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Formulation and evaluation of emulgel containing Sucralfate and Dimethicone; A novel transdermal pharmaceutical dosage form

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ABSTRACT: Considering the lack of oxygen, prolonged pressure, and infection are common features of most chronic wounds, especially in pressure ulcers, finding a way to heal the wounds at the early stages is essential. Various pharmaceutical dosage forms are used to act against the infection and prevent the development of this painful condition. The present study aimed to prepare and evaluate topical emulgel formulation for use in the treatment of patients with grade I and II bedsore. The formulation was prepared through the oil in water (o/w) emulsion of organic and aqueous parts to prepare a depot for the transfer of the hydrophobic drug. Emulgel contains sucralfate (Suc), as the epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) could increase the wound healing rate in these patients. The results showed that the emulgel was stable, homogenous non-discolored, and had a constant pH. In terms of microbial content, it was within the acceptable range of pharmacopeia, and the preservatives had a suitable performance. The product had good spreadability. Rheological studies demonstrated that the prepared formulation had thixotropic properties, which is one of the most critical indicators in the design of semi-solid pharmaceutical forms. Topical emulgel containing Suc can be used as an effective pharmaceutical dosage form to treat grade I and II bedsore due to its innovative pharmacological form and the benefits of this drug form along with the moisturizing, softening, and protecting properties of dimethicone and unique properties of Suc.

KEYWORDS: Topical formulation; Dimethicone; Emulgel; Pressure Ulcer; Sucralfate.

1. INTRODUCTION

Pressure ulcers are localized chronic wounds caused by constant pressure on the underlying tissues or pressure along with friction and shear in bedridden patients. The recurrence of pressure ulcers in hospitalized patients strongly affect the patient's quality of life and place a heavy financial burden on families and healthcare systems [1, 2]. Besides, long-term hospitalization of immobile patients leads to an increase in the possibility of nosocomial infections, tissue necrosis, amputations and, even mortality [3]. Despite significant advances in wound healing, treating pressure ulcers is still a challenge due to the sustained mechanical pressure, increase in temperature, and bacterial infections in soft tissue.

Emulgels as the novel dermal drug delivery systems are remarkable because of their special properties such as the controlled release behavior, longer durability on the skin, thixotrophicity, better loading capacity, removability, long shelf-life, and a pleasing appearance [4, 5]. Emulgels or gellified emulsions are the emulsions that their solution parts change to the hydrogel portion. Their cross-linked networks trapped the drug molecules which ultimately lead to controlling drug release behavior [6].

Suc as the basic aluminum salt of sucrose octasulfate is a drug with antiulcer properties [7, 8] which is significantly safe [9] and approved by the Food and Drug Administration (FDA) for duodenum [10]. It has been used as a cytoprotective agent to treat gastrointestinal ulcers for more than three decades [11-14]. The application of Suc for topical wounds includes stress ulcers [15], radiation dermatitis [16], anal fistulotomy wounds [7], bedsores [17, 18], second and third-degree burns [19], and recurrent aphthous stomatitis [20] was then confirmed. Suc accelerates the wound healing process with increased bioavailability of growth factors. Suc by forming a protective layer on the wounds, prevention of apoptosis, reducing the production

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of oxygen free radicals, and eventually, stimulate the synthesis of prostaglandin E2 applies its accelerative effects [11, 14, 16, 21, 22].

Preparing topical formulations for wound healing requires a thorough knowledge of various wounds and their healing process. The condition of each wound is different from other wounds; sometimes a specific formulation that is effective on one wound may have the opposite effect on another wound. This study aimed to prepare a topical formulation containing Suc and Dimethicone to be used in the treatment of patients with stage I and II bed ulcers, taking into account the specific conditions of pressure ulcers.

