

Original Article

DESIGN AND DEVELOPMENT OF NOVEL MICROEMULSION BASED TOPICAL FORMULATION OF HESPERIDIN

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ABSTRACT

Objective: Bioflavonoid hesperidin is used primarily to assist treatment of capillary disorders like hemorrhoids and varicose veins, by reducing capillary permeability. There are certain limitations to the use of these bioflavonoid in the pharmaceutical formulations because of their physical properties like limited aqueous solubility, poor bioavailability and high oral dose. Therefore in the present research work micro emulsions of hesperidin were developed for improving solubility and bioavailability using topical route.

Methods: Micro emulsions of hesperidine were prepared by plotting pseudo ternary phase diagram using eucalyptus oil, Tween 80, AccononCC6 and water. The micro emulsion with optimized droplet size, polydispersity index and ex vivo diffusion ability was then converted into the ointment for ease of application. Droplet size and Polydispersity index were obtained by using Photon correlation spectroscopy. Optimized o/w microemulsion of hesperidin composed of eucalyptus oil 20%, Tween-80/Acconon CC-6(2:1) 33% and water was then converted into ointment using optimized ointment base. The microemulsion loaded ointment was then characterized for physicochemical parameters. Ex-vivo permeation and *In-vivo* bioavailability studies were carried out to check the r. release profile of drug from the prepared formulations.

Results: It was observed from ex-vivo permeation studies that flux value for optimized microemulsion was found to be 8.971 $\mu\text{g}/\text{ml}/\text{cm}^2$ as compared to pure hesperidin (1.230 $\mu\text{g}/\text{ml}/\text{cm}^2$). This formulation was then converted into ointment by using optimized base containing PEG-6000, PEG-400 and Cetyl alcohol. This microemulsion based ointment passed all the characterization tests (droplet size analysis using PCS, *ex-vivo* permeation studies, *in-vivo* bioavailability studies etc.) as well as remained stable for the period of 3 mo during stability studies as per ICH guidelines. The bioavailability studies of hesperidin microemulsion based ointment in rats showed 3 fold statistically significant ($p < 0.001$) improvement in bioavailability as compared to microemulsion when applied topically.

Conclusion: Thus it can be concluded that components of microemulsion and ointment are contributing to improve bioavailability of hesperidin.

Keywords: Hesperidin, Bioflavonoid, Venous disease, Microemulsion, Topical, *In-vivo* bioavailability.

INTRODUCTION

Hesperidin is the major flavanone glycoside in sweet orange and lemon obtained as an abundant byproduct of Citrus cultivation [1, 2]. Hesperidin has antioxidant, anti-inflammatory, hypolipidemic, vasoprotective, and cholesterol-lowering properties [3] and it plays important role in the inhibition of enzymes involved in several diseases like phospholipase A2, lipoxygenase [4]. It is known to reduce permeability and fragility of capillary walls [2]. The bioavailable formulations of this bioflavonoid may prove to be an effective treatment for many blood vessel disorders like hemorrhoids, varicose veins, venous stasis etc. In all these diseases proper therapeutic treatment is not widely available. As a result patient suffers silently until the disease aggravates to the level of surgery. For improving therapeutic efficacy of hesperidin it is required to identify the problems associated with its bioavailability in order to develop various formulations which can prove to be effective to treat moderate symptoms of venous diseases at an early stage.

Hesperidin is reported to be unstable at gastric pH where it undergoes hydrolysis into aglycone hesperetin and enzymatic degradation. Moreover, probably due to its crystalline state, this flavonoid is slightly soluble in water [5], a characteristic which leads to a very low dissolution rate and an irregular absorption from oral solid dosage forms in the gastrointestinal tract (oral bioavailability < 25%) [2]. Literature reports few attempts made to improve oral bioavailability of hesperidin by preparing oral formulation containing spray dried gastro-resistant microparticles [6].

It is also evident from the literature that when poorly soluble actives have bioavailability problems through oral route, the molecules show good permeability through lipophilic membranes like skin [7]. Rate limiting step for drug permeation is poor drug dissolution i.e.

dissolution of actives from formulation is not fast enough to replace permeated molecules which affects the permeation rate [8]. As only dissolved fraction of drug in the vehicle can enter the skin, solubility of the drug in the vehicle is an important aspect in the formulation of topical dosage forms [9].

