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FORMULATION, PHYSICOCHEMICAL CHARACTERIZATION AND IN VITRO EVALUATION OF WATER-IN-OIL MICROEMULSION CONTAINING VITAMIN E FOR TOPICAL APPLICATION

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ABSTRACT

Vitamin E is an antioxidant agent with low aqueous solubility and bioavailability which has been used to protects the skin from atopic dermatitis and various deleterious effects due to solar radiation by acting as a free-radical scavenger. The aim of the present study was to formulate, to characterize and in vitro evaluate the microemulsion containing vitamin E at 0.1% for topical skin application. In order to design suitable microemulsion system, appropriate amounts of isopropyl myristate, tween 80, propylene glycol and water able to encapsulating high vitamin E amount were earlier defined by our research group and herein characterized. Physicochemical properties such as particles size, polydispersity index, zeta potential and encapsulation efficiency were measured. The particles size, polydispersity index, zeta potential was obtained by dynamic light scattering method and the analysis indicated an average of particles size 250 nm, a low PDI index and negative zeta potential. In this work, the investigated vitamin E-loaded microemulsion and unloaded microemulsion (control) were unable to induce cell cytotoxicity in the concentration range below 0.75mg/mL of vitamin E after 24h cell exposure.

1. INTRODUCTION

Topical application of drugs provides accessible entry of them through the skin, being an alternative route for local and systemic drug effects with a decrease in the side effects related to the other routes of administration. The use of nanotechnology compared to conventional formulations has been shown to offer advantages in the treatment of cutaneous diseases, which have been achieved through nanocarriers, which are satisfactory and promising results, mainly related to the overlap of the cutaneous barrier. The application of nanotechnology compared to conventional formulations offers several advantages which are drug increased solubility, protection against degradation, increased skin diffusion and retention of the drug carried (Wu et al., 2009).

However, the skin remains the largest barrier to skin penetration due to the stratum corneum layers (Depieri et al., 2015). Stratum Corneum in the presence of atopic dermatitis (AD) was related to skin hydration degree decreased and transepidermal water loss (TEWL) increased (Lin et al., 2013) and consequent impairment of the skin barrier function (Elias et al., 2014).

The AD is a common chronic skin inflammatory disease. The pathogenesis of AD is attributed to both epidermal barrier dysfunction and chronic Th2 inflammation into the skin. As a result, this impairment of the skin barrier is considered a primary event in AD pathogenesis (Elias et al., 2011). Perturbed barrier function largely contributes to the allergic sensitization to both protein antigens and staphylococcal superantigens. Moreover, the inflammation underneath the barrier can alter the differentiation of epidermis, leading to disrupted barrier function. Therefore, it has been proposed that early interventions to repair the epidermal barrier with the use of appropriate soaps, emollients, or moisturizers may be useful in the control of this chronic disease as well as the prevention of its progression (Elias et al., 2011).

Vitamin E (VitE) is an antioxidant agent used for more than 50 years in dermatology (Figure 1). VitE was first described in 1922 by Herbert M Evans and Katherine Bishop in 1936, it was biochemically characterized and named tocopherol such as Greek: “tocos” meaning offspring and “phero” meaning to bring forth (Evans and Bishop, 1922). The antioxidant activity of tocopherols is mainly due to their ability to donate their phenolic hydrogens to lipid free radicals, inhibiting oxidation process (Kamal-Eldin et al., 1996).

Topical vitamin E has emerged as a popular treatment for a number of skin disorders owing to its antioxidant properties (Tanaka et al., 1993) Tsoureli-Nikita et al. (2002) reported a single-blind, placebo-controlled study in atopic dermatitis, by in which 96 patients were treated with either placebo or oral vitamin E (400 IE/day) for 8 months. They found an improvement and near remission of AD and in the vitamin E-treated group a 62% decrease in serum IgE levels. Vitamin E decreases serum levels of IgE in atopic subjects. The correlation between vitamin E intake, IgE levels, and the clinical manifestations of atopy indicate that vitamin E could be a therapeutic tool for atopic dermatitis. Most of the antiaging creams contain from 0.5% to 1% of vitamin E. One of the most popular applications of vitamin E is the treatment of burns, surgical scars, and wounds. However, studies looking at the efficacy of vitamin E in the treatment of burns and scars have been disappointing (Chiu et al., 2003; Ashamalla et al., 1988).

