Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 8, 2014

Original Article

TOPICAL BENZOPHENONE-3 MICROEMULSION-BASED GELS: PREPARATION, EVALUATION AND DETERMINATION OF MICROBIOLOGICAL UV BLOCKING ACTIVITY

ALIA A. BADAWI[1], NABAWEYA ABD EL-AZIZ[2], MAHA M. AMIN[1], NERMIN M. SHETA[2]

[1]Department of Pharmaceutics, Faculty of Pharmacy, Cairo University[2], Department of Pharmaceutics, Faculty of Pharmacy, 6th October University
Email: maha.amin@pharma.cu.edu.eg

Received: 12 Jul 2014 Revised and Accepted: 05 Aug 2014

ABSTRACT

Objective: Microemulsions (MEs) have been developed as active vehicles for sunscreens being physical sunscreens on their own.The aim of this study is to incorporate Benzophenone-3 (BZ-3), a chemical sunscreening agent into MEs resulting in a synergistic effect on its protective characteristics.

Methods: Screening of BZ-3 in different oils, surfactants and cosurfactants available for topical delivery was carried out. A full factorial study design (3.2^2) was adopted to study the effect of three independent variables namely;BZ-3, oil, and S/CoS_{mix}(1:1) concentrations on the *in-vitro* SPF value of the prepared ME gel formulae.

Results: Formulae having SPF>30 were further evaluated regarding *in-vitro* permeation test. Finally, an *in vitro* microbiological assay was adopted to compare the survival percent of E.coli against UV exposure. Formula F11IPM ME gel consisting of 3% BZ-3 and 10% Isopropyl myristate as oil together with 60% w/w Tween80/Cremophore RH40 mix (1:1)as surfactant and cosurfactant respectively was chosen as the optimum formula having an *in vitro* SPF of 33.15±0.91,lowest permeation of 192±16.13µg/cm^2 after eight hours and microbiological UV blocking activity of 88±5.84%which is not significantly different from the standard Spectra Ban plus (SPF=30) having a survival percent of 80±3.04%.

Conclusion: This study illustrated the potential use of microemulsified BZ-3 delivery system to improve the SPF of BZ-3.

Keywords: Benzophenone-3, Microemulsion gel, *In vitro* SPF, Spreadability test, Permeation test, Sunscreen efficacy test, Microbiological UV blocking activity.

INTRODUCTION

Microemulsions are optically transparent, thermodynamically stable dispersions of oil and water stabilized by surfactant, usually in combination with a cosurfactant[1-5]. Microemulsions can be differentiated into three types: oil-in-water, water-in-oil and bi continuous depending on the type of dispersed and continuous phases. Hydrophilic drugs solubilize mainly in water phase inthe water-in-oil microemulsion droplets and hydrophobic drugs in oil droplets of oil-in-water microemulsions[6].

Microemulsions provide several advantages over conventional topical formulations such as; their spontaneous formation as they can be prepared easily by simple mixing of particular components in suitable ratios at ambient temperature, thereby suitable for the preparation of thermo-sensitive drugs giving rise to thermodynamic stable systems over a wide range of pH's and ionic environments[2-7]. The major difference between coarse emulsions versus MEs is that the interfacial tension in ME is very low compared to that in coarse emulsion leading to spontaneous formation, small droplet size of the dispersed phase and thermodynamic stability of the produced ME system. Conventional emulsions may exhibit excellent kinetic stability, but they are subjected to thermodynamic instability and will eventually phase separate [8].

Microemulsions are of considerable importance as they have found practical applications in different fields, such as: industrial, chemical, petroleum, food, cosmetic as numerous applications of cosmetic MEs include skin-care, hair care, sunscreen[2] and personal care products [9].

Sunscreen products are primarily designed to protect the skin from the harmful effects of solar ultraviolet radiations (UVR) such as photoaging and skin cancer, and to minimize various photosensitivities and phototoxicities, they contain molecules or molecular complexes that can absorb, reflect, or scatter UV photons, in order to prevent, ameliorate, or even repair solar-induced skin damage[10,11].

Conventional sunscreen compositions are usually in the form of a liquid, either a lotion or a cream. The carrier water evaporates and leaves a thin film of active ingredients and excipients deposited on the skin protecting it from UV radiation. The liquid applications of these sunscreen compositions are often greasy to touch. Also, the distribution of the active ingredients on the skin is uneven. The user must wait for the liquid in the sunscreen compositions to dry. There is a continuing need in the market for products with an improved performance spectrum against the harmful effects of the sun [12].

During the formulation of sunscreen preparations, the most important seems to be the impact of the formulation on skin penetration as many studies have shown that the currently used sunscreens can cause adverse skin and systemic reactions arising from their lipophilic nature leading to their bioaccumulation in humans giving rise to allergic and irritant contact dermatitis, phototoxic and photoallergic reactions [13].

Microemulsions have the ability to deliver larger amounts of water and topically applied agents into the skin than water alone or other traditional vehicles such as lotions or creams because they act as a better reservoir for a poorly soluble drug through their capacity for enhanced solubilization [14].

Benzophenone-3 (BZ-3) is a widely used lipophilic, wide spectrum chemical sunscreening agent with UVB, some UVA and some UVC absorbing properties[15] which allow the selection of this compound as a major component of many cosmetic[16] formulations in the market, such as lipcares, sunscreen lotions, creams, or emulsions. It is considered the most common cause of an allergic or photoallergic contact dermatitis[13]. Despite this large use there is published data to its potential skin penetration, thus the vehicle used is a major factor influencing percutaneous absorption as it may enhance or block the movement of the UV-filter through the skin [17-21].

The aim of this study is to prepare and evaluate ME gels with o/w composition as vehicles for the incorporation of the chemical

sunscreening agent (Benzophenone-3)adopting simple technique producing non greasy, emollient sunscreen topical gels having high SPF value, low skin permeation and favorable cosmetic properties, thereby allowing more patient compliance by enhancing the sunscreening efficacy and reducing skin and systemic adverse effects of the drug.

MATERIALS AND METHODS

Benzophenone-3(BZ-3), (ISP)(USA); Isopropyl myristate (IPM); Olive oil; Black seed oil (BS); Liquid paraffin; Jojoba oil; Coconut oil (of analytical grade and used without further purification); Tween 80 (T80) and Tween 40 (T40) (Merck-schuchardt, Germany); isopropyl alcohol (IPA); propylene glycol (PG); polyethylene glycol (PEG) 400; Chloroform, Methanol and methylene chloride (Analar India Mumbai); Cremophor RH 40 (BADF, Germany); Potassium dihydrogen phosphate and Disodium hydrogen phosphate, (Merck-schuchardt, Germany), Sodium chloride, (Merck-schuchardt, Germany); Deionized double distilled water; Surgical Transpore tape ®(3M Australia Pty Ltd.,Australia); Sodium Nitroprusside &Phosphotungestic acid (PTA) (Adwic. El-Nasr pharmaceutical chemicals Co., Abu-Zaabal, Cairo, Egypt); Carbomer 940 (BF Good rich company, Cleveland, Ohio, USA); Triethanolamine (TEA) (Nouresh' shark Company, 10th of Ramadan city, Egypt); newly born rat skin (one week age) were brought from the animal house of applied research center for medicinal plant ARCMP, Egypt; Nutrient broth; Nutrient agar (NA) was the product of Lab M™ (Lab M limited Topley House, 52 wash Lane, Bury, Lancashire BL 96 AS, UK); *Escherichia coli* (ATCC 87064); Spectra Ban plus 30® (SPF label claim *in-vivo*=30)(Stiefel, England); Disinfectant (70% isopropyl alcohol).