Therefore in the present research work attempt has been made to develop a novel topical formulation of hesperidin having better solubility in the vehicle as well as good permeation ability through the skin to show venoprotective effect. It was thought that topical formulation will improve the patient compliance as a chronic treatment for mild to moderate symptoms of venous diseases.

It is a challenge to develop a topical formulation with enhanced permeation ability, high drug-loading capacity and less skin irritation. Microemulsions have been visualized as superior drug carriers because of its several permeation enhancement mechanisms such as an increased concentration gradient and thermodynamic activity towards skin as well as the permeation enhancement activity of the components [10].

High solubilization capacity of microemulsion helps in improving bioavailability of poorly soluble drugs. Literature reports microemulsions as efficient vehicles for the topical delivery of poorly soluble drugs such as triptolide and estradiol [10, 11].

In the present work hesperidin o/w microemulsions were prepared using various oils and were characterized for droplet size as well as other physical parameters and were converted into ointment so that they gain suitable viscosity for topical application of hesperidin.

The *in-vitro* permeation study was carried to evaluate the effect of microemulsion components and ointment bases on permeability of the drug through excised rat skin. *In-vivo* permeation studies were

carried out on rats to determine bioavailability of hesperidin by topical application.

MATERIALS AND METHODS

Materials

Hesperidin was purchased from Aaisland Chemical Products, Aurangabad, India. Soyabean oil, eucalyptus oil were acquired from Research lab Fine chemical industries, Mumbai. Tween 80, PEG-1500, PEG-400 and Cetyl alcohol were obtained from Loba Chemie, Mumbai, India. Acconon CC-6 (ethoxylated caprylic and capric glycerol esters) was a gift sample provided by Abitec Corporation, Janesville, WI. USA.

Formulation of o/w microemulsion of hesperidin

Various oils like soybean oil, eucalyptus oil, surfactants and co surfactants were screened for hesperidin solubility in order to formulate hesperidin o/w micro emulsion. Suitable concentration

ranges of components for the existence in micro emulsion region were obtained by constructing pseudo ternary phase diagrams fig. (1) Using water titration method at ambient temperature.

According to the micro emulsion regions in the phase diagrams formulations were prepared (table 1) and were characterized for transmittance and visual clarity, viscosity, droplet size and electrical conductivity (table 3). Micro emulsions passing characterization tests were selected for drug loading.

Drug loading was carried out by adding excess amount of hesperidin to each of the selected micro emulsion (5 ml) in a stoppered vial. The vials were then equilibrated at 37 ± 1.0 °C in a water bath shaker (Remi, Mumbai India.) for 72 h and subsequently centrifuged at 3000 rpm for 15 min. The supernatant was filtered through a 0.45 µm membrane filter. The concentration of hesperidin was determined by UV spectrophotometer at 285 nm. Each experiment was carried out in triplicate and results were expressed as mean \pm standard deviation (table 2).

Table 1: Composition of microemulsion formulations

% Composition	Formulation code (Quantities in percent)			
	E 1	E 2	E 3	E 4
Eucalyptus oil	20	25	10	5
Tween 80	22	20	24	28
Acconon-cc-6	11	10	12	14
Water	47	45	54	53

Table 2: Drug loading of optimized micro emulsions

Formulation code	Drug loading (mg/ml)
E 1	18.6 \pm 0.7
E 2	13.8 \pm 0.2
E 3	11.3 \pm 0.3
E 4	11.7 \pm 0.5

(mean \pm SD, n=3) E1-(micro emulsion containing 20% eucalyptus oil, s-cos ratio 2:1), E2 (micro emulsion containing 25% eucalyptus oil, s-cos ratio 2:1), E3 (micro emulsion containing 10% eucalyptus oil, s-cos ratio 2:1), E4 (micro emulsion containing 5% eucalyptus oil, s-cos ratio 2:1)

Drug loaded microemulsions were subjected to droplet size analysis by using Photon Correlation Spectrometer (DTS version 5.10 beta 1, serial No. MAL501131, Malvern Instruments Ltd.). Samples were loaded into 1 cm² cylindrical cuvettes and placed in a thermostated scattering chamber. The aperture of the photomultiplier tube was set at 50 nm.

