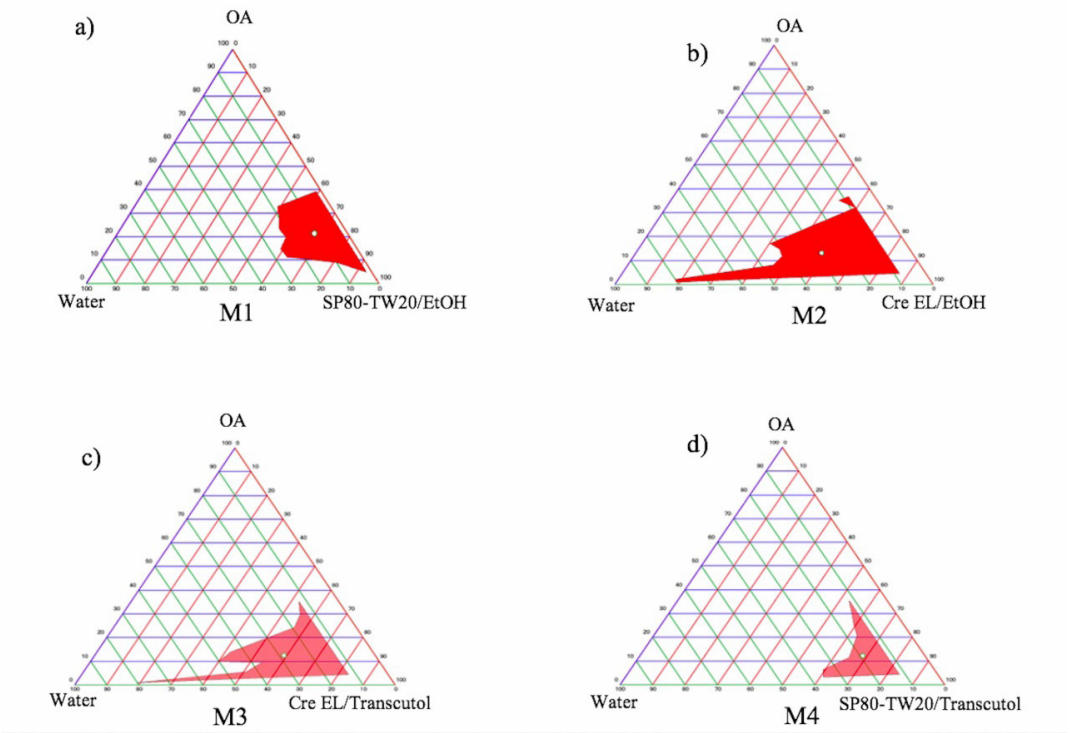

FIGURE 1 - The pseudo-ternary phase diagrams of the microemulsion. (a) The pseudo-ternary phase diagram of the microemulsion composed of OA, SP80, TW20, ethanol and water (w/w). (b) The pseudo-ternary phase diagram of the microemulsion composed of OA, Cre EL, EtOH and water (w/w). (c) The pseudo-ternary phase diagram of the microemulsion composed of OA, Cre EL, Transcutol HP and water (w/w). (d) The pseudo-ternary phase diagram of the microemulsion composed of OA, SP80, TW20, Transcutol HP and water (w/w).

Characterization of microemulsions

The physicochemical characterization of microemulsions intended to be used in dermal applications is an important issue which should be condsidered during formulation stage (Çağlar *et al.*, 2019). The characterization parameters and results of the microemulsions are listed in Table III.The pH values of the formulations can be criticized as appropriate for dermal application as they were between 4.84±0.01 and 5.42±0.01 (Ali, Yosipovitch, 2013; Okur *et al.*, 2019). Nanotechnology is still the emerging application field for pharmaceutical technology. Nanomaterials are claimed to be promising in the medical field especially as imaging agents or local drug carriers due to their large surface area, improved tissue penetration, and controlled drug delivery (Siafaka *et al.*, 2019). Thus, the droplet size of a carrier is significant value when topical drug delivery systems are studied considering that vehicles of sizes under 300 nm can deliver their ingredients tothe deep skin layers. The prepared MEs present sizes under 185 nm, revealing that can potentially deliver ETF delivery into the skin. PDI values measured between 0.257±0.047 and 0.429±0.044 determining homogeneity. Zeta potential values showed that all formulations were almost neutral as they were between 0.0028±0.0006 and 0.8886±0.4229. This means no precipitation was expected.

The refractive index measurement was carried out by refractometer and found to range between 1.3985±0.003 ($M1_{ETF}$) to 1.4386±0.0008 ($M4_{ETF}$).The viscosity measurement was carried out by AND Vibro Viscometer; viscosity calculated as 6.59±0.01 in case of $M1_{ETF}$ and 415.67±7.64 for $M4_{ETF}$.