We hypothesized that emulgels as the dual control release system can be a stable vehicle for poorly water-soluble drugs [8, 23]. The addition of dimethicone to the formulation creates a synergistic effect by Suc, because of its special coating and emollient properties in wound healing. We first investigated the stability of formulation comprised of spreadability study, rheological study and viscosity determination, pH determination, homogeneity, and organoleptic examination. We also report the preservative challenge test to evaluate the product shelf life.

2. RESULTS AND DISCUSSIONS

2.1. Selection of optimum emulgel

Different formulations were prepared using various components. The prepared formulations were examined in appearance, consistency, physical and chemical stability, etc. After removing the unstable formulations with undesirable appearance, the final formulations were selected as the top formulations listed in Table 1.

Table 1. Amounts of emulgel formulation compositions (% w/v).

Formulation Number	Dimethicone	Paraffin	PG	CMC	Chloroform	Suc	Span 60 & Tween 80	Methyl Paraben	Water
F1	-	25	5	2	5	-	5	0.1	57.9
F2	-	23	5	2	5	-	5	0.1	59.9
F3	1.5	23.5	5	2	-	-	5	0.1	62.9
F4	1.5	18.5	5	2	5	-	5	0.1	62.9
F5	1.3	16	4.3	1.7	4.3	13	4.3	0.1	55
F6	5.8	11	4.1	1.5	4	16.6	4	0.1	52.9
F7	7.3	13	5.2	2	5.2	16.1	2	0.1	49.1
F8	7	20	7	2	5	15	5	0.1	39
F9	7	20	7	3	4	7	5	0.1	46.9

Among the stable prepared formulations, F9 was selected as the optimal formulation due to its more suitable viscosity, better spreadability, more desirable appearance, and more dimethicone along with stability. The F9 was further studied for any change in physicochemical properties by placing in germinator at 40 °C and 75% RH.

2.2. Drug content

The amount of Suc in the sample was calculated on the first, 30th, and 60th days to examine the stability of the prepared formulation. The amount of Suc in the formulation can be calculated using the calibration curve equation [$y = 0.029x-0.0062$ ($R^2 = 0.997$)], where x is the concentration of Suc (mg. ml^{-1}), and y is the

solution absorbance at 260 nm. Finally, drug concentration was calculated from the difference between emulgel as the blank and Suc loaded emulgel. The emulgel drug content on days 1, 30, and 60 were 34.62, 34.625, and 30.903, respectively. By comparing these values, no significant changes were observed, indicating the stability of Suc over a two-month period ($p < 0.05$).

2.3. Stability

Stability is an important qualitative aspect in the formulation of new pharmaceutical products. The main purpose of drug stability tests is to provide a reasonable guarantee that products will remain at an acceptable level in terms of safety, quality, and effectiveness during the period they are available in the market for patient access. Stability tests should be performed on the final formulation supplied to the patient under actual drug storage conditions, which vary according to the climatic conditions of each country and region. Because of the long shelf life of pharmaceutical products, which is often several years, stability tests are not plausible in such a period. Therefore, in the stage of preparation and evaluation of formulations, accelerated alternative conditions are used. Stress tests use strict storage conditions. These tests are of great value in predicting the stability of pharmaceutical products [24]. The obtained results are given in Table 2.

Table 2. Physico-chemical evaluation of optimal emulgel formulation.

	Day 1	**Day 30**	**Day 60**
Appearance	Semi-solid, smooth	Semi-solid, smooth	Semi-solid, smooth
Color	White	no color change	no color change
Odor	Odorless	Odorless	Odorless
Consistency	Excellent	Excellent	Excellent
Phase separation	None	None	None
Oily feel	Absent	Absent	Absent
Stickiness	Absent	Absent	Absent
Grittiness	Absent	Absent	Absent
pH	7	6.9	6.9
Average spreadability (g.cm /sec)	25.77±4.13	24.27±2.21	22.90±2.34
Drug content (w/w%)	6.9	6.92	6.16

2.3.1. Organoleptic properties

The physicochemical properties of the prepared formulation were evaluated for two months (Table 3). The semi-solid Suc-emulgel was bright white with a shiny appearance and remained stable without color change after two months. It was smooth without any stickiness, grittiness, and oily feel due to the pharmaceutical form of emulgel.