Ex-vivo skin permeation studies of all the drug loaded formulations were carried out using Franz diffusion apparatus fitted with the abdominal skin of excised male Sprague-Dawley rats weighing 200-250 g. Saturated solution of hesperidin in pH 7.4 phosphate buffer was used as a control for comparison with ex-vivo permeation rates of hesperidin microemulsions. Data obtained from the analysis was further analysed to determine the permeation rate at steady state (Jss, µg/cm²/h) and Permeability coefficient (Kp cm/h).

Preparation of micro emulsion based ointment (MBO) of hesperidin

Optimized microemulsion formulations were converted into ointment for attaining suitable viscosity which subsequently improves applicability of the formulation. For the preparation of MBO, water soluble ointment bases were used which consists of carbowaxes. The most suitable carbowax ointment bases contain heavy and light molecular-weight polyethylene glycols. Ointment base formulae containing mixture of various grades of Polyethylene glycols (PEGs) were physically evaluated.

The formula showing better physical properties and stability was selected for incorporation of microemulsion (table 4). Ointment base containing Polyethylene Glycol 1500 (47.5%), Polyethylene Glycol 400 (47.5%) and Cetyl Alcohol (5.0%) was prepared by fusion

method [12]. The hesperidin microemulsion was added to the ointment base (1 ml/g) on an ointment slab and mixed using spatula to ensure uniform mixing.

Characterization of microemulsion based ointment

Evaluation of pH and viscosity

The pH values were evaluated at 25 °C by using a LI120 digital pH meter (Elico, pH meter, Type 003). The pH of the formulations was measured by mixing 5 g of MBO in 45 ml of water. The pH of the formulations should be compatible with that of the skin in order to avoid skin irritation.

Viscosity of the MBO was measured using Brookfield DV-II+Pro viscometer. Experiments were carried out in triplicate for each sample, and the results are expressed as an average \pm standard deviation.

Analysis of drug content

Accurately weighed 1g of MBO sample was transferred to 10 ml volumetric flask and volume was adjusted using Dimethyl Sulphoxide. The resulting solution was filtered using 0.45 µm filter.

1 ml of filtrate was pipette out and was transferred to 50 ml volumetric flask to make up the volume using phosphate buffer (pH 7.4). This solution was then analyzed at 285 nm using UV Spectrophotometer to determine the content of hesperidin in the formulation.

Extrud ability

Extrudability of MBO formulations was determined using a closed collapsible tube containing 20 g of MBO. The tube was pressed firmly at the crimped end and a clamp was applied to prevent any

rollback. The cap was removed and the MBO was extruded until the pressure was dissipated.

Spread ability

Spread ability of the MBO formulation was determined by an apparatus which consists of a wooden block with pulley at one end. A rectangular ground glass was fixed on this block. An ointment (about 3 g) was placed on this ground plate and was sandwiched using another glass plate having the dimension as that of the fixed ground plate which was provided with the hook. 1 Kg weight was placed on the top of the plate for 5 min to expel air and to provide a uniform film of the ointment between the plates. The top plate was then subjected to pull off 80 g weight with the help of string attached to the hook and the time (in seconds) required by top plate to cover a distance of 10 cm was noted. A shorter interval indicates better spread ability [13]. Spread ability is given in unit g. cm/sec and was determined by the following formula-

$$S = M \times L / T$$

Where, L= length moved by glass slide

T= Time in sec

M=Weight in pan

S= Spread ability

Ex-vivo permeation study

Ex-vivo skin permeation studies on micro emulsion and MBO formulations were performed using Franz diffusion apparatus fitted with the abdominal skin of excised male Sprague-Dawley rat weighing 200-250 g. Before using rat skin was cleaned and washed with saline. It was mounted on the receptor chamber of the diffusion cell assembly having effective diffusion area of 3.14 cm² which was filled using 20 ml of pH 7.4 phosphate buffer saline (PBS). Accurately weighed 1g of formulation was applied on the epidermal surface of the mounted rat skin. The receptor fluid was continuously stirred using small magnetic bar and the temperature was maintained at 37°C±1°C. An aliquot of 1 ml of sample was withdrawn after each interval and the same amount of fluid was replaced using fresh phosphate buffer pH 7.4. Collected samples were filtered through 0.45µm filter and hesperidin was quantified by using UV spectrophotometer at 285 nm. Saturated solution of hesperidin in pH 7.4 phosphate buffer was used as a control since topical marketed formulation of hesperidin was not available. Each experiment was carried out in triplicate and the results were expressed as an average±standard deviation (fig. 2, table 6).

