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Development and photoprotective effect of a sunscreen containing the antioxidants *Spirulina* and *dimethylmethoxy chromanol* on sun-induced skin damage

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ABSTRACT

The literature claims that incorporation of antioxidants into sunscreens provides additional skin photoprotection by scavenging free radicals formed due to sun radiation, but there are limited *in vivo* studies that support this hypothesis. This study aims to examine whether addition of antioxidants to a broad-spectrum sunscreen increases its photoprotective effect in real-use conditions. Sunscreen formulations composed of stable UV filters (Tinosorb® S, Tinosorb® M, Uvinul® APlus, and Uvinul® T150) alone or in combination with antioxidants (*Spirulina* and dimethylmethoxy chromanol-loaded solid lipid nanoparticles) were developed and their appearance, odor, rheological behavior, Sun Protection Factor (SPF), and UVA protection were analyzed. Next, it was conducted a 3-month, single-blind clinical study with 44 healthy subjects (30–50 years). Before and 28, 54, and 84 days after twice-daily self application of the sunscreens on the face, the stratum corneum water content, transepidermal water loss, dermis echogenicity, and skin elasticity and pigmentation were measured. At the end of the study period, the volunteers answered a questionnaire containing terms related to sensory characteristics of the formulations. All formulations were stable and exhibited non-Newtonian and pseudoplastic behavior, *in vivo* SPF 30, and good UVA protection. Antioxidant supplementation to the sunscreen formulation significantly improved the skin pigmentation, the collagen degradation on the dermis and thereby the skin net elasticity after 84 days of treatment compared to the sunscreen alone. Concerning safety, all formulations were considered non-irritant according to the sensorial analyses, whose results agreed with the clinical study findings.

1. Introduction

UV radiation, which represents approx. 6–7% of the total amount of solar radiation that reaches the earth's surface, accounts for most of the sun-induced damages to the skin. UVB radiation is mostly absorbed in the epidermis and can directly damage DNA and cause sunburn and skin cancer after long-term exposure. UVA reaches deep dermal layers and participates in the generation of reactive oxygen species (ROS). These highly reactive molecules damage not only DNA but also the collagenous extracellular matrix of connective tissues, disrupt dermis integrity, and play crucial roles in photoaging, skin cancer, immunosuppression, and induction of sun tanning and pigmented spots (Natarajan et al., 2014; Polefka et al., 2012). Photoaging is the superposition of chronic UV-induced damage and intrinsic skin aging; up to 95% of the visible signs of skin aging result from exposure to sunlight (Agbai et al., 2014).

The regular use of sunscreens prevents sunburn and photoaging and reduces the risk of UV-related skin cancer (Hughes et al., 2013, Wu et al., 2011). Several studies have reported that sunscreens diminish the sun-induced ROS formation by nearly 50%, and suggested that incorporation of antioxidants into their formulation provides additional benefits to the skin by scavenging the formed ROS (Wu et al., 2011, Matsui, 2009, Haywood et al., 2003). Scientists have recently reported the addition of resveratrol, rutin, and other natural ingredients to sunscreen formulations as an alternative for preventing photochemical damage (Oliveira et al., 2016a, 2016b, Rigo et al., 2015, Pérez-Sánchez et al., 2014, Wu et al., 2013). However, there are few *in vivo* studies that demonstrate the effect of this association on human skin. The development of sunscreen formulations containing antioxidants that preserve the effectiveness of both in the final product is still a challenge.

Medicine and food industry researchers have extensively studied *Spirulina*, a blue-green algae belonging to the class cyanobacterium, due to its anti-inflammatory and antioxidant effects after dietary supplementation (Wu et al., 2016), but they have given little attention to the

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benefits of its topical application. Our research team has recently demonstrated that formulations containing *Spirulina* extract (at 0.1% w/w) are compatible with the skin and exert immediate effects on the skin microrelief and hydration (Delsin et al., 2015; Neto et al., 2014). In order to improve this effect, in this study it is proposed the association of *Spirulina* with Dimethylmetothoxy chromanol (DMC). DMC is a new synthetic γ-tocopherol analogue with high antioxidant property that scavenges ROS and reactive nitrogen species (RNS) *in vitro*, an advantage compared to other currently available antioxidant agents. Despite their high and broad antioxidant activity, DMC is an unstable molecule after incorporation into sunscreen formulations limiting its application.

A large number of nanostructured systems that have already been developed and applied to cosmetic products provide increased stability, reduced degradation, sustained and prolonged release, and reduced toxicity of the active ingredients (Oliveira et al., 2016a, 2016b; Shetty et al., 2015; Lacerda et al., 2011; Pardeike et al., 2009). Solid lipid nanoparticles (SLN) are usually composed of solid lipids, surfactants, and water, with a hydrophilic shell and a hydrophobic lipid core, which is solid at room temperature and allows the encapsulation of hydrophobic compounds (Zhai and Zhai, 2014). As SLN improve UV absorbance, they also greatly contribute to sunscreen development (Wissing and Müller, 2001).