2.3.2. Homogeneity

Optical microscopy was used for two months to appraise possible changes in the morphology and homogeneity of the F9 formulation (**Figure** 1). All images demonstrated similar morphologies with well-dispersed spherical droplets.

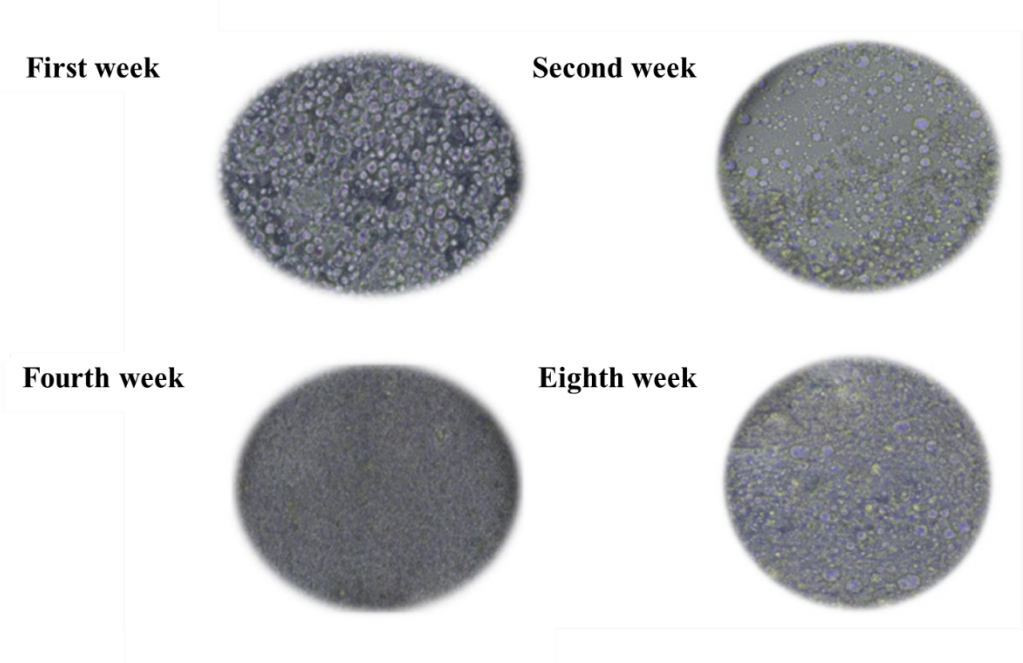

Figure 1. Microscopic images at 40x magnification of the F9 formulation during eight weeks.

2.3.3. pH value

pH is one of the prominent factors in the preparation of skin products and should be evaluated [25], [26]. The acidic nature of the skin was first described by Hesus et al. in 1892. Since then, numerous studies have confirmed this observation and measurement of skin pH [27]. The use of formulations with very high or very low pH can damage the skin, so a moderate pH value should be used for topical administration [28]. Due to the sensitivity of patients with bed ulcers, the suitable pH for preparing topical formulations was selected close to neutral to reduce the risk of side effects [29]. So, the pH of the prepared formulation was set at a value of 7. The pH value did not change after two months.

2.3.4. Viscosity and rheological properties

According to USP, the apparent viscosity of semi-solid pharmaceutical products should be evaluated at the beginning of production and during shelf life since viscosity is the critical factor in drug release from formulation [30]. There is an inverse relationship between viscosity and drug release rate, and as the viscosity increases, the drug diffusion decreases [31].

Figure 2 shows the results of drawing the apparent viscosity of the sample versus shear rate. The emulgels indicated that with increasing rotation speed of the equipment, the viscosity of the formulations reduced; non-Newtonian, pseudoplastic flow, and thixotropic behavior. Thixotropy is the recovery of the formulation to its original structure due to movement and increase in viscosity at rest [32]. The thixotropic behavior of the emulgel is due to the presence of CMC in the composition. CMC solutions identified as strongly time-dependent ingredients [33-36].