Stability studies

Three batches of each formulation were filled in sealed glass containers and kept in stability chamber (Remi, India) at 40±2 °C (75±5% RH) and 25±2 °C (60±5% RH) for three months. The stability of the formulations was checked periodically with respect to appearance, pH, viscosity, spread ability, extrude ability and drugcontent.

In-vivo bioavailability study

The animal study protocol was reviewed and approved by the Institutional Animal Ethical Committee, Gurunanak College of Pharmacy (Regd no. 675/02/C/CPCSEA, Dated-13/09/07), RTM Nagpur University, India. Male Wistar strain rats of both sexes were selected for the study. All the experimental rats were housed in cages, with access to food and water ad libitum until use. 24 h prior to the experiment the dorsal skin of rats were shaven to remove hair and washed with physiological saline solution. Animals were grouped as Group1, Group 2 and Group 3 containing 12 animals each. Accurately weighed 1 g of micro emulsion (drug content 0.8%, w/w), MBO (drug content 0.8%, w/w) was applied on the dorsal surface of the rat skin (1.76 cm²) for Group 1 and Group2 respectively. Ointment containing thoroughly mixed hesperidin powder was used as a control and was applied to animals in Group 3 (1g equivalent to 0.8%w/w hesperidin). One animal was used as a blank control. After application of formulations, blood samples were withdrawn by retro-orbital technique at regular intervals. Each time

1-1.2 ml of blood was withdrawn and allowed to clot. The blood samples were then centrifuged for 10 min at 4000 rpm. The separated plasma was transferred to clean micro-centrifuge tubes and stored at -20 °C. The amount of hesperidin in the samples was estimated using HPLC.

HPLC analysis of plasma sample

Hesperidin concentration in plasma sample was determined by HPLC using (Agilent 1000 series USA), an Agilent G1314A Variable wavelength detector set at 285 nm and column Eclipse XDB-C18 (5 µm, 150 mm×4.6 mm). The mobile phase was a mixture of methanol: water (40:60), pH adjusted to 4.5 by acetic acid. The mobile phase was filtered through a 0.45 µm membrane filter and pumped from the filter reservoir at a flow rate of 1 ml/min which yielded a column back pressure of 110-120 bars. The run time was set at 5 min and the volume of injection was 20 µl. The column was equilibrated for at least 30 min with the mobile phase running through the system before injecting the drug solution. Then the phase separated sample was diluted with the mobile phase filtered through sample filter and injected into the column. The eluent was monitored by isocratic elution at 285 nm [14].

Pharmacokinetic analysis

The area under the plasma concentration-time curve from the time of drug administration to the last quantifiable concentration (AUC_{0-∞}) was calculated by linear trapezoidal integration.

Skin irritancy study

To assess the skin-sensitizing potential of hesperidin micro emulsion and MBO skin irritancy studies were carried out on rats. The formulations were applied on dorsal side of shaven skin and the site of application was occluded with gauze and covered with a non-sensitizing micro-porous tape. Erythema values for formulations with and without pretreatment with abrading ointment were recorded. The patch was removed after 24 h and the score of erythema was recorded as follows: no erythema-0; mild erythema-1; moderate erythema-2; severe erythema-3. Eventually, the total scores for irritation test in each condition were calculated using the following equation [15].

$$\text{Average irritation scores} = \frac{\text{Erythematic reaction scores} + \text{dropsy reaction scores}}{\text{Amount of animals}}$$

RESULTS AND DISCUSSION

Formulation of o/w micro emulsion

It was observed from the results of screening studies that hesperidin showed maximum solubility in Eucalyptus oil (26.27±0.235 mg/ml). Out of various surfactants and co surfactants non-ionic surfactant Tween-80 and co surfactant Acconon CC-6 showed highest solubility of hesperidin (6.374±0.278 mg/ml and 6.731±0.267 mg/ml respectively).