Screening of BZ-3 in different vehicles

Solubility of BZ-3 in various oils namely; Isopropyl myristate (IPM), Olive oil, Black seed oil (BS), Liquid paraffin, Jojoba oil, Coconut oil; two surfactants: Tween 80 (T80) and Tween 40 (T40) and two cosurfactants: isopropyl alcohol (IPA) and Cremophor RH 40 (Crem RH 40) was determined[8, 22].

An excess amount of BZ-3 was added to each oil, surfactant and cosurfactant in 2 mL stoppered vials and shaken reciprocally at 37°C ± 1.0 for 72 hours to get equilibrium [23].The mixtures were removed from the shaker and centrifuged for 30 min at 2500 rpm to remove the excess undissolved BZ-3. The supernatants were then filtered through millipore filter 0.45 µm and the drug concentration in the filtrate was determined using a UV spectrophotometer (UV-1601 PC, Schimadzu, Kyoto, Japan) at λ_{max} 288 nm after appropriate dilution with either methylene chloride in case of oil or methanol in case of surfactants and co surfactants[24].

Construction of ME pseudo-ternary phase diagrams

Four pseudo-ternary phase diagrams for the two selected oils (IPM and BS) were constructed using spontaneous emulsification method (or water titration method) by mixing oil, S/CoS_{mix}in two w/w ratios (1:1 & 3:1) and water at certain weight ratios into glass vials, mixed for 2-3 min using Vortex mixer (VM-300, Gemmy industrial Corp, Taiwan), then incubated at room temperature for 72 hours till equilibrium. After equilibrium, the mixtures were assessed visually [23].ME Gels are also claimed for those clear and highly viscous mixtures that did not show a change in their meniscus after being tilted to an angle of 90ºC[25].

Factorial design study plan (3.2^2)

The study design involved the investigation of the effect of three independent variables namely; BZ-3 concentration (1, 2, 3%w/w), oil concentration (10, 20%w/w) & S/CoS_{mix}(1:1) concentration (40, 60%w/w) on the *in-vitro* SPF of BZ-3 from the different prepared gel formulae.

Preparation of BZ-3 ME gels

In order to prepare BZ-3 loaded MEs, the appropriate oil, S/CoS_{mix} weight ratios were weighed in glass vials, then the specified amount of BZ-3 was accurately weighed, added to the mixture and vortexed. Finally water was added drop by drop at ambient temperature and vortexing was continued for 5 min, the resultant MEs were stored for 72 hours at room temperature till equilibrium before further investigations [8, 22].Clear viscous were classified as gels[8].

Evaluation tests of the prepared BZ-3 ME gels

Visual inspection

The prepared gel formulae were examined for optical clarity, fluidity, homogenity and phase separation (syneresis).

Examination under cross-polarized microscope

The prepared ME gels were examined under cross-polarized microscope (Olympus, Japan) for the absence of birefringence to exclude liquid crystalline systems [26].Part of ME gel was placed between a cover slip and a glass slide and then examined under polarized light [22].

pH measurements

The pH of 10% w/w aqueous solution of each of the prepared ME gels [8] was measured by pH meter (Hanna-213, Portugal). The solutions were prepared by dissolving 1 g of each ME gel formulae in 9 g of double distilled water using magnetic stirrer [27].

Test for spreadability

This test was carried out by pressing 0.5 g of each of the prepared ME gel formulae between two slides of glass and left for about 5 min where no more spreading was expected by the help of known weight. The diameter of the formed circle was measured and taken as comparative values for spreadability [28].

Thermodynamic stability

To assess the thermodynamic stability [27]of the different prepared ME gels, three tests were carried out as follows:

Centrifuge stress test

The prepared ME gels were centrifuged at 3000 rpm for 30 min and then examined for liquefaction and phase separation. Formulae that did not show phase separation were subjected to a cooling-heating cycle[29].

Cooling-heating cycle

The prepared ME gels were submitted to a total of three complete cycles, each cycle consisting of 24 hours at 5ºC followed by 24 hours at 45ºC. Formulae that did not show phase separation were considered for freeze thaw stress test[30].

Freeze thaw stress test

The prepared ME gels were submitted to a total of three complete cycles, each cycle consisting of 24 hours at 25ºC followed by 24 hours at -5ºC [30].

In-vitro measurement of SPF and UVA-PF of the prepared BZ-3 ME gels

The principle of this method is the measurement of the spectral transmission of UVR through a sample placed on a tape with or without the sunscreen to be evaluated. By using this method, the applied radiation provides a continuous spectral power distribution in a range between 290 and 400 nm and measure the transmittance every 5 nm increment [31, 32].The *in vitro* SPF is calculated as follows:

$$\mathbf{SPF} = \sum_{290}^{400} \mathbf{E}(\boldsymbol{\lambda}) \in (\boldsymbol{\lambda}) / \sum_{290}^{400} \mathbf{E}(\boldsymbol{\lambda}) \in (\boldsymbol{\lambda}) / \mathbf{PF}(\boldsymbol{\lambda}) \quad \textit{(Eq.1)}$$

Considering the UVA wavelength range (320-400 nm) and using the terms of SPF equation, the *in-vitro* UVA protection factor could be calculated from the same equation but instead of substitution from 290-400 nm for SPF, the wavelength range from 320-400 nm was used instead to get UVA-PF[31].

At least three samples should be prepared for each sunscreen to be measured. At the same time, a reference sample of Transpore Tape® should be prepared from the same section of the roll. To prepare the sunscreen samples, a small fine needle syringe was used to transfer the sample (2 mg/cm^2) for 4 cm^{2}surface area Quartz cuvette, the

slide was placed on an analytical balance and the samples were distributed on the sample plate by dotting the sunscreen on the slide and noting the weight[32-34].

The sunscreen-coated slide was removed from the balance and the sunscreen was distributed over the entire surface by slowly and deliberately rubbing the surface of the slide with a single finger-cot-coated finger for 20-30 strokes (approximately 20s). The sample should then be put aside to dry and let the emulsion break for at least 10 min before measurements begin. Failure to allow this dry-down period will result in inaccurate SPF measurements[32-34]. All BZ-3 ME gel formulae were compared to placebo ME gel bases (free from BZ-3) and also to conventional sunscreen gel Spectra Ban plus 30®(containing BZ-3).

Based on *in vitro* SPF and UVA-PF results, candidate formulae:F11IPM (3% BZ-3, 10% IPM oil, 60 % S/CoS_{mix}(1:1), 27% water) and F11BS (3% BZ-3, 10% BSoil, 60 % S/CoS_{mix} (1:1), 27% water) showing the highest *in vitro* SPF were subjected to further investigations.