Electrical conductivity has been widely utilized for the analysis of MEs microstructure (Abd-Allah, Dawaba, Ahmed, 2010). Herein, conductivity measurement was performed using Milwaukee MW801 conductometer and conductometer prob. It was found at 10.5±0.1 of $M1_{ETF}$ and 17.93±0.45 for $M4_{ETF}$.

TABLE III - Characterization of the developed blank and ETF-loaded microemulsion formulations (mean ± SD, n=3)

Formulation/ parameters	pH	Droplet size (nm)	PDI	Zeta potential (mV)	Refractive index	Conductivity (mS/cm)	Viscosity (cP)
M1	4.93±0.021	9.276±0.35	0.257±0.047	0.0028±0.0006	1.393±0.001	20.03±0.208	5.87±0.0153
$M_{1ET}F$	4.84±0.006	7.523±0.375	0.429±0.044	0.0084±0.0018	1.3985±0.003	17.93±0.451	6.59±0.0058
M2	5.22±0.000	8.183±0.65	0.351±0.03	0.261±0.0041	1.4032±0.001	16.87±0.306	113.67±1.5275
$M2_{ETF}$	5.17±0.012	109.8±1.572	0.314±0.046	0.0554±0.0036	1.4095±0.009	15.13±0.208	76.87±0.1528
M3	5.36±0.000	162±1	0.334±0.0076	0.8886±0.4229	1.4237±0.001	11.73±0.153	213.33±0.5774
$M3_{ETF}$	5.42±0.010	184±8.185	0.332±0.0076	0.6004±0.4432	1.43±0.003	10.50±0.100	415.67±7.6376
M4	5.35±0.012	183.6±0.141	0.232±0.0457	0.8504±0.3815	1.433±0.005	19.77±0.208	178.33±4.1633
$M4_{ETF}$	5.35±0.000	125.45±3.18	0.319±0.0199	0.6882±0.4358	1.4386±0.008	17.80±0.361	216.33±1.5275

Stability studies of microemulsions

Blank and ETF loaded microemulsions were examined at 4±2°C, 25±2°C and 40±2°C to check their stabilities. It has been reported that physical stability of MEsis dependent on particle size and particle size distribution which are critical boundaries on in-processcontrol and especially inqualityassurance (Vicentini *et al.*, 2011). It was revealed that there was not significant difference in droplet size of tested MEs for the duration of 12 months storage at 4±2°C and 25±2°C. ETF concentration was still above 75% during 12 months of storage. The concentration change is given in Figure 2. The value of viscosity was also stable. In addition, ETF loaded microemulsions showed no changes in physical appearance after centrifugation and freeze-thaw cycles.

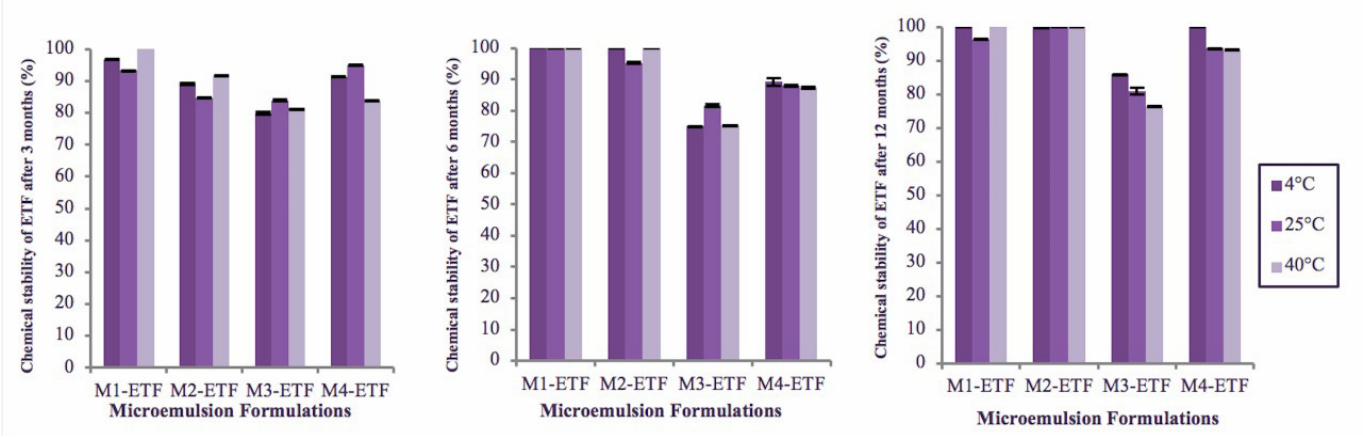

FIGURE 2 - ETF amount change for 3, 6 and 12 months storage at 4 ± 2°C, 25 ± 2°C and 40 ± 2°C.