On the other hand, topical vitamin E was found to be effective in granuloma annulare as a promising cosmetic appearance of scars (Baumann et al., 1999) as well as anti aging treatment (Jenkins et al., 1986). In addition, topical application of the gel containing 2% phytonadione, 0.1% retinol, 0.1% vitamin C, and 0.1% vitamin E has been seen to be fairly or moderately effective in reducing dark under-eye circles, especially in cases of hemostasis (Mitsuishi et al., 2004).

In the present work, we evaluated a topical ME based on isopropyl myristate as a promising vitamin E delivery system for AD treatment. For this, physicochemical properties such as particles size, polydispersity index, zeta potential, encapsulation efficiency, differential scanning calorimetry and *in vitro* cell cytotoxicity were measured.

2. MATERIALS AND METHODS

2.1 Materials

Vitamin E (α-tocopherol) were gently provided from Quântica Farmacia de Manipulaçao (Catanduva-SP, Brazil), polysorbate 80 and propylene glycol were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Water was purified using the Millipore Milli-Q® Water System (Millipore Corporation, Bedford, USA). Amicon® Ultra centrifugal filter devices were purchased from Millipore (Darmstadt, Germany). All other chemicals were of analytical or HPLC reagent grade and purchased from Merck (Darmstadt, Germany).

2.2 Microemulsion Preparation

The microemulsion was prepared as previously reported by Zhinan Mei et al., 2003. Mixtures of isopropyl myristate (IPM), polysorbate 80 (Tween 80), propylene glycol, and water were obtained. The surfactant: cosurfactant (Tween 80 and propylene glycol) were blended in a 5:1 mass ratio to obtain the surfactant mixture. The IPM and distilled water were then added. Vitamin E (0.1% w/w concentration) was incorporated in surfactant mixture of the ME by stirring with a magnetic stirrer for 30 min. Finally, the components were sonicated in an ice bath at 22.5 kHz for 4 min.

2.3 Physicochemical characterization

2.3.1 Size, polydispersity index and zeta potential by dynamic light scattering

The mean diameter and particle size distribution of unloaded and loaded lipid nanocarriers prepared were determined using a dynamic light scattering system (Zetasizer, NanoZS, Malvern, UK) containing a laser system of 4mW He-Ne, operating at a wavelength of 633 nm. Measurements were taken in a 173 ° detection angle and the measurement position within the cuvette was automatically determined by the software. The data represent the average values from three separate measurements. For this procedure, samples were first diluted in 1 mM Kcl or water (1:400, v/v) and the measurements were performed at 25ºC. Measurements of the particle electrophoretic mobility were carried out using the same instrument. The equipment performs an average of 12 determinations for each analysis. The data represent the average values from three separate measurements.

2.3.3. Calorimetric measurements

Differential scanning calorimetry (DSC) measurements were made using a Jade DSC model (Perkin Elmer, Waltham, Massachusetts, USA) and Pyris™ software (Perkin Elmer, Waltham, Massachusetts, USA) for data processing. Five to ten micrograms of each formulation were placed in an aluminium pan under nitrogen atmosphere (3 Kgf/cm^2) and submitted to DSC analyse. A similar empty pan was used as the reference. The temperature range of analysis was first heating from 30 °C to 75 °C at a rate of 10 °C/min, then cooling from 75 °C to 30 °C at a rate of 10 °C/min. The measurements were made to evaluate any possible phase transition after incorporation of vitamin E (0.1%) into microemulsion system.