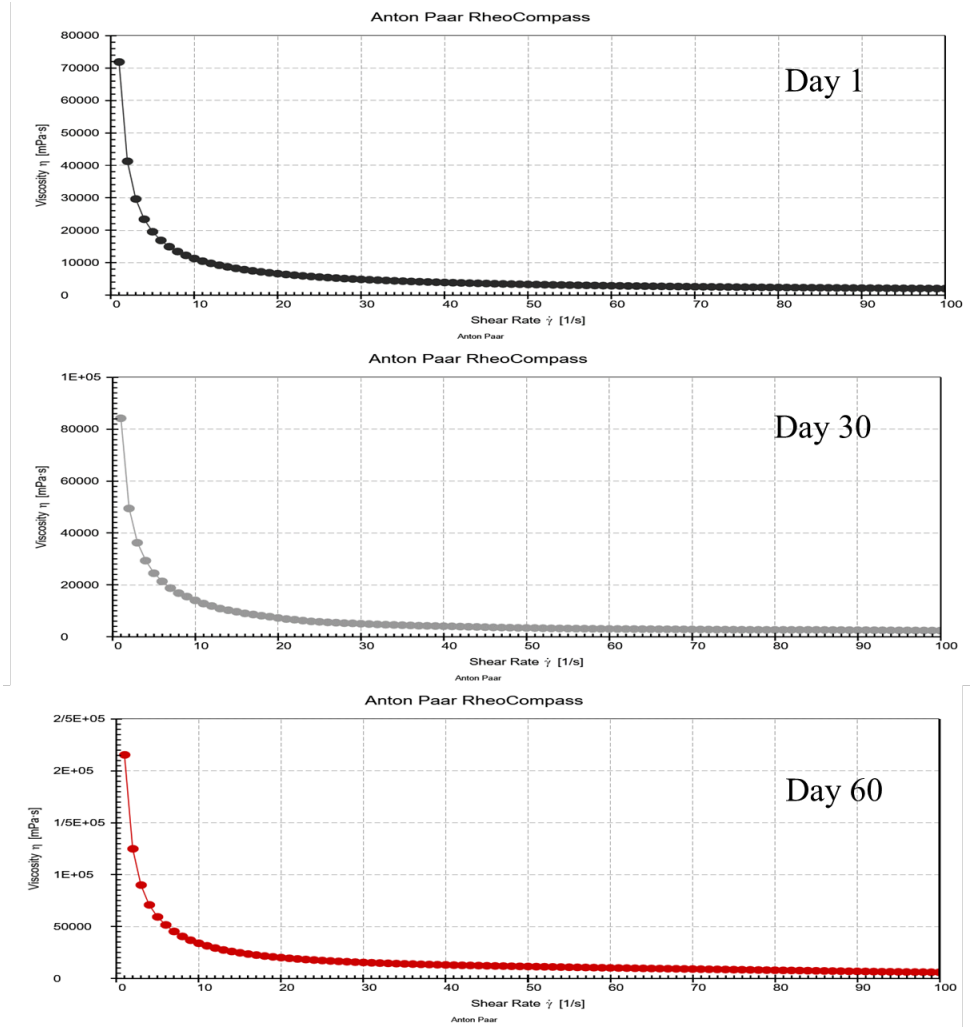

Figure 2. The viscosity of final emulgel formulation at different shear rates during two months.