Ex vivo studies (permeation and penetration studies)

$M1_{ETF}$, $M2_{ETF}$, $M3_{ETF}$ and $M4_{ETF}$ microemulsions were prepared for ex vivo penetration and permeation study. Mice abdominal skins were used. The permeation percentage of ETF through the mice skins into the receptor phase and penetrated ETF in the skin after 24 h is shown in Table IV. After 24 hours, the highest penetration detected for $M1_{ETF}$ was 63% and for $M2_{ETF}$ was 47%.

The penetrated ETF amount was found at 63% for $M1_{ETF}$ and 25% for $M2_{ETF.}$ It can be said that these were the highest and lowest value. It can be concluded that $M1_{ETF}$ performed better in permeation and penetration study.

According to Mura's study (2011), the permeation percentage difference between $M3_{ETF}$ and $M4_{ETF}$ occurred due to Transcutol HP amount in the microemulsion. It is believed that as Transcutol HP concentration is increased, the drug could reach deeper in the skin layers (Mura *et al.*, 2011). The main mechanism of Transcutol HP as permeation enhacer is to increase the partition parameter of the drug into the skin. This could bedue to the close solubility parameter of Transcutol HP with skin (Haque, Talukder, 2018). In another study, researchers proved that Cre EL enhanced permeation of active compound (Abd-Elsalam, El-Zahaby, Al-Mahallawi, 2018). The same correlation was also found between $M1_{ETF}$ and $M2_{ETF}$.

TABLE IV - Ex vivo permeation and penetration studies result of $M1_{ETF}$, $M2_{ETF}$, $M3_{ETF}$ and $M4_{ETF}$ formulations

Formulation Codes	% Permeation from skin (after 24 h)	% Penetration to skin
$M1_{ETF}$	42.894 ± 3.96	63.963 ± 0.012
$M2_{ETF}$	47.884 ± 2.30	25.912 ± 0.201
$M3_{ETF}$	24.856 ± 3.85	30.399 ± 0.170
$M4_{ETF}$	35.466 ± 0.73	26.812 ± 0.111

Cell cytotoxicity

When novel drug carriers are developed, their potential to be cytotoxic should be examined by various methods. In vitro cell cytotoxicity assay can predict the *in vivo* possible toxicity of the developed carriers. Herein, the MEs were tested for their cytotoxicity against RAW 264.7 cell lines via MTT assay (Table V). The concentration of MEs, which did not significantly affect cell viability, were further chosen and used for the subsequent nitrite assay. IC_{50} values of formulations, which stands for the inhibition of cell growth by 50%, were as follows: Formulation $M1_{ETF}$:2.10±0.04‰ (v/v), Formulation $M2_{ETF}$:0.54±0.02‰ (v/v), Formulation $M3_{ETF}$:0.52±0.01‰ (v/v), Formulation $M4_{ETF}$:1.03±0.02‰ (v/v). As it was expected, the developed MEs were not quite cytotoxic in desirable dilution, and they can be safely applied for dermal delivery. The obtained data were expected since the used chemical substances are non-toxic. Similar results were reported by Okur et al. who studied the possible cytotoxicity of ME based gels loaded with fusidic acid on RAW 264.7 cell lines and examined them as dermal burn wound healing systems (Okur *et al.*, 2020a).

TABLE V - Effects of formulations ($M1_{ETF}$, $M2_{ETF}$, $M3_{ETF}$ and $M4_{ETF}$) on the viability of LPS-treated RAW 264.7 macrophages at different concentrations (‰ v/v)

Cell viability % (Relative to medium control) (Mean ± SD)		
Medium Control		**100.0 ± 2.40**
Dilution of the tested sample (v/v)		
Formulation $M1_{ETF}$	0.16‰	105.7 ± 5.4
	0.32‰	107.9 ± 4.3
	0.63‰	107.3 ± 2.2
	1.25‰	109.4 ± 7.9
	2.50‰	23.74 ± 5.5
Formulation $M2_{ETF}$	0.04‰	100.6 ± 1.9
	0.08‰	103.0 ± 2.5
	0.16‰	105.7 ± 0.8
	0.32‰	104.1 ± 2.7
	0.63‰	19.0 ± 5.2
Formulation $M3_{ETF}$	0.04‰	103.5 ± 3.4
	0.08‰	105.3 ± 4.9
	0.16‰	104.7 ± 7.0
	0.32‰	101.5 ± 3.4
	0.63‰	20.80 ± 1.1
Formulation $M4_{ETF}$	0.08‰	106.4 ± 5.8
	0.16‰	101.6 ± 2.8
	0.32‰	111.3 ± 6.6
	0.63‰	108.1 ± 7.9
	1.25‰	16.9 ± 3.1