2.3.4 Measurement of encapsulation efficiency

The encapsulation efficiency (EE) was performed by ultrafiltration process using Amicon® Ultra centrifugal filter devices (Millipore, Darmstadt, Germany). Exactly 0.5 mL of vitE-

loaded microemulsion system was added to the filter unit followed by centrifugation at 6000 rpm (Centrifuge 5430R, Eppendorf, Hamburg-Eppendorf, Germany) for 5 min at 22 °C. The non entrapped vitamin were collected in the ultrafiltrate (M_{FD}) since a molecular weight cut-off of 50 kDa for filter was used, and then, it was quantified by validated HPLC assay as well the total vitamins amount from microemulsion (M_{TD}). EE was calculated by the follow formula: EE (%) = (M_{TD} - M_{FD})/ M_{TD} x 100.

2.3.5 HPLC Assay

Identification and quantification of vitamin E were carried out by High Performance Liquid Chromatography (HPLC) following previously reported by Alencastre and colleagues, 2006. HPLC method used a Shimadzu system (Kyoto, Japan) with UV-visible (UV-vis) detector to 290 nm and Lichnospher®100 RP-18 (10 cm length, 5 µm, 4 mm LiChrospher; Merck, Darmstadt, Germany) as chromatographic column and isocratic mobile phase was methanol–water (99:01, v/v) previously degassed with helium gas. The analyses were performed at room temperature (25°C, approximately), with mobile phase flow rate of 1.2 ml min^{-1} and injecting 50 µL. The standard curve was linear from 2.5 to 100 µg/mL of vitamin E in methanolic solutions showing linear correlation coefficient (r) greater than 0.99 and CV% lowers than 10%. The microemulsion containing vitamin E was diluted and destroyed with Methanol and the vitamin was quantified by HPLC. Finally, the method was validated following the established parameters such as selectivity, linearity, intra-day precision and accuracy, lower limit of quantification (LLOQ), and lower detection limit (LLOD) (The European Agency for the Evaluation of Medicinal Products, 1996; Shabir, 2003). The method precision and accuracy were assessed from 9 determinations of different Vitamin E concentrations: low, medium and high such was 10, 40 and 100µg/mL evaluated in 3 different days. Each replica was prepared independently. The Precision and accuracy were expressed as coefficient of variation (CV) and relative error in percentage (RE,%), respectively. The acceptance criterion was variations values lower than 15%.

2.4 In vitro studies

2.4.1 Cell line

The HaCaT cells line were cultured in 75 cm^2 flasks with high glucose Dulbecco's Modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum and antibiotics (100 IU/mL of penicillin, streptomycin and 250 ng/mL amphotericin-B) at 37 ºC in which the CO2 level was kept constant at 5%.

2.4.2 Cell viability assay

The cell viability was assayed by resazurin reduction assay following the instructions of the manufacturer's protocol. Cells were seeded in 96-well culture plate at 10^4 /well for the HaCaT cell line 24 hours before treatments. Serial dilutions of samples were freshly prepared in 0.01mM phosphate buffer solution (pH 7.4) in the range of 3 to 0.09 mg/mL of Vitamin E. Cells were treated for 24 hours, thus the cells were washed twice with complete DMEM and incubated with 90 µL of DMEM and 10 µL of the resazurin reagent for 4 hours. Fluorescence intensities were measured at 540 nm in a SYNERGY-HT multiwell plate reader, Bio-Tek (USA) using KC4 software. Untreated cells were used as a control with 100% viability. The relative cell viability (%) compared to control cells was calculated by [abs] sample/ [abs] control × 100.

2.5 Statistical Analyses

Results obtained in this work were presented as mean ± standard deviation (SD) of three independent experiments. Linear regression was performed as a function of time from area peak for vitamin E concentration using three samples for each concentration. Coefficient of determination (r^2) for analytical curve of vitamin E was given with the linear regression function. Data were statistically analyzed by One-way analysis of variance (ANOVA) followed by the Newman-Keuls Multiple Comparison Test to compare all studied groups, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ using the SigmaPlot software.

3. RESULTS AND DISCUSSION

Formulated Vitamin E-loaded ME proposed in this work had a nanometric particle size, negative potential zeta and high loaded efficacy and it was suggested as a potential dermal delivery system for Vitamin E.