This study aims: (i) to develop stable sunscreen formulations containing UV filters (Tinosorb® S, Tinosorb® M, Uvinul® APlus, and Uvinul® T150) alone or in combination with the antioxidants *Spirulina* and DMC-loaded SLN; (ii) to examine the *in vitro* and *in vivo* photoprotective effects of the developed formulations; and (iii) to assess whether the addition of antioxidants to a broad-spectrum sunscreen increases its photoprotective effect in real-use conditions. Different skin parameters were analyzed in healthy volunteers including pigmentation, barrier function, mechanical properties, and dermis echogenicity, and applied a “check-all-that-apply” (CATA) questionnaire to compare the clinical results with the volunteers' perception of the formulations efficacy.

2. Material and Methods

Polaxamer 407 and Tween 80 (polysorbate 80) were purchased from *via* Farma Ltda (São Paulo, SP, Brazil). Purified beeswax with an esterification index of 70% and melting point of 54 °C (batch number 153475) was supplied by Labsynth Ltda (São Paulo, SP, Brazil). All other chemicals used were of analytical grade.

The UV-filters *bis*-ethylhexyloxyphenol methoxyphenyl triazine (Tinosorb® S), methylene *bis*-benzotriazolyl tetramethylbutylphenol (Tinosorb® M, 50.0% of active ingredient), diethylamino hydroxybenzoyl hexyl benzoate (Uvinul® APlus), and ethylhexyl triazone (Uvinul® T150) were provided by BASF Personal Care and Nutrition (Monheim, Germany). Dimethylmethoxy chromanol (Lipochroman®, hydrophobic) was acquired from Lipotec SAU (Barcelona, Spain). *Spirulina* dry extract (Blue Green Algae®) was provided by Ouro Fino Agronegócios (Ribeirão Preto, SP, Brazil). This hydrophilic extract is rich in proteins (50–70% of its dry weight), and contains the amino acids methionine, glycine, and lysine; polysaccharides (8–14% of its dry weight) composed mainly of glucose, galactose, mannose, and ribose; lipids (about 6% of its dry weight), including γ-linolenic acid; and high concentration of pigments, including β-carotene (pro-vitamin A) and vitamin B (Neto et al., 2014).

2.1. Preparation and Characterization of DMC-loaded SLN

DMC-loaded SLN were prepared by the hot melt microemulsion technique reported by Freitas et al. (2015), with modifications. Beeswax (5% w/v) and DMC (0.5% w/v) were melted in a beaker in a thermostatic water bath at 60.0 ± 2.0 °C. Tween 80 (1.0% w/v) and Polaxamer 407 (2.0% w/v) were mixed with deionized water at 90.0 ± 5.0 °C and added slowly to the molten lipid mixture under magnetic stirring until the predetermined volume (q.s. to 100 mL). The resulting mixture was dispersed during 5 min, at 60.0 ± 2.0 °C and 20,000 rpm, using an Ultra-turrax – T25 Digital homogenizer (IKA Works Inc., EUA). Finally, the resulting nanoemulsion containing DMC was rapidly cooled to room temperature in an ice bath and stored at 4.0 ± 2.0 °C.

Twenty-four hours and 54 days after preparation of DMC-loaded SLN, their average size, zeta potential, and polydispersity index (PdI) were determined by dynamic light scattering using a ZetaSizer Nano Series instrument (Malvern Instruments, UK). The SLN samples were appropriately diluted with Ultrapure water (Milli Q, Millipore Inc., USA) at a 1:3 ratio (SLN dispersion:water). The surface appearance and shape of DMC-loaded SLN were determined 24 h after preparation, using an atomic force microscope model SPM-9600 (Shimadzu Co., Kyoto, Japan). The SLN samples were also diluted 1:200 using ultrapure water and spread onto thin mica plates.

2.2. Encapsulation Efficiency

The encapsulation efficiency of DMC-loaded SLN was determined indirectly by quantifying the total amount of DMC not trapped within SLN (free DMC) 24 h and 30 days after preparation and storage at 4.0 ± 2.0 °C. Aqueous insoluble free DMC was separated from the dispersion by centrifugation at 12,857 *g* for 30 min. The free DMC that could be soluble in the aqueous phase was separated from the dispersion by ultrafiltration (Amicon® Ultra 100 K, Millipore, Merck S/A, Ireland) followed by centrifugation at 12,857 *g* for 30 min. Free DMC was quantified by HPLC as described in Section 2.3.

The entrapped DMC content was determined by subtracting the total amount of free DMC (soluble and insoluble in the aqueous phase) from that initially added to the dispersion. DMC encapsulation efficiency (E.E. %) was calculated using Eq. (1):

$$EE(\%) = \frac{A_{total\ DMC} - A_{free\ DMC}}{A_{total\ DMC}} \times 100 \quad (1)$$

where A is the amount of DMC (mg).

2.3. DMC Quantification by HPLC

DMC was quantified by reverse-phase HPLC, as described by Nyeborg et al. (2010), with modifications, using the HPLC system UltiMate 3000 (Thermo Scientific®, São Paulo, Brazil) equipped with a pump, a system controller, a variable-wavelength UV/Vis detector, an auto-injector, the Chromeleon® software (version: 7.0), and a reverse-phase C18 column (Lichrospher® 100 RP-18, Merck Millipore Brazil, São Paulo, Brazil, particle size 5 µm, 1250 × 4.0 mm) maintained at 40 °C. Ten µL of the sample was injected and elution was