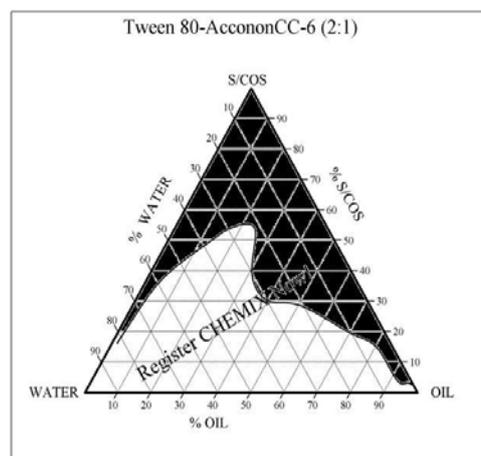


Fig. 1: Pseudoternary phase diagram of the system containing Eucalyptus oil, Tween 80 and Acconon CC-6 (2:1)

Therefore these components were used to plot pseudo ternary phase diagrams using various ratios of Tween-80 and Acconon CC-6. From fig. (1) it was observed that in phase diagram constructed using 2:1 ratio of surfactant-co surfactant larger isotropic region existed. As a result micro emulsion formulations were chosen as shown in table (1) from phase diagram plotted using Eucalyptus oil and surfactant: co surfactant ratio (2:1).

Characterization of microemulsions

All the prepared microemulsion formulations passed visual clarity test and showed 100% transmittance as seen from table (3). It was observed that formulation E1 has viscosity 40.2 cps and droplet size

33.12±2.31 nm which is very small as compared to the other formulations. All the formulations passed electrical conductivity test confirming formation of o/w microemulsion. Polydispersity index suggests uniformity of droplet size within all the formulations. pH values of all the formulations were nearly in the neutral region suitable for topical application.

Drug loading of micro emulsions

As all the above formulations passed the evaluation tests and having droplet sizes below 100 nm. These formulations were loaded with the drug for further evaluation. Formulation E1 showed highest drug loading (18.6±0.7 mg/ml) as seen from table (2).

Table 3: Physicochemical parameters of t micro emulsion formulations

Formulation Code	Electrical Conductivity ($\mu\text{S}/\text{cm}$)*	Viscosity (cps)*	Droplet size (nm)*	Polydispersity Index	pH
E 1	173.5±1.3	40.2±1.2	33.12±2.3	0.3	6.4
E 2	106.9±1.4	37.5±1.5	96.8±1.1	0.5	6.7
E 3	104.2±2.0	58.3±0.1	85.76±1.1	0.9	6.2
E 4	132.2±2.3	46.1±0.9	78.64±1.1	0.5	6.6

*All values are represented as mean±SD, n=3.

Table 4: Physicochemical parameters of the drug loaded micro emulsions

Formulation Code	Viscosity (cps)	Droplet size (nm)*	Polydispersity Index
E 1	37.9	49.10±1.3	0.3
E 2	39.4	106.13±2.1	0.3
E 3	59.6	95.67±2.8	0.7
E 4	60.7	84.89±2.9	0.3

*All values are represented as mean±SD, n=3

Characterization of drug loaded micro emulsions

Measurement of viscosity, droplet size and polydispersity

Drug loaded microemulsion formulations were subjected to determination of viscosity, droplet size and droplet size distribution i.e. polydispersity index to know the effect of addition of drug to the microemulsion formulations. Results are shown in table (4). It was observed that viscosity as well as droplet size increased due to addition of the drug. As the drug incorporated in the microemulsion is less water soluble drug it must be finding its site for solubilization in the oil droplets.

Also the solubilizing power of the microemulsion components the unique structural organization of the microemulsion can add solubility regions and thus increasing the loading capacity of microemulsion [16]. This might be the possible reason for increase in droplet size of the o/w microemulsion as a result of drug loading.