3.1 Preparation and characterization of microemulsions

Microemulsion developed and characterized herein was a water/oil microemulsion prepared with the components commonly acceptable for dermal use (Table 1) using a mixture of surfactant, co-surfactant, oil and aqueous phase. The vitamin was added in oil phase of the microemulsion. The Microemulsion are considered an effective delivery system to improve dermal delivery of both lipophilic and hydrophilic drugs (Esposito et al., 2003). In the last decade, Packer et al. (2002) reported that skin exposure to UV resulted in a significant potentiation of the UV-induced vitamin E (VitE) depletion, suggesting that VitE is efficiently quenching Reactive Oxygen Species (ROS) generated during UV skin exposure. In addition,

the use of topical VitE showed decreased number of sunburnt cells due to protection from free radicals (direct skin protection pathway), and increased epidermal thickness (indirect skin protection pathway), (Thiele et al., 2001).

As topical VitE had displayed photoprotection against acute and chronic UV-induced skin damage, such as inflammation/hyperpigmentation and skin cancer, respectively, it is widely used to protect the skin before UV exposure (Godic et al., 2014). However, VitE is light-sensitive which can rapidly degrade when exposed to UV, leading to decreased VitE levels in the topical creams or solutions (Argimón et al., 2017; Alencastre et al., 2006). Taking this into account, we assume that Vitamin E delivered from a microemulsion delivery system could to preserve their activity and improved the treatment efficacy and be a promising tool for dermal delivery of vitamin E to the deeper layers of the skin.

Thus, the vitamin E-loaded ME formulated and characterized in this work had a size, polydispersity and zeta potential determined by DLS and expressed Z-average size were between 126 and 131.7 nm and negative zeta potential (Table 2). The vitamin E addition had significantly affected these values. An increase from approximately 130 to 250nm of particle size was observed after vitamin E encapsulation. In addition the ME encapsulation efficiency was close to 100% for vitamin E at 0.01%. This high encapsulation efficiency is a positive feature of the ME herein developed and suggest protection against photodegradation of vitamin E from ultraviolet (UV) radiation exposition when applied to skin (Rozman B, Gasperlin M, 2007) and promote more drug concentration at the application's local (Barry, B. W., 2006).

Quantification of Vitamin E was procedure by HPLC using a selective, sensitive and precise analytical method earlier reported by our research group (Salomao et al., 2017). The linearity of VitE was obtained in methanolic solution at the range of 2.5 to 100 µg. mL^{-1} (Figure 2) and the results of HPLC validations parameters were summarized in Table 2. Retention time of Vitamin E was approximately 5 minutes and it was considered promising since allowed analyzing a large number of samples in a short period of time.

Differential scanning calorimetry measurements were procedure to evaluate the thermal properties such as melting point and enthalpy (crystalline structure) of the vitamin E, the lipids, the polymers and ME, as well as, the interactions between components. It was investigated at controlled temperature to evaluate any possible phase transition after

incorporation of the Vitamin E. the Figure 2 showed thermal behaviors of the individual components and the vitamin E-loaded ME system. Thermograms of pure components such were propylene glycol and Tween 80 reveals narrow endothermic peaks at 120 and 180°C, approximately, suggesting the crystalline state of the materials (Aliberti et al., 2017). Moreover, when vitamin E-loaded ME system was formed, these endothermic peaks were minimized due crystalline state amorphization (Maria TS et al., 2013).

3.2 *In vitro* studies

The low cell cytotoxicity induced by Vitamin E loaded microemulsion were evaluated in vitro using a human skin cell line HaCaT (Jancula et al., 2013). HaCat was chosen for this proposal since this is an immortal keratinocyte cell line from adult human skin and the gold standard to simulate the healty skin cells (Fabris et al., 2006; Jancula et al., 2013; Macedo et al., 2014; łukasz lamchet al.,2016).

In this work, the microemulsions in presence or not of vitamin E were unable to induce cell cytotoxicity in the concentration range below 0.75mg/mL of vitamin E during 24h cell exposure. This result is promising and this is in agreement with other reported (George et al., 2018).

Tables and Figures

Table 1: Composition of Microemulsion (%).