Evaluation of the selected BZ-3 ME gels

Sunscreen efficacy testing

Sodium nitroprusside solution method was used for *in vitro* sunscreen efficacy testing using UV lamp.A 0.05% w/w solution of sodium nitroprusside in distilled water was prepared and 40 mL of this solution was placed in a petriplate covered with cellophane membrane to which 2 g of each of the selected ME gel formulae was spread uniformly over the membrane as a layer. In the first two plates, 2 g of F11IPM and F11BS were applied over the cellophane membrane. A third petriplate containing sodium nitroprusside solution was not covered with cellophane membrane to expose it directly to UV lamp (VILBER LOURMAT-4.LC France)[15].A fourth petriplate was left in the dark for comparison. The first three petriplates were exposed to UV lamp for 2 hours.

Sodium nitroprusside in aqueous solution is colorless photosensitive substance [34] and the light of the UV lamp causes its degradation yielding prussian blue color and nitric oxide (NO). After exposure to UV lamp, the samples were analyzed using spectrophotometric measurements to determine the stability of sodium nitroprusside with most emphasis on increase in the absorbance at 390–395 nm with degradation. A comparative study was carried out to determine the most efficient ME sunscreen formula having the highest protection confirmed by the appearance of less intense blue color formation [15].

Refractive index

The refractive index (η) of a medium measures the extent of interaction between electromagnetic radiation of light and the medium through which it passes. Refractive index is the net value of the components of a micro emulsion and indicates the isotropic nature of the formulation. The refractive index of each of the two selected BZ-3 ME gel formulae(F11IPM,F11BS)was measured by Higler and Walt's Refractometer (M46.17/63707, England) by placing one part of the formulation on a slide at 25°C[27, 35]. The average value ± SD of three readings was taken.

In-vitro BZ-3 permeation studies

Ethical clearance was obtained from the institutional animal experimentation committee at Cairo University before the study. The full body skin of newly born rat (one week aged hairless rat) was excised and used [36, 37]. The subcutaneous tissue was removed surgically if present and the dermis side was wiped with isopropyl alcohol to remove adhering fat. The cleaned skin was washed with distilled water and stored in the deep freezer until further use (for not more than one month). The skin was brought to room temperature and cut into circular patches 5 cm diameter upon use.

Permeations of BZ-3 from the two selected ME gel formulae (F11IPM,F11BS) through newly born excised rat skin were carried out according to the following procedure: Exactly constant amount of the prepared ME gel (0.7 g) was spread over the surface of a glass slide of 8.03 cm^2surface area and then covered with the newly born rat skin. The glass slide and the rat skin were held together by waterproof plaster and equally spaced plastic clips [8].This assembly was placed at the bottom of the dissolution vessel containing 300 ml of 1% w/v Tween 80 in PBS solution (pH =7.4). 1% Tween 80 was added into the dissolution media to maintain the sink condition [15].

The permeation test was carried out for eight hours at 37±1°C. The stirring rate was adjusted at a speed of 100 rpm. Every hour aliquot of 5 ml of the medium was withdrawn and replaced with equal volume of fresh medium to maintain a constant volume. The concentration of BZ-3 was determined spectrophotometrically at the pre determined λ_{max}. All experiments were run in triplicates and the results were expressed as the average mean values±SD, and compared to the corresponding BZ- carbapol gel having the same concentration.

The amount of BZ-3 permeated through the skin per unit surface area (μg/cm^2) was plotted as a function of time. The drug flux (permeation rate) at steady state (J_{SS}) was calculated from the slope of the straight line. The correlation coefficient (R^2), $t_{50\%}$ (time to 50% drug permeated) and the amount of drug permeated after 8 hours ($Q_{8\ hrs}$) were also determined for each formula. Permeability coefficient (K_P) was calculated from the following equation:

$K_p=J_{ss}/C_o$ ***(Eq.2)***

Microbiological method for the assessment of the UV blocking activity

Commercially bought *E.coli* was diluted using normal saline (0.9 w/w % NaCl in double distilled sterile water). Four different serial dilutions of *E.coli* were made: 10^{-1}, 10^{-2}, 10^{-3}& 10^{-4} to select the optimium number of colonies that could be cultured per plate after 24 hours incubation at (37±1ºC) in the incubator in an inverted position in the dark. The bacterial counts were carried out using standard pour plate method [38, 39].From the previous screening test, the dilution showing white, uniform, clearly and freely distributed, easily counted, not overlapped colonies will be selected for the "Decimal Reduction Time" (DRT) determination for further investigations.

The prepared plates were then exposed to UV lamp having wavelength of 365 nm. In order to sterilize the radiation area and allow the lamp to reach a stable state, the lamp was operated for at least 30 min before irradiating the petriplates in the lab. The optimal distance between the UV lamp and the dish was reported to be 30-35 cm [40].

For control three non irradiated petriplates were kept in dark (C_{dark}) while the other plates were irradiated at different time intervals 0, 3, 5, 10, 20, 40, 60, 80, 100 and 120 min (3 plates per each UV light exposure duration), then both irradiated and non irradiated plates were incubated in the dark in an inverted position for (24 hours) at 37±1°C inside the incubator.

The survival of bacterial cells and its ability to form colonies on a solid medium following irradiation assume that every colony is founded by a single cell and that the cell must have been alive in order to grow and form a colony [41].Calculating the number of colonies per plate was expressed in the form of colony forming units (CFU) and Log of survivals was plotted versus exposure time.

The resistance value (or death kinetics) of a bioburden (BI) was characterized in terms of decimal reduction time (DRT) or D-value, which is the exposure time required, under specified set of conditions, to cause one log_{10} or 90% reduction of the initial population (N_o, bioburden) of viable BI in the suspension [42].

The decimal reduction time (DRT) or D-value is used as the microbiological parameter for sunscreen efficacy and was estimated from the corresponding regression equation graphs on which the logarithms of the survivors were plotted against time [43]. The D-value at 37±1°C was determined from the negative reciprocal of the slopes of the regression lines, using the linear portions of the survival curves (log_{10} CFU/ml versus time of exposure to UV

radiation at constant temperature)[42].The D-value was determined from the inactivation kinetic curve given by the equation:

$t = D \times (\log N_o - \log N_f) = D \times n$ ***(Eq.3)***

$\log N_f = \log N_o - 1/D \times t$ ***(Eq.4)***

Where D = D-value (min) at specified conditions, N_o = bioburden of the chosen bacterium; N_f = surviving population after an exposure time t (min) and n = ($\log N_o - \log N_f$) = $\log_{10}$ reduction of a bioburden; N_o= bioburden; N_f= survival population; D-value = decimal reduction time; (-1/D) = slope.