Ex-vivo skin permeation studies

The permeation ability of various microemulsions was evaluated by carrying out *ex-vivo* permeation studies. It was observed from the results that formulation E1 showed highest flux value (8.971 $\mu\text{g}/\text{cm}^2/\text{h}$) as compared to other o/w eucalyptus oil microemulsion formulations of hesperidin ($p < 0.05$). The reason might be higher drug loading in the formulation resulting in maintenance of concentration gradient for the diffusion of hesperidin through rat skin. Also droplet size of this formulation was smallest i.e. 49.10 nm. Presence of droplets of the microemulsion of only a few nanometers in size has a significant contribution to the percutaneous penetration of drug [9]. From the above results formulation E1 (containing 20% eucalyptus oil and 33% Tween 80: Acconon CC-6 mixture 2:1) was selected for the preparation MBO.

Preparation of microemulsion based ointment (MBO)

In order to improve topical applicability of the prepared hesperidin micro emulsion as well as to further improve permeation rate various water soluble ointment bases were investigated. As the drug is embedded in oil droplets of the o/w micro emulsion choice of

water soluble base was thought to be appropriate for incorporating micro emulsion in the base to adjust the consistency and applicability of the microemulsion.

Combination of PEG 6000: PEG 400: Cetyl alcohol (47.5%:47.5%:5%) and combination of PEG 1500: PEG 400: Cetyl alcohol (47.5%:47.5%:5%) were found to be the best as their consistency and texture was excellent. Ointment bases were prepared by fusion method. To the prepared ointment base microemulsion was added (1 ml/g) and mixed on ointment slab using spatula.

Physicochemical characterization of MBO

The excellent the extrude ability the better is the applicability of the formulation. Spread ability denotes the extent of area to which the readily spreads on application to the skin or the affected part. The efficacy of the formulation or the bio-availability of the ointment also depends on the spread ability value. The higher the value of spread ability of the ointment the higher the bioavailability of the formulation [17]. It was observed from table (5) that formulation MBO1 (PEG6000:PEG400: Cetyl alcohol 47.5%:47.5%:5%) has excellent extrude ability and spread ability as well as suitable pH for the topical application. Therefore this formulation was selected for further evaluations. Drug content of MBO formulations ranged between 96-98%.

Ex-vivo permeation study of MBO

Ex-vivo permeation studies on prepared MBO formulations were carried out using excised rat skin to study the influence of components of ointment base on the permeation profile of hesperidin micro emulsion.

Therefore release rates of MBO were compared with optimized hesperidin micro emulsion E1 and saturated solution of hesperidin in pH 7.4 phosphate buffers was used as a control. It was observed from fig.(2) and table (6) that formulation MBO1 has highest permeation rate 26.91 $\mu\text{g}/\text{cm}^2/\text{h}$ as compared to MBO2, micro emulsion E1 ($p < 0.001$) and control. The high permeation rate of MBO1 could be attributed to high drug loading resulting in the

maintenance of high concentration gradient across the skin as well as the presence of Cetyl alcohol in ointment base which enhances penetration of topically applied drugs by physically altering the epidermis. It acts as a permeation enhancer by altering the intercellular lipid shape and extracting lipids from stratum corneum which increases penetration [18]. Polyethylene glycol base used in the formulation is suitable for biological applications because it is soluble in water and therefore it enhances the solubility of

hydrophobic drugs [19]. Also the MBO acts as a drug reservoir where drug is released from the inner phase to the outer phase and then further onto the skin [10]. Very small size of oil droplets in the MBO which contains drug could easily cross the skin barrier to release drug to the required site. Presence of eucalyptus oil in the micro emulsion which acts as a permeation enhancer might be one more reason for enhancement of permeation rate of drug through the skin [20, 21].

Table 5: Composition and physicochemical parameters of formulated MBO

Formulation code	Composition of ointment base	ME*	pH	Viscosity** (cps)	Spread ability**(g. cm/sec)	Extrudability
MBO1	PEG6000:PEG400:Cetyl alcohol (47.5%:47.5%:5%)	E 1	7.2	18,000±5.00	6.1±0.02	+++
MBO 2	PEG 1500: PEG 400: Cetyl alcohol (47.5%:47.5%:5%)		6.7	14,410±12.00	5.5±0.1	++

* ME-Micro emulsion,+++Excellent,++Good, E1-Optimized hesperidin micro emulsion, -**All values are represented as mean±SD, n=3.