2.3.5. Spreadability

Spreadability, the ability to spread the formulation on the skin as the uniform layer, is one of the essential features of topical formulations that can play a significant role in patient compliance and therapeutic efficacy [37]. The results obtained from measuring the spreadability of the sample showed that the product has a good spreadability. It has been observed that the spreadability value of emulgel was recorded from 25.77 ± 4.13 to 22.90 ± 2.34 (mm^2g^{-1}) during two months which had no significant difference. It may be due to solvent evaporation from a formulation that increased the viscosity. There was an inverse relation between viscosity and spreadability [38, 39]. It seems the use of propylene glycol as a moisturizer provides optimal spreadability for the product and improves the appearance of the product aesthetically [40].

Microbial quality control

2.4.1. Preservative challenge test

The presence of pathogens in topical dosage forms is a high risk for consumer health, and it should be monitored. A preservative challenge test was used to evaluate the efficiency of preservatives in non-sterile topical products. The optimal formulation was examined in the case of the total aerobic bacterial and the combined yeast count four weeks after production.

Regarding that the prepared emulgel formulation is in the second class of pharmaceutical formulations in terms of microbial control based on USP [41], the maximum acceptable value for total aerobic bacterial and the total combined yeast count were defined as below;

In the case of aerobic bacteria microorganism, the bacterial counting on 14th day should not be less than two units of reduction in the logarithm (200-CFU reduction) compared to the initial counting, and the

counting on the 28th day should not be more than the 14th day. Moreover, the total combined yeast count should be no increased on the 14th and 28th days compared to the first day [42].

The results of the preservative challenge test with culturing the microorganisms proposed by the USP are reported in Table 3. Emulgels reduced viable *E. coli, P. aeruginosa, S. aureus, C. albicans,* and *A. brasiliensis*. Given that the preservative challenge test results met all the acceptable criteria, it is concluded that the preservatives of the product have the necessary efficiency in inhibiting harmful microorganisms and have a good performance.

Experiments related to special culture media show that optimal and specific conditions are provided for the growth of particular microorganisms. If these microorganisms are present in the product and have not shown themselves in basic cultures, they will grow in this experiment. Based on the negative results, it is concluded that the preservative used in the emulsion has a suitable role in maintaining microbial quality.

Table 3. The results of the preservative effectiveness test (PET) for various microorganisms (CFU/ml is reported for grow of organisms).

	dilution / Time(day)	Microbial Count (cfu/ml)			Log Reduction (from first day count)		
		0.001	0.0001	0.00001	0.001	0.0001	0.00001
Escherichia coli	1	$75 * 10^3$	$4 * 10^4$	$4 * 10^5$	-	-	-
	14	$4 * 10^3$	$1 * 10^4$	Nil	1.57	0.6	5.6
	28	Nil	Nil	Nil	4.87	4.6	5.6
Pseudomonas aeruginosa	1	$2 * 10^3$	$2 * 10^4$	$1 * 10^5$			
	14	$1 * 10^3$	Nil	Nil	0.33	4.3	5
	28	Nil	Nil	Nil	3.33	4.3	5
Staphylococcus aureus	1	$200 * 10^3$	$50 * 10^4$	$10 * 10^5$	-	-	-
	14	$6 * 10^3$	$1 * 10^4$	Nil	1.53	1.69	5
	28	Nil	Nil	Nil	5.3	5.69	5
Candida albicans	1	$4 * 10^3$	$1 * 10^4$	$4 * 10^5$	-	-	-
	14	Nil	Nil	Nil	3.6	4	5.6
	28	Nil	Nil	Nil	3.6	4	5.6
Aspergillus brasiliensis	1	$11 * 10^3$	$1 * 10^4$	$1 * 10^5$	-	-	-
	14	Nil	Nil	Nil	4.04	4	5
	28	Nil	Nil	Nil	4.04	4	5

2.4.2. Microbial limits test (MLT)

The acceptable range for microbiological quality of non-sterile pharmaceutical forms (topical use) based on USP is shown in table 4 [43, 44].The sample was free of *S. aureus* and *P. aeruginosa*. The counting of the colonies formed in the incubated samples shows none grow in bacteria and fungi. As a result, all the above conditions have been met, and the sample has not been infected. Considering the result of the microbial tests, it was found that the product was in an acceptable range of pharmacopeia in terms of microbial content and had a high microbiological quality.