The D-value was used as the optimum time required for the microbiological assay to determine the *in-vitro* UV blocking ability of the selected sunscreen formulae. According to the previously done screen tests, the optimum predetermined number of colonies and time were selected as the standard conditions to precede the UV blocking activity [40, 44]. This was carried as follows: The petriplates were prepared with the predetermined bacterial count. The solidified plates were then properly sealed from both sides by an adhesive tape to prevent the opening of the petriplates while handling. Then each petriplate was placed on the electrical balance and an aliquot of 2 mg/cm^2 of the product were used to be distributed on petriplates by dotting the sunscreen on the petriplate's lid surface and noting the weight. The sunscreen-coated petriplates were removed from the balance and the sunscreen is distributed over the entire surface by slowly and deliberately rubbing the surface of the slide with a single finger-cot-coated finger [45, 46]. The sample should then be put aside to dry and let the emulsion break for at least 20 min before exposing them to UV light. After the exposure of each product to the previously mentioned procedures, the test dishes together with the three control petriplates which were kept in the dark (C_{dark}) and three control irradiated petriplates without sunscreen protection (C_{UV}), were placed in the incubators (37±1°C) in an inverted position for 24hours[42]. The percent survival of the bacteria was then calculated by counting the number of colonies of *E.coli* that survived after exposure to UV light divided by the total number present multiplied by 100[44]as follows:

$$\text{Percent survival} = \frac{\text{Average colony count with sunscreen (experimental)}*100}{\text{Average colony count in the dark (control)}} \quad \textit{(Eq.5)}$$

All experiments were run in triplicates and the results were expressed as the average mean values (±S.D), statistical analysis was carried on the percent survival of bacteria between the different tested formulae using One-Way Analysis of Variance *(ANOVA)* for multiple comparisons at (∗*P*=0.05). Statistical analysis was performed using Statview version 4.53 computer program.

RESULTS AND DISCUSSION

Screening of BZ-3 in different vehicles

Table (1) summarizes the solubility of BZ-3 in various oils, nonionic surfactants and cosurfactants which was determined at 25ºC.Based on the solubility studies, BS and IPM were chosen to represent the oily phases, T80 and Crem RH 40 were selected as surfactant and co-surfactant respectively for construction of the pseudoternary phase diagrams exhibiting the significantly highest Bz-3 solubility (∗p< 0.05).

Table 1: Solubility of BZ-3 in different vehicles at 25±0.5ºC

Vehicles	BZ-3 Solubility (mg/ml)
Isopropyl myristate	89±3.0
Black seed	90±5.0
Olive oil	64±9.0
Jojoba oil	51±9.0
Coconut oil	28 ±7.0
Liquid paraffin oil	10 ±0.4
Isopropyl alcohol	103±3.0
Cremophor RH 40	173±4.0
Tween 80	266 ±2.0
Tween 40	209±0.5

Four pseudoternary phase diagrams for the two selected oils (IPM & BS) were constructed with two S/CoS_{mix} ratios namely (1:1 and 3:1 w/w). Figures (1&2) show the four different phase diagrams with both clear liquid and viscous ME gel points, from which gel areas were selected.

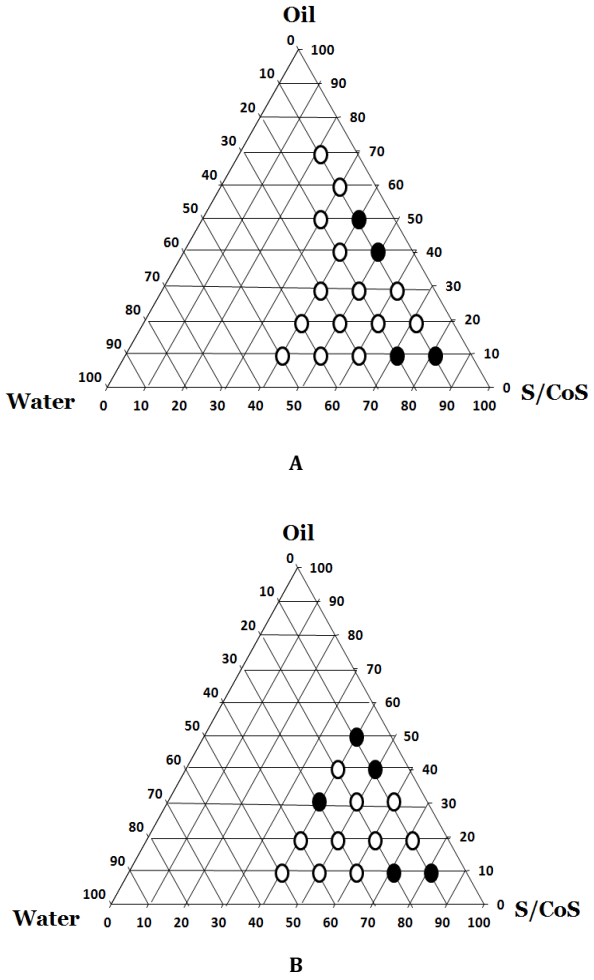

Fig. 1: Pseudo-ternary phase diagrams of BZ-3 ME systems containing: A) IPM as oil / T80 &Crem RH 40 as S/CoSmixratio 1:1 (w/w), B) IPM as oil / T80 &Crem RH 40 as S/CoSmix ratio 3:1 (w/w)

Dark spots represent liquid ME areas-white spots represent ME gel areas.

Increasing S/CoS_{mix} ratio from 1:1 to 3:1 w/w results in a decrease in the microemulsion region.This may be attributed to the reduction of flexibility of the surfactant/co-surfactant layer resulting in a more rigid surfactant films which in turn reducing the range of existence of MEs. Similar outcomes were observed by Hathout et al (2010)[47]who worked on oleic acid, Tween 20, Transcutol and water, where the ratio S/CoS_{mix}(1:1) was found to be optimum regarding the monophasic area and the ease of ME formation.

Microemulsion Gel areas were selected being considered as final formulae [8] with a shiny appearance and good skin feeling effect upon application. Gels also improve patient compliance compared to other conventional creams or emulsions which produce white layer due to the presence of heavy oils or drying agents [48].

Gels are also aqueous preparations providing cooling sensation; ideal for oily skin or individuals exerting high activity [49].Being gels, they also hinder the permeation into systemic circulation leading to superficial effect on the skin [50].

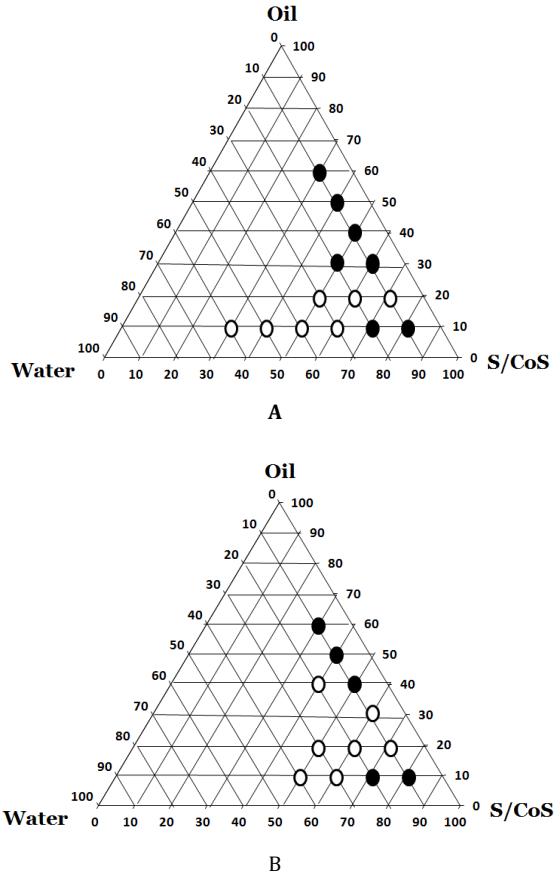

Fig. 2: Pseudo-ternary phase diagram BZ-3 ME systems containing: A) BS as oil/T 80 & Crem RH 40 as S/CoSmix ratio 1:1 (w/w), B) BS as oil/T80 & Crem RH 40 as S/CoSmix ratio 3:1 (w/w) Dark spots represent liquid ME areas-white spots represent ME gel areas.