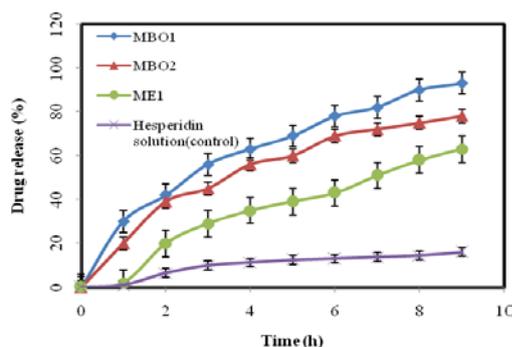


Fig. 2: Permeation profiles of hesperidin MBO formulations and micro emulsion---All values are represented as (mean±SD, n=3)

(47.5%:47.5%:5%) as a base,MBO2-Micro emulsion Based Ointment containing PEG 1500: PEG 400: Cetyl alcohol (47.5%:47.5%:5%), E1-optimized micro emulsion containing 20% eucalyptus oil, 22%

Tween 80, 11% Acconon CC-6 and 47%water. Control-saturated solution of hesperidin in pH 7.4 buffer.

Stability studies of MBO

From the study it was found that all the formulated MBO were stable at 40±2 °C (75±5% RH) and 25±2 °C (60±5% RH) for 3 mo as seen from table (7). No drastic change in color, appearance and pH was observed. The drug content of the final formulation was found to be in the acceptable limit i.e.96-99%.

Table 6: Permeation parameters of MBO

Formulation Code	Jss (µg/cm ² /h)	Kp× 10 ⁻³ (cm/h)
MBO 1	26.9	1.4
MBO 2	19.1	1.0
E1	8.9	0.5
Control	2.2	0.1

MBO1-Micro emulsion Based Ointment containing PEG6000: PEG400: Cetyl alcohol.

Table 7: Stability study data of MBO formulations

Formulation code	Time (month)	pH	Appearance	Viscosity (cps)	% Drug content
MBO 1 40±2 °C (75±5% RH)	0	7.2±0.2	CLEAR	18,000±1000	100.0
	1	7.11±0.1	CLEAR	17,900±500	98.90±0.5
	2	7.08±0.16	CLEAR	17,600±350	97.03±0.30
	3	7.08±0.00	CLEAR	17,600±600	97.00±0.10
MBO 1 25±2 °C (60±5% RH)	0	7.2±0.2	CLEAR	18,000±350	100.0
	1	7.08±0.12	CLEAR	17,800±1000	98.34±0.1
	2	7.01±0.21	CLEAR	17,580±350	97.19±0.5
	3	7.00±0.13	CLEAR	17,510±400	97.10±0.1
MBO 2 40±2 °C (75±5% RH)	0	6.7±0.21	CLEAR	14,410±700	100.0
	1	6.5±0.12	CLEAR	14,300±500	98.57±0.5
	2	6.5±0.12	CLEAR	14,250±250	97.08±0.35
	3	6.5±0.3	CLEAR	14245±250	96.78±0.5
MBO 2 25±2 °C (60±5% RH)	0	6.7±0.2	CLEAR	14,410±400	100.0
	1	6.6±0.2	CLEAR	14,320±300	97.82±0.15
	2	6.5±0.12	CLEAR	14100±100	96.73±0.30
	3	6.5±0.2	CLEAR	14100±100	96.00±0.10

-All values are represented as mean±SD, n=3

In-vivo bioavailability studies

The results of the bioavailability study as shown in fig.(3), table (8) indicate that hesperidin is released and permeated well from the MBO when applied topically as compared micro emulsion E1. The C_{max} of hesperidin after topical application of hesperidin MBO and micro emulsion was found to be 30.3 and 11.3 µg/ml respectively.

MBO1-Micro emulsion Based Ointment containing PEG6000: PEG400: Cetyl alcohol

(47.5%:47.5%:5%) as a base, ME1-optimized micro emulsion containing 20% eucalyptus oil, 22%Tween80,11%AccononCC-6 and 47%water, Ointment(pure hesperidin)-containing 18.5 mg of hesperidin), Control-saturated solution of hesperidine in pH 7.4 buffer.

-All values are represented as mean±SD, n=3

Significant difference in the C_{max} values was observed for MBO as well as micro emulsion and simple ointment. The T_{max} value for all

the formulations was found to be 4h, since the route of administration was topical for all the formulations. The overall mean value of AUC (0-8) for hesperidin MBO was significantly greater than the microemulsion of hesperidin demonstrating improved bioavailability when microemulsion was converted into ointment.