Component	Unloaded ME	VitE-loaded ME
Water	10	10
Isopropyl myristate	40	40
polysorbate 80: propylene glycol (5:1)	50	50
Vitamin E	_____	0.1

Table 2: Physicochemical Properties of Blank and Vit E -loaded Microemulsions.

Formulations	Particle Size (nm)	Polydispersity	Zeta potential	EE
Unloaded ME	129.1 (±2.61)	0.419 (±0.009)	-10.04 (±0.5)	---
Vit E-loaded ME	256.6 (±18.47)	0.427 (±0.12)	-8.03 (±0.32)	90%

Results are represented as mean ± SD (n = 3)

ME=microemulsion, Vit E=vitamin E

[a]The concentration of Vitamin E in the ME was 0.1% (w/w)

Table 3: Summary of HPLC validation parameters for Vitamin E.

Validation parameters			
Linearity			
Concentration range	2.5; 5.0; 10; 20; 40 and 100 µg/mL		
Regression equation	y = 2321.5x+2893.5		
Correlation coefficient	r^2 = 0.9983		
Precision/Accuracy (n = 9)			
Concentration levels	10 µg/mL	40 µg/mL	100 µg/mL
VitE mean ± SD; CV%	10.41±0.10; 0.97	44.98±0.08; 0.19	100.99±4.96; 4.98
Accuracy (RE, %)	4.12%	12.45%	1%
Sensitivity			
LLOD* (µg/mL)	1.25		
LLOQ** (µg/mL)	2.50		

*lower limits of detection; ** lower limit of quantification.

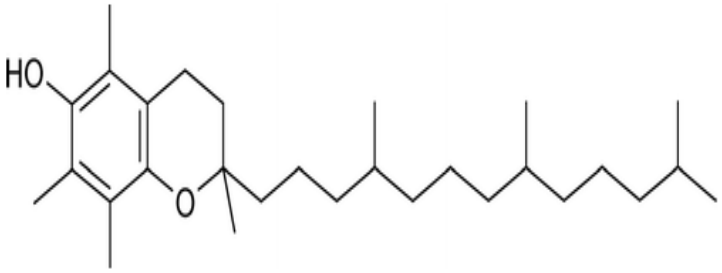

Figure 1: Molecular structure of vitamin E.

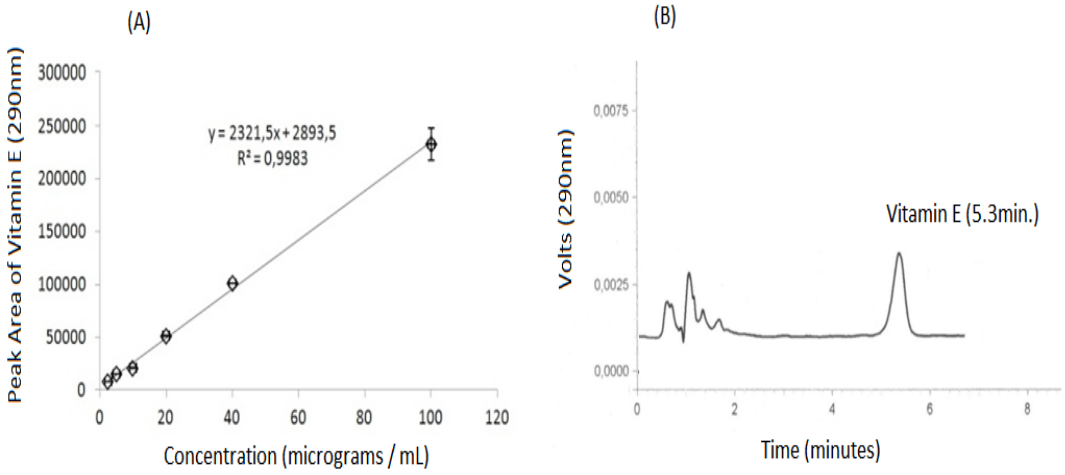

Figure 2: Quantification of Vitamin E by HPLC: (A) Linear regression of the Vitamin E in methanol solution showing (y = 2321.5x+2893.5) and correlation coefficient about 0.99, (B) Chromatogram of Vitamin E in methanol solution at 10 micrograms/mL. The chromatographic method was developed using an RP-18 column, methanol: water (99:01, v / v) as mobile phase, flow rate 1.2 mL.min^{-1} and detector operating at 290 nm.