Preparation of BZ-3 ME gels

Hundreds of formulations could be prepared from the clear ME area of each phase diagram, but only gel areas were selected to prepare twelve formulae for each oil adopting 3.2^2 factorial design study plan. Compositions of the different prepared BZ-3ME gel formulae were given in Tables (2&3).

Evaluation of the different prepared BZ-3 ME gels

Visual and cross-polarized microscope examination

Visual inspection of the different prepared BZ-3 ME gels showed clear homogeneous systems of gel consistency with no phase separation[51].Isotropic material, such as ME, in contrast to anisotropic liquid crystals, will not interfere with the polarized light and the field view remains dark under the polarized microscope. Examination under cross-polarized microscope showed that all the prepared formulae appeared dark when viewed between cross polarizer, indicating isotropic properties of the ME[51].

pH measurements

The pH of the prepared ME gel formulae should fall within the physiological accepted range for dermal and transdermal preparations (4-7 pH units) in order to be non-irritant and safe for dermal applications[8, 22].Results of pH values was found to be in the range of (6.64±0.09 - 7.09±0.03 units) for ME gels prepared using IPM oil and in the range of (5.85±0.05 - 6.27±0.03 units) for those prepared using BS oil, indicating that the pH of the different prepared BZ-3 ME gel formulae is within the required range.

Test for spreadability

The spreadability is an important criteria for uniform and ease of application of topical preparations, it was measured in terms of the average diameter of the spread circle of the prepared ME. Spreadability values for all the prepared BZ-3ME gel formulae varied between (2.47±0.05 to 7.83±0.17 cm) and (2.36±0.04 to 5.36±0.12 cm) for IPM and BS respectively. The larger the diameter, the better the spreadability[52].These findings were with accordance with Soliman et al (2010)[8] who worked on IPM as oily phase during the preparation of Celecoxib ME.

Table 2: Composition of the different prepared BZ-3 IPM ME gel formulae.

Composition (%w/w)	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
BZ-3	1	1	1	1	2	2	2	2	3	3	3	3
IPM	10	20	10	20	10	20	10	20	10	20	10	20
S/Cos $_{mix}$	40	40	60	60	40	40	60	60	40	40	60	60
Water	To 100 gm											

Table 3: Composition of the different prepared BZ-3 BS ME gel formulae.

Composition (%w/w)	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
BZ-3	1	1	1	1	2	2	2	2	3	3	3	3
BS	10	20	10	20	10	20	10	20	10	20	10	20
S/Cos $_{mix}$	40	40	60	60	40	40	60	60	40	40	60	60
Water	To 100 gm											

Thermodynamic stability ofBZ-3 ME gels

All the prepared BZ-3 ME gel formulae were subjected to different stress tests such as centrifugation and freeze thaw cycle tests to assess their physical stability. Freeze thaw cycle test applies stress on the ME at temperature below freezing as the formation of ice crystals in an o/w type of ME may cause elongation and flatten to oil particles. In addition, the lipophilic portion of the surfactant could lose its mobility while the hydrophilic portions are simultaneously dehydrated due to the freezing action of water. As the sample is thawed, water is released and travels rapidly through ME [8].

If the system can recover itself before coalescence occurs, the system will be considered stable.

All the prepared BZ-3 ME gel formulae showed no phase separation as well as no changes in their physical appearance such as turbidity or creaming. These results indicate that all the prepared ME gelsare physically and thermodynamically stable systems formed at a particular concentration of oil, surfactant and water, that makes them stable with no phase separation, creaming or cracking [53].

***In-vitro* measurement of SPF and UVA-PF of BZ-3 ME gels**

Figures (3 & 4) show the values of *in vitro* SPF and UVA-PF of the different prepared BZ-3 ME gel formulae.

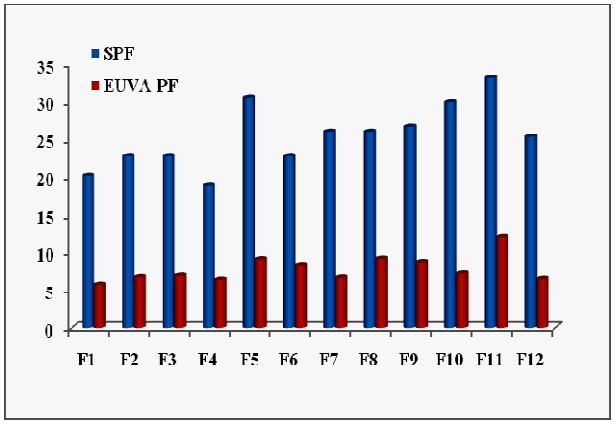

Fig. 3: Sun protection factor (SPF) and UVA- protection factor of the different prepared BZ-3 ME gel formulae using IPM as oil.

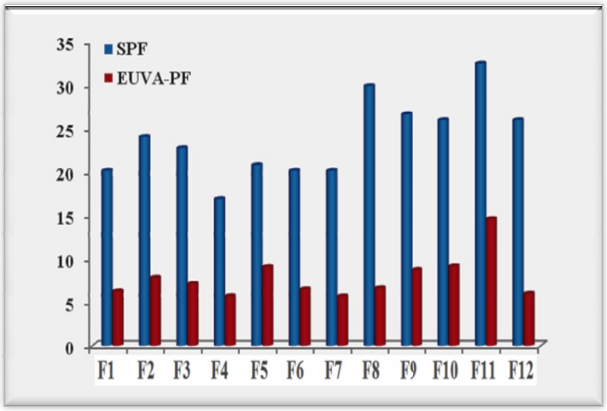

Fig. 4: Sun protection factor (SPF) and UVA- protection factor of the different prepared BZ-3 ME gel formulae using BS as oil.

Regarding IPM oil,it was found that increasing the concentration of BZ-3 from 1% to 3% w/w, results in a significant increase in the SPF of BZ-3 (***p*<0.0001) thereby, an increased level of protection was reported by increasing the concentration of the filter [54]as high concentration of BZ-3 increases the amount of light absorbed by the formulae leading to further skin protection. This might be due to the solubilization of the drug in the ME vehicle giving rise to an even distribution producing products with SPF>15, comparing with conventional carbopolgels having the same BZ-3 concentration whose SPF ≤ 2 (Figures 5&6).

Regarding the effect of increasing S/CoS$_{mix}$(1:1)concentration from 40% to 60% w/w, no significant difference (*p*>0.682) in the SPF values was observed, while upon increasing the IPM oil concentration from 10% to 20% w/w, a significant reduction (***p*<0.0001) in the SPF was observed as illustrated in Figures 5&6.

Concerning BS oil,it is clear that upon increasing the concentration of BZ-3 from 1% to 3% w/w, the SPF of BZ-3 was significantly increased; an increased level of protection was reported by increasing the concentration of the filter as previously discussed [54] (Figures 5&6).