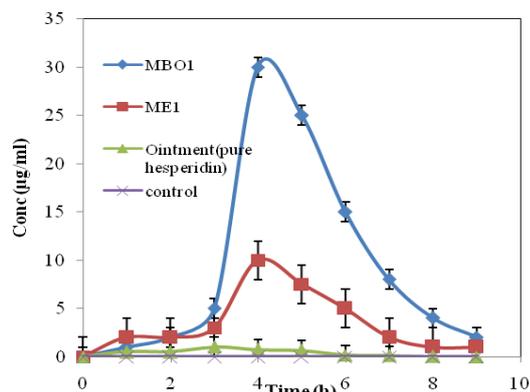


Fig. 3: Plasma drug concentration-time profile of hesperidin MBO and micro emulsion

It is clearly evident from the results that components of micro emulsion and MBO have significant contribution in improving the

bioavailability of hesperidin. It is also reported in the literature that hesperidin itself enhances epidermal permeability barrier homeostasis to certain extent due to stimulation of epidermal proliferation, differentiation, as well as lamellar body secretion [22]. Therefore it can be concluded that topical formulations of hesperidin with improved bioavailability can be prepared by adopting this novel technique for the treatment of venous diseases i.e. to assist the treatment of capillary disorders like haemorrhoids and varicose veins by reducing capillary permeability.

Skin-irritancy studies

The results of skin irritation test are shown in table (9). The intensity criterion of skin irritation followed the protocol that scores of <0.5 meant no irritation, 0.5-3 for slight irritation, >6 showed severe irritation and others were moderate irritation. It was observed that when MBO and micro emulsion formulations were applied to the normal rat skin no irritation observed i.e. all the scores were below 0.5. Whereas when formulations were applied to the damaged rat skin micro emulsion formulation (ME) showed slight irritation. The possible reason might be the decreased metabolic capability for damaged skin induces accumulation of surfactant mixture and oil phase, which led to occurrence of irritation reaction [22]. Therefore, it could be concluded that MBO showed no irritation to intact skin under the conditions of the study and these findings might be due to the change of micro emulsion property after adding PEG ointment base. The network structure formed and the increase of the viscosity decreased the contact chances between skin and micro emulsion. Thus it can be said that the skin irritation by MBO was negligible.

Table 8: Pharmacokinetic parameters of hesperidin MBO, microemulsion and ointment containing hesperidin powder (each containing 18.5 mg of hesperidin)

Pharmacokinetic parameter	MBO	Microemulsion	Ointment
C _{max} (µg/ml)	30.3±0.1	11.3±0.2	3.5±0.1
T _{max} (h)	4.0±0.5	4.0±0.01	4.0±0.01
AUC(0-8)(µg/ml)	66.7±0.2	43.26±0.1	12.1±0.1
Relative Bioavailability (%)	154.3±0.1	----	

-All values are represented as mean±SD, n=3

Table 9: Average response scores of skin irritation for single application

Animal group F. code		1 h	2 h	3 h	4 h
Normal skin	ME	0.6	0.25	0.0	0.0
	MBO	0.25	0.0	0.0	0.0
Damaged skin	ME	0.8	0.4	0.0	0.0
	MBO	0.4	0.1	0.0	0.0

-All values are represented as mean, n=3.

CONCLUSION

A novel micro emulsion based topical formulation of hesperidin with suitable viscosity and spread ability was formulated which showed significant improvement in permeation parameters ex-vivo as compared to control. Eucalyptus oil in micro emulsion has important role to play in solubilization as well as permeation enhancement of hesperidin when applied topically. Conversion of micro emulsion to MBO (micro emulsion based ointment) showed significant improvement in *in-vivo* bioavailability as compared to micro emulsion. Therefore it is evident that components of ointment significantly contributed to the efficient topical delivery of hesperidin. Therefore this novel formulation gives insight to the topical use of hesperidin with improved bioavailability for the treatment of moderate symptoms of venous disease where hesperidin reduces capillary permeability.

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CONFLICT OF INTERESTS

Declared None

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