Nitrite assay

MEs were studied for their inhibitory properties opposed to LPS-induced nitrite production in RAW 264.7 cell lines. The comparison of anti-inflammatory activities of the formulations with the reference molecule, indomethacin (100 µM), are shown in Figure 3. Nitrite inhibition (%) of formulations $M1_{ETF}$, $M2_{ETF}$, $M3_{ETF}$ and $M4_{ETF}$, at their highest studied concentrations, was calculated at 51.3%, 73.7%, 55.9% and 52.1% respectively. Formulation $M2_{ETF}$ exhibited the highest anti-inflammatory activity compared to the indomethacin treated group (57.6%). It can be said that the developed MEs present anti-inflammatory effects in LPS induced RAW 264.7 macrophage in a dose dependent manner. Similar data reported by Okur *et al.* (Okur *et al.*, 2020a).

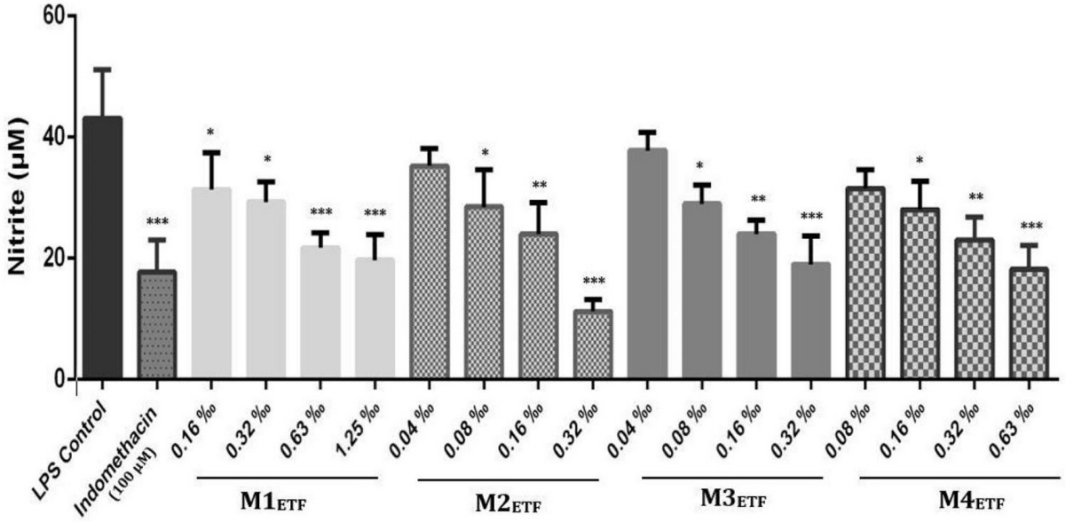

FIGURE 3 - The amount of nitrite in LPS-induced RAW 264.7 macrophages. Values are expressed as the mean ± SD of various experiments. *$p < 0.05$. **$p < 0.01$ and ***$p < 0.001$ indicate a significant difference from the LPS-stimulated control group.

CONCLUSION

The present study demonstrates that the microemulsions can be applied as an alternative system for the enhancement of poorly water-soluble drugs like ETF. Solubility assays exhibited that oleic acid, almond oil, and castor oil led to the greatest solubilization of ETF, and consequently oleic acid was chosen as oil phase. From the constructed ternary phase diagrams, the optimal microemulsions were selected for further analysis. The optimized microemulsion containing oleic acid, Cremophor EL, ethanol and distilled water was transparent with low viscosity, appearing the droplet size of 109.8±1.572 nm. From the stability studies, it was confirmed that the optimal microemulsion was stable for 12 months. As a result of ex vivo studies, the optimized formulation showed the maximum permeation through the skin and showed the minimal penetration amount to the skin. At last, M2ETF was found to be the most effective to inhibit nitrite production. As it is explained before, this result indicates that M2ETF presents the highest anti-inflammatory effect, among others. In conclusion, this study showed that etofenamate could be delivered successfully via dermal administration route when it is loaded to microemulsion formulation.

ACKNOWLEDGEMENTS

The authors would like to thank the Recordati and Gattefosse for providing the etofenamate and Transcutol HP. Authors wish to thank Prof. Dr. Dilek Telci of Yeditepe University, Faculty of Engineering, Department

of Genetics and Bioengineering (İstanbul, Turkey) for providing the used RAW 264.7 cell line.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Received for publication on 10th March 2020
Accepted for publication on 13th July 2020