It was also found that 40% S/CoS$_{mix}$ (1:1) concentration gave an overall significant increase (***p*<0.0001) in the SPF over 60% w/w concentration,while it was found that 10% oil concentration gave no significant difference (*p*>0.986) in the SPF over20 % w/w as shown in Figures (5&6).

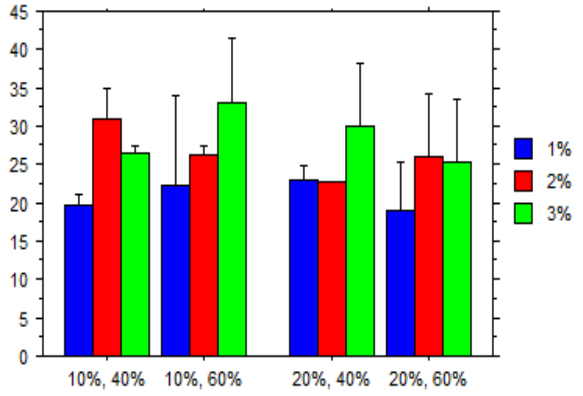

Fig. 5: Interaction bar plot for the effect of BZ-3 (1, 2, 3%), S/CoS mix (40, 60%) and IPM oil (10, 20%) concentration on SPF values of the different prepared BZ-3 ME gel formulae using IPM as oil

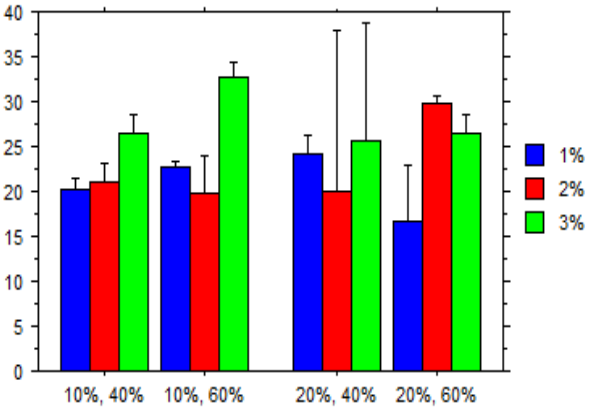

Fig. 6: Interaction bar plot for the effect of BZ-3 (1, 2, 3%), S/CoSmix (40, 60%) and BS oil (10, 20%) concentration on SPF values of the different prepared BZ-3 ME gel formulae using BS as oil.

Evaluation tests for the selected BZ-3 ME gel formulae

According to the factorial outcomes, two formulae were selected one from each oil having the highest SPF value, their composition were given in Table (4)and were subjected to further investigations as follows:

Table 4: Composition of the two selected BZ-3 ME gel formulae.

Formulae Code	BZ-3 concentration (%w/w)	Oil type	Oil concentration (%w/w)	S/CoS$_{mix}$ (1:1) (%w/w)	
F11 IPM	3	Isopropyl Myristate (IPM)	10	Tween 80 /Crem RH40	640%
F11 BS	3	Black Seed (BS)	10		660%

Table 5: Absorbance of sodium nitroprusside solution (0.05% w/v) degraded at 395 nm after exposure to UV lamp for 2 hoursfollowing application of the two selected BZ-3 ME gel formulae.

Petriplates covered with	Absorbance of the degraded sodium nitroprusside solution.
F11 IPM	0.527±0.083
F11 BS	0.880±0.030
Control (Covered with cellophane membrane)	1.516±0.038

Sunscreen Efficacy Testing

The absorbance data of the degraded sodium nitroprusside solution measured at 395 nm after exposure to UV lamp for 2 hours for the two selected BZ-3 ME gel formulae (F11IPM and F11BS) and the control were given in Table (5). It is clear that F11 IPM gave the best sunscreen efficacy ($*p<0.05$) expressed by the lowest absorbance value equals to 0.527±0.083 compared to 0.880±0.030 and 1.516±0.038 for F11BS and control respectively

Refractive Index

The values of refractive index for F11IPM and F11BSwere:1.435±0.001 and 1.439±0.000 respectively. A value of refractive index less than 1.476 is a good indication that the two selected BZ-3 ME gel formulae are transparent and optically isotropic[55].

***In-vitro* BZ-3 permeation studies through excised newly born rat skin**

*In-vitro*BZ-3 permeations were carried out through excised newly born rat skin over a period of eight hours in 1% Tween 80 in PBS solution (pH=7.4) at 37± 1℃ for the two selected BZ-3 ME gel formulae(F11IPM, F11BS) and compared to the corresponding carbopol gel having the same concentration of BZ-3.

In-vitro permeation parameters were given in Table (6), where the amount permeated per unit area was plotted against time (hr). The drug flux (permeation rate) at steady state (J_{SS}) was calculated from the slope of the straight line. Permeability coefficient (K_P), correlation coefficient (r^2), $t_{50\%}$ (time required for 50% of the drug to be permeated) and the amount of drug permeated after eight hours ($Q_{8\ hrs}$) were also determined for each formula and compared to the corresponding BZ-3 carbopol gel having the same concentration.

Table 6: Permeation parameters of the two selected BZ-3 ME gel formulae after eight hours compared to the corresponding BZ-3 carbopol gel.

Formulae	r^2	Flux (J_{SS}) (µg/cm²/hr)	Kp (cm/hr)	$t_{50\%}$ (hr)	Amount permeated (µg/cm²)	Percent Permeated (%)
F11 IPM	0.976	20.81	0.297	61.40	192.00	7.31
F11 BS	0.959	37.74	0.539	33.80	315.00	12.00
3%BZ-3 Carbopol Gel	0.980	40.21	0.574	31.96	338.43	12.89

It is clear from the results of BZ-3flux at steady state (J_{SS}), permeability coefficient (K_P), $t_{50\%}$ and the percentage of BZ-3 permeated ($Q_{8\ hrs}$) of the two selected formulae (F11IPM, F11BS) that F11IPM formula shows the lowest permeation profile outcomes ($**p<0.0001$) as shown in Table (6).Therefore, F11IPM will be taken for further investigation by assessment of its microbiological UV blocking activity.

Microbiological UV blocking activity

After incubation of the four different serial dilutions (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) of *E. coli* inoculums for 24 hours at a temperature of 37±1℃ as previously discussed, $10^{-4}$was selected as the suitable dilution containing the suitable number of colonies that could be cultured per plate.

Table (7) shows the number of colony forming unit per mL and log survival count of bacteria exposed to UV light at 365 nm at different time intervals. After different exposure time intervals, the number of colonies survived reflects the effect of UV bactericidal activity according to the duration of exposure.

A decrease in the number of surviving colonies was observed with the progressive increase in the UV exposure time. The initial number of bacteria (N_o) exposed to the UV light at time zero is around 428.00±14.14 CFU/ml, while at the end of the experiment it was found to be around 67.50±3.53 CFU/ml.

Table 7: Colony forming unit per ml (CFU/ml) and log survivor count of *E. coli* bacteria exposed to UV light at 365 nm at different time intervals.