Table 4. Acceptance Criteria for Microbiological Quality of Nonsterile Dosage Forms [43].

Route of Administration	Total Aerobic Microbial Count (CFU/g or CFU/ml)	Total Combined Yeasts/Molds Count (CFU/g or CFU/ml)	Specified Microorganism(s)
Oromucosal use Gingival use Cutaneous use Nasal use Auricular us	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 ml) Absence of *Pseudomonas aeruginosa* (1 g or 1 ml)

Which is interpreted as follows:
10^1 cfu: maximum acceptable count = 20;
10^2 cfu: maximum acceptable count = 200;
10^3 cfu: maximum acceptable count = 2000; and so forth [43, 44].

3. CONCLUSION

Suc-based emulgel, for the first time, was developed for pressure ulcer treatment. The formulation's stability, rheological, drug content, and Physico-chemical properties were investigated, and the preservative challenge test was also determined. Preliminary stability studies showed that the incorporation of Suc in emulgels did not interfere with its Physico-chemical properties, and total formulation has excellent stability. The Suc acts as a physical barrier on the skin, protects the skin, prevents its moisture loss, and accelerates the wound healing process. Owing to the unique properties of Suc-emulgel such as thixotropic property, suitable shelf life, optimal spreadability, good stability, and the feasibility of mass production, the new pharmaceutical transdermal formulation could be a good product for scaling up the pharmaceutical industry.

4. MATERIALS AND METHODS

4.1. Materials

Suc, Dimethicone, Carboxy Methyl Cellulose (CMC), Hydroxy Propyl Methyl Cellulose (HPMC), , Propylene glycol (PG), and Span 60 were purchased from Sigma-Aldrich. Chloroform, Tween 80, Methylparaben, Tryptic Soy Broth (TSB), Sabouraud dextrose broth (SDB), Tryptic Soy Agar (TSA), Soybean Casein digest Agar, MacConkey Broth, Mannitol Salt Agar, Cetrimide Agar, and other chemicals were obtained from Merck.

4.2. Preparation of emulgel

Various formulations with different gelling agents such as HPMC, and CMC were prepared by varying the amount of emulgel compositions to achieve the optimal type and range of use. CMC was used as gel-forming agent at the final series of formulations, PG as Humectant, Dimethicone as protection agent, Span 60 & Tween 80 as a surfactant, Methyl Paraben as a preservative, Suc as an active ingredient, and Paraffin as the oil phase. Based on the study in the available pharmaceutical formulations, according to FDA approval, the amount of Suc and dimethicone used in topical formulations was 3-25% and 1-30%, respectively [45]. Table 1 demonstrates the range of other formulation ingredients (%w/v) based on the "Handbook of Pharmaceutical Excipients"[46].

The emulgel formulations were prepared by mixing oil phase and gelling agant contained water as organic and aqueous parts, respectively [47, 48]. The process was done as follow:

a- Oil phase: At organic phase preparation, the weighted amount of span 60 was gradually added to paraffin and let completely dissolved using a stirrer at 300 rpm at 70 ℃. Then Certain amounts of dimethicone and chloroform were added to the mixture. Based on the hydrophobic nature of Suc, it was added to the organic phase.

b- Aqueous phase : The aqueous phase was prepared by dissolving propylene glycol and tween 80 in the PBS buffer (at pH=6) while stirring at 25 ℃. CMC as gelling agent was added to the stirring solution. Finally, the solution was warmed up to 70℃

c- The organic phase was added dropwise to the aqueous phase and stirred at 70 ℃ for 20 min. Then the mixture was homogenized by homogenizer at 1000 rpm for 10 min. (It is important to note that both the aqueous and organic phases should have the same temperature at this step.)