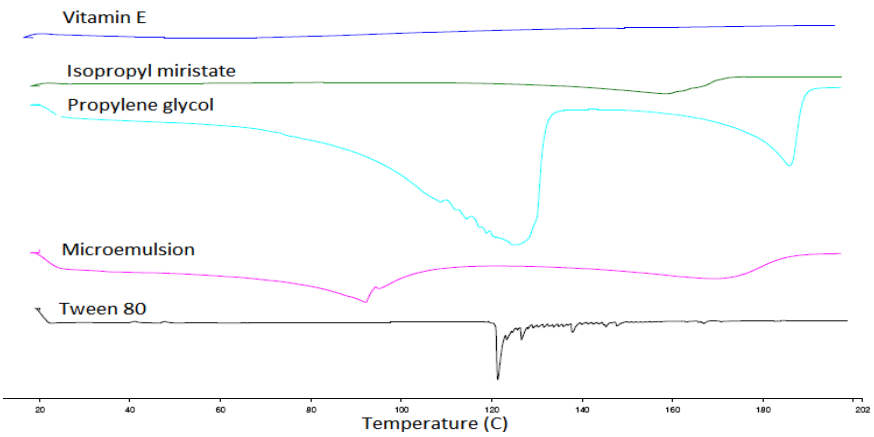

Figure 3: Differential scanning calorimetry thermograms of Microemulsion containing Vitamin E and its individual components.

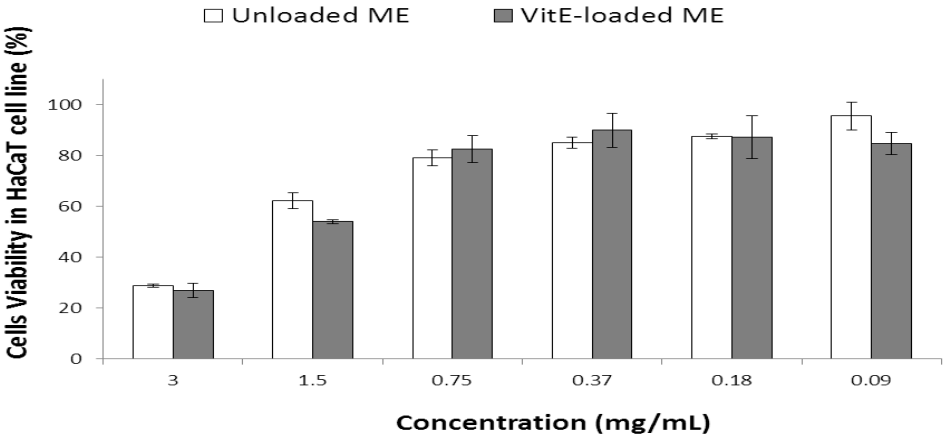

Figure 4: HaCaT cell viability results determined by resazurin uptake assay for the microemulsions with or without vitamin E after 24h of incubation.

4. CONCLUSION

In the current research a Vitamin E-loaded w/o microemulsion was developed for topical application as a vehicle for UV-protecting activity of Vitamin E when dermal applied. Microemulsion based on isopropyl myristate was efficiently developed to high encapsulation rate of vitamin E and appropriate amount of propylene glycol and tween 80 were defined as the surfactant and co-surfactant phase. Physicochemical characterization reveals particle size about 250nm and low PDI value. Moreover, Vitamin E-loaded microemulsion presented little cytotoxicity against HaCaT cell line. Taken together these results, our study illustrated that the proposed Vitamin E-microemulsion delivery system has a good potential for use in topical application for antioxidant dermal therapy. However, future assessments should

clarify the benefits of this microemulsion to delivery of vitamin E into the deeper layers of the skin.

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