Time (minutes)	CFU/ml (±S.D)	Log (Number of survivals)
0	428.00±14.14	2.63
3	245.00±7.07	2.38
5	236.00±22.62	2.37
10	197.00±24.04	2.29
20	148.00±16.97	2.17
40	116.00±5.65	2.06
60	93.50±2.12	1.97
80	89.00±8.48	1.94
100	79.00±1.41	1.89
120	67.50±3.53	1.82

Similar outcomes were obtained by Milosevic et al (2011)[56]and Gomaa et al (2006)[40] who found that the decrease in number of bacteria was observed after irradiation, this reduction increased upon increasing the duration of exposure and that the survival of bacteria is influenced significantly by: the initial bacterial culture count, the duration of exposure and interaction between duration and bacterial culture.

Figure (7) shows the logarithms of the survivals plotted against exposure time.

The decimal reduction time (DRT) was determined from the negative reciprocal of the slopes of the regression lines, using the linear portions of the survivals curves (log10 CFU/ml versus time of exposure to UV radiation[40].

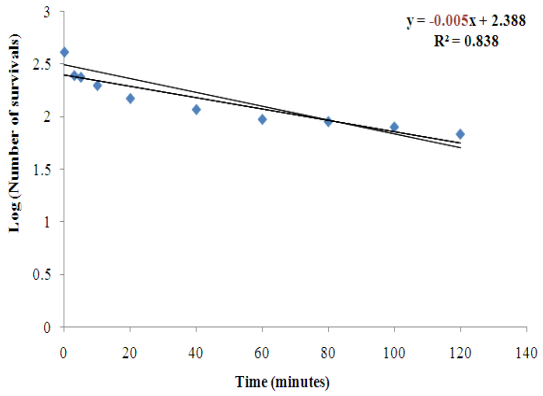

Fig. 7: Log survivals/time graph for *E.coli* following the exposure to UV light at 365 nm at different time intervals at 37±1°C.

The decimal reduction time was found to be equal to 200 min, which means that the bacterial viable count will be reduced to 90% of its value after 200 min of exposure to UV radiation. This D-value was taken as the time at which the comparison between the tested sunscreening product (F11IPM) and the market product (Spectra Ban) will take place.

It is clear from Table (8) that in case of non irradiated plates (C_{dark}), the total number of colonies is the same, which means 100±6.03% survival, while in case of control irradiated without the use of sunscreening agent (C_{UV}), the total number of colonies shows only 14.03±1.40% survival. The percent survival of *E. coli* bacteria is calculated by counting the number of colonies that survived after exposure to UV light divided by total number present multiplied by 100.

Table 8: Percent survival of *E.coli* bacteria after application of F11 IPM and the market product Spectra Ban plus (SPF30) following exposure to UV light at 365 nm for 200 minutes at 37±1°C

Formula code	*E.coli* Survival percent (±S.D.)
F11 IPM ME gel	88±5.84
Control (C_{dark}) (Non-irradiated and without sunscreening agent)	100±6.03%
Control (C_{UV}) (Irradiated and without sunscreening agent)	14.03±1.40%
Spectra Ban plus SPF30	80±3.04

The survival percent of F11 IPM ME gel (SPF=33) was found to be equal to 88±5.84% which is not significantly different from the standard Spectra Ban plus (SPF=30)having a survival percent of 80±3.04%, confirming that both formulae are effective with higher UV blocking activity.

CONCLUSION

The developed BZ-3 ME gel formula (F11IPM) shows a great potential for improving the *in-vitro* SPF of BZ-3 due to its synergistic effect as a vehicle while decreasing its skin permeation. The *in-vitro* microbiological method to test the UV blocking activity of the optimized formula is simple and reliable showing acceptable *in-vitro*/*in-vivo* correlation, therefore can be used as a preliminary test for the development of sunscreen formulations.

CONFLICT OF INTERESTS

Declared None

REFERENCES

1. El-Badry M, Fetih G, Shakeel F. Comparative topical delivery of antifungal drug Croconazole using liposome and micro-emulsion based gel formulations. J Drug Deliv 2014;21(1):34-43.
2. Montenegro L, Carbone C, Puglisi G. Vehicle effects on *in-vitro* release and skin permeation of octylmethoxycinnamate from microemulsions. Int J Pharm 2011;405:162-8.
3. Paul BK, Moulik SP. Uses and applications of microemulsions. J Curr Sci 2001;80:990-1001.
4. Tenjarla S. Microemulsions:an overview and pharmaceutical applications. J Crit Rev Ther Drug Carrier Syst 1999;16:461-521.
5. Park KM, Kim CH. Preparation and evaluation of flurbiprofen-loaded microemulsion for parenteral delivery. Int J Pharm 1999;18:173-9.
6. Lawrence MJ, Rees GD. Microemulsion-based media as novel drug delivery systems. Adv Drug Del Rev 2000;45:89-121.
7. Tsai YH, Lee KF, Huang YB, Huang CT, Wu PC. *In-vitro* permeation and *In-vivo* whitening effect of topical hesperetinmicroemulsion delivery system. Int J Pharm 2010;388:257-62.
8. Soliman SMH, Abd El-Rehim AEA, El-Gazayerly ON, Abd El-Malak NS. Formulation of microemulsion gel systems for transdermal delivery of celecoxib:*in vitro* Permeation, anti-inflammatory activity and skin irritation tests. J Drug Discov Therap 2010;4(6):459-71.
9. Boonme P. Application of microemulsions in cosmetics. J Cosmet Dermatol 2007;6:223-8.
10. González S, Lorente MF, Calzada YG. The latest on skin photoprotection. J Clin Dermatol 2008;26:614-26.
11. Seite S, Fourtanier AM. The benefit of daily photoprotection. J Am Acad Dermatol 2008;58:160-66.
12. Wankhade RP, Pundir AR, Bhalerao SS. Microemulsion as Novel Cosmeceutical Drug Delivery System. J Pharm Cosmetol 2012;9(2):78-92.
13. Lautenschlager S, Wulf HC, Pittelkow MR.Photoprotection. J Lancet 2007;370:528-37.
14. Grampurohit N, Ravikumar P, Mallya R. Microemulsions for Topical Use-A Review. Ind J Pharm Edu Res 2011;45:100-7.
15. Patel M, Jain SK, Yadav AK, Gogna D, Agrawal GP. Preparation and Characterization of Oxybenzone-Loaded Gelatin Microspheres for Enhancement of Sunscreening Efficacy. Drug Deliv 2006;13:323-30.
16. Hernàndez JRV, Goymann CCM. Artificial Sun Protection:Sunscreens and Their Carrier Systems. J Curr Drug Deliv 2006;3:405-15.
17. Treffel P, Gabard B. Topical bioavailability of sunscreen agents in stratum corneum:effect of vehicle and time. J Pharm Res 1996;13:770-4.
18. Hagedorn-Leweke U, Lippold BC. Absorption of Sunscreens and Other Compounds Through Human Skin *in Vivo*:Derivation of a Method to Predict Maximum Fluxes. J Pharm Res 1995;12:1354-60.
19. Hayden CG, Roberts MS, Benzon HAE. Systemic absorption of sunscreen after topical application. J Lancet 1997;350:863-4.
20. Yener G, Hadgraft J, Pugh WJ. Penetration of two sunscreens in various formulations through a synthethic membrane as compared to human skin *in vitro*. J Acta Pharm Turc 1998;40(1):27-32.
21. Gupta VK, Zatz JL, Rerek M. Percutaneous absorption of sunscreens through micro-Yucatan pig skin in vitro. J Pharm Res 1999;16:1602-7.
22. Badawi AA, Nour SA, Sakran WS, El Mancy SS. Preparation and evaluation of microemulsion systems containing salicylic acid. J AAPS Pharm Sci Tech 2009;10:1081-84.
23. Shakeel F, Baboota S, Ahuja A, Ali J, Aqil M, Shafiq S. Nanoemulsions as vehicles for transdermal delivery of aceclofenac. J AAPS Pharm Sci Tech 2007;8:E1-E9.
24. Such H, Jun HW. Physicochemical and release studies of Naproxen in poloxamer gels. Int J Pharm 1996;129:13-20.
25. El Maghraby GM. Transdermal delivery of hydrocortisone from eucalyptus oil microemulsion:Effects of cosurfactants. Int J Pharm 2008;355:285-92.