4.3. Calculating required HLB

The Hydrophilic Lipophilic Balance (HLB) value of the system was calculated to anticipate the formulation behavior. Based on the HLB, the values below and above ten indicate the lipophilic and hydrophilic nature of the emulsifier, respectively. Required HLB (RHLB) is the amount of surfactant required to make a stable emulsion. The RHLB was calculated using the following formula:

$$HLB=\frac{Wp\times HLBp+Wd\times HLBd}{Wp+Wd} \quad \text{[Eq. 1]}$$

Where the *Wp* and *Wd* were the weight of paraffin and dimethicone, respectively, *HLBp* the assigned HLB values for paraffin, and *HLBd* the assigned HLB values for dimethicone [49].
The estimated HLB for the emulsifier system was 9.48 ,which is an optimum ratio for the best emulsification. It has been well established that using two surfactants to achieve the desirable HLB of the system is more appropriate than using one surfactant. The percentage of each emulsifier is calculated based on Equation (2):

$$\%\ (A) =\frac{100(RHLB- HLBLow)}{HLBHigh- HLBLow} \quad \text{[Eq. 2]}$$

$$\%\ (B) = 100 - \%\ (A)\ \%$$

Where A and B are emulsifiers, HLB Low is the HLB of lipophilic surfactant, and HLB High is the HLB of hydrophilic surfactant [50, 51].

4.4. Evaluation of optimal formulation

4.4.1. Drug content

To determine the amount of drug in the optimal formulation, a drug-loaded emulgel (500 mg) was soaked in a centrifuge tube containing 10 ml of a mixture of NaOH 2.2N and H_2SO_4 4N (1:1) and vortexed at medium speed for five min. The sample was then placed in an ultrasonic bath at 30 °C for another five minutes and immediately transferred to a vortex mixer. The pH of the sample was raised to 2.5 by adding appropriate amounts of 0.1N NaOH solution. After stabilization of the pH, the sample was centrifuged at 5000 rpm for five minutes, and the supernatant was passed through a 0.45-micron filter. The amount of drug in the sample was determined by spectrophotometry using the calibration curve at a wavelength of 260 nm [52].

4.4.2. Stability studies

The emulgel samples were submitted to two-month cycles kept in an incubator at a temperature of 40±2 °C and relative humidity of 75±5% for accelerated studies according to the instructions of USP and ASEAN guidelines [53, 54]. Stress tests are performed at 10° C above the temperature used in the accelerated test, and the relative humidity of 75% or more is performed in a shorter period [55]. As a result, the optimal formulation was placed in stress conditions for two months at 40 °C, 75% relative humidity, and evaluated each month for drug content, organoleptic characteristics, homogeneity, pH, and viscosity.

4.4.3. Organoleptic examination

The emulgel formulations were visually evaluated for appearance, color, phase separation, and consistency. The odor of emulgel was checked by smelling it directly. Oily feel, stickiness, and grittiness were evaluated by spreading on the skin [56-58].

4.4.4. Homogeneity

To evaluate the homogeneity of the formulation, a thin layer of gel was placed on a microscope slide, and its microscopic images at 40x magnification were examined within two months using the Euromex model optical microscope.

4.4.5. pH determination

The pH of the prepared formulation in germinator (accelerate testing) with temperature conditions of 40±2 °C, and 75±5% relative humidity was measured by a Metrohm digital pH meter on the first day, the first week, second week, fourth week, and eighth week.

4.4.6. Rheological Study and Viscosity Determination

The viscosity of the formulation was measured at one-month intervals for two months using a rheometer Anton Paar model MCR 502 with a 25 mm spindle.

4.4.7. Spreadability study

The spreadability of prepared emulgel was measured using the device proposed by Multimer et al. (1956) and the Parallel-plate method [59].

It comprises two slides with 7.5 × 2.5 cm which one of them is attached and fixed to a wooden board, and the other is movable. The moving board is tied to a string that runs over a spool and is attached to a weight. After placing one gram of emulgel between the two slides, a weight of 100 g was placed on the upper slide for 1 to 2 minutes to remove the trapped air between the two slides and create a uniform layer of emulgel. The weight on the upper slide was removed, and the slide was pulled by a weight of 30 g attached to the string and the spool. The required time for moving the slid a distance of 6.5 cm was recorded. The obtained number was placed in the following equation, and the spreadability was calculated.

$$S = \frac{M.L}{T} \qquad \text{[Eq. 3]}$$

Where the M is the weight tied to the upper slide, L is the length of slides, and T is the time taken to separate the slides.

4.5. Microbial quality control

According to United States Pharmacopeia (USP) and European Pharmacopoeia, the Preservative effectiveness test and Microbial Limits Test were performed to evaluate the microbial quality of the prepared formulation [60, 61].

4.5.1. Preservative effectiveness test (PET)

Preservatives are substances that are typically added to pharmaceutical products to extend shelf life [62]. This test was used to evaluate the preservative systems and microbial stability. According to the instructions, this test was performed on five species of microorganisms including *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans,* and *Aspergillus Brasiliense's*. For the conventional PET, 10^5–10^6 cells/ml were individually inoculated after growth of each in casein soy broth for 6 h at 36 ± 1 ℃ (bacteria) or sabouraud Dextrose broth at 26 ± 1 ℃ (fungi) in 1 g sample aliquots. Immediately following inoculation, serial dilutions from the sample were prepared for colony counting and transferred to agar plates. After incubation at 37 ℃ for 3–7 days (bacteria) and 25±1 ℃ for 3–7 days (fungi), colonies were counted and the values were expressed in a colony-forming unit (CFU)/ml. The CFU were performed for all bacteria and fungi, according to the following equation;

CFU (number of bacteria/ml) = Number of colonies on plate × reciprocal of the dilution of sample (or the dilution factor of the plate) [Eq. 4]

The preservative-free formulation was used as a control to evaluate the viability of microorganism and their ability to grow in the product and prove the antimicrobial effect of preservative ingredients. Table (5) describes the microorganisms used and the appropriate culture conditions for each of them [42, 44].

4.5.2. Microbial limits test (MLT)

In this study, the Pour Plates method was used to identify microorganisms. Briefly, 1 ml of the sample or diluted sample was transferred to a sterile petri dish, then agar (usually 10 ml) was added to the culture medium. The plate rotated slowly to mix the sample with the agar. The agar was left to become rigid. The samples were then incubated for the appropriate condition shown in Table 2.

To further ensure the absence of fastidious microorganisms, the product was cultured in the unique media of these microorganisms, which may be identified under the conditions described in the USP.

Table 5. Appropriate culture conditions for microorganisms used in PET.

Organism	Suitable Medium	Incubation Temperature	Inoculum Incubation Time	Microbial Recovery Incubation Time
Escherichia coli	*Soybean Casein Digest Broth, Soybean Casein digest Agar*	32.5 ± 2.5°C	18 to 24 hours	3 to 5 days
Pseudomonas aeruginosa	*Soybean Casein Digest Broth, Soybean Casein digest Agar*	32.5 ± 2.5°C	18 to 24 hours	3 to 5 days
Staphylococcus aureus	*Soybean Casein Digest Broth, Soybean Casein digest Agar*	32.5 ± 2.5°C	18 to 24 hours	3 to 5 days
Candida albicans	*Sabouraud Dextrose Agar, Sabouraud Dextrose Broth*	22.5 ± 2.5°C	44 to 52 hours	3 to 5 days
Aspergillus brasiliensis	*Sabouraud Dextrose Agar, Sabouraud Dextrose Broth*	22.5 ± 2.5°C	6 to 10 days	3 to 7 days

4.6. Statistical analyses section

All quantitative results were obtained from triplicate samples. Every data point was expressed as mean ± SD. The analysis was carried out using SPSS 11.5 software and included one-way ANOVA and t-test. The confidence level set for all analyses was 95% ($p<0.05$).

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