26. Djordjevic L, Primorac M, Stupar M. *In-vitro* release of diclofenacdiethylamine from caprylocaproylmacrogolglycerides based microemulsions. Int J Pharm 2005;296:73-9.
27. Azeem A, Rizwan M, Ahmad FJ, Iqbal Z, Khar RK, Aqil M *et al*. Nanoemulsion components screening and selection:a technical note. J AAPS Pharm Sci Tech 2009;10:69-76.
28. Nour SA, Shalaby SH, Afify NN, Abd El-Aal S, Mekhael MK. Formulation and evaluation of econazole nitrate emulgels. J Drug Res Egypt 2002;24:63-71.
29. Ambade KW, Jadhav KR, Gambhire MN, Kurmi SD, Kadam VJ, Jadhav KR. Formulation and evaluation of flurbiprofenmicroemulsion. J Curr Drug Deliv 2008;5:32-41.
30. Brime B, Moreno MA, Frutos G, Ballesteros MA, Frutos P. Amphotericin B in oil-water lecithin-based microemulsions: formulation and toxicity evaluation. J Pharm Sci 2002;91:1178-85.
31. Diffey BL, Robson J. The influence of pigmentation and illumination on the perception of erythema. J Photodermatol Photoimmunol Photomed 1992;9:45-7.
32. María CS, Revilla E, Miramontes E, Bautista J, Martínez AG, Romero E *et al*. Protection against free radicals (UVB irradiation) of a water-soluble enzymatic extract from rice bran. Study using human keratinocyte monolayer and reconstructed human epidermis. J Food Chem Toxicol 2010;48:83-88.
33. Vettor M, Perugini P, Scalia S, Conti B, Genta I, Modena Tet al. Polylactidenanoencapsulation to reduce photoinactivation of a sunscreen agent. Int J Cosmet Sci 2008;30:219-27.
34. Kaidbey KH, KligmanAM. Laboratory methods for appraising the efficacy of sunscreens. J Soc Cosmet Chem 1978;29:525-36.
35. Pathan IB, Setty CM. Stability evaluation of tamoxifen citrate nanoemulsion containing Cremophor RH 40 as surfactant. J Acta Pharm Sci 2011;53:127-34.
36. Yener G, Incegül T, Yener N. Importance of using solid lipid microspheres as carriers for UV filters on the example octylmethoxycinnamate. Int J Pharm 2003;258:203-7.
37. Yang J, Wiley CJ, Godwin DA, Felton LA. Influence of hydroxypropyl-b-cyclodextrin on transdermal penetration and photostability of avobenzone. Eur J Pharm Biopharm 2008;69:605-12.
38. American A, Washington DC. American Public Health Works Association and Water Pollution Control Federation;1989.p.
39. Munshi HA, Saeed MO, Green TN, Al-Hamza AA, Farooque MA, Ismail ARA. Impact of UV irradiation on controlling biofouling problems in NF-SWRO desalination process. International Desalination Association (IDA) World Congress: Conference Singapore; 2005.
40. Gomaa YAMM. Master Sc Thesis, Faculty of Pharmacy, Alexandria University;2006.p. 65, 74, 109, 128, 165, 188, 200, 205, 213.
41. Roszakt DB, Colwell RR. Metabolic activity of bacterial cells enumerated by direct viable count. J Appl Environ Microbiol 1987;53:2889-983.
42. Mazzola PG, Penna TCV, Martins AMS. Determination of decimal reduction time (D value) of chemical agents used in hospitals for disinfection purposes. J BMC Infect Dis;2003. http://www.biomedcentral.com/1471-2334/3/24.
43. White HR. The Heat Resistance of Streptococcus faecalis. J Gen Microbiol 1953;8:27-37.
44. Amin MM, Sheta NM, Abd El Gawad NA, Badawi AA. Preparation and characterization of Benzophenone-3 loaded polymeric nanoparticles of lactide-co ε-caprolactone as drug carriers. J pharm Res Opin 2012;2:28-41.
45. Abd El-Malak NS. PhD Thesis, Faculty of Pharmacy. Cairo University;2006.
46. Sanad RA, Abd El Malak NS, El Bayoomy TS, Badawi AA. Formulation of a Novel Oxybenzone-Loaded Nanostructured Lipid Carriers (NLCs). J AAPS Pharm Sci Tech 2010;11:1684-94.
47. Hathout RM. PhD Thesis, Faculty of Pharmacy, Ain Shams University;2010. p.45.
48. Schuch AP, Menck CFM. The genotoxic effects of DNA lesions induced by artificial UV-radiation and sunlight. J Photochem Photobiol B 2010;99:111-6.
49. Sambandan DR, Ratner D. Sunscreens:An overview and update. J Am Acad Dermatol 2011;64:748-58.
50. Chen H, Chang X, Weng T, Zhao X, Gao Z, Yang Y *et al*. A study of microemulsion systems for transdermal deliveryoftriptolide. J Control Rel 2004;98:427-36.
51. Gannu R, Palem CR, Yamsani VV, Yamsani SK, Yamsani MR. Enhanced bioavailability of lacidipine via microemulsion based transdermal gels:Formulation optimization, ex *vivo* and *in-vivo* characterization. Int J Pharm 2010;388:231-41.
52. Desai KGH. Enhanced skin permeation of rofecoxib using topical microemulsion gel. J Drug Dev Res 2004;63:33-40.
53. Shafiq S, Shakeel F, Talegaonkar S, Ali J, Baboota S, Ahuja A *et al*. Formulation Development and Optimization Using Nanoemulsion Technique:A Technical Note. J AAPS Pharm Sci Tech 2007;8:E1-E6.
54. Forestier S. Rationale for sunscreen development. J Am Acad Dermatol 2008;58:S133-S8.
55. Saukharev AR, Wojciechowska J. Microemulsion as potential ocular drug delivery systems: phase diagrams and physical properties depending on ingredients. J Acta Pol Pharm 2005;62:465-71.
56. Milosevic D, Solaja MM, Lj NTT, Stijepic MJ, Glusac JR. The survival of Escherichia coli upon exposure to irradiation with non-coherent polychromatic polarized light. J Vet Med 2011;56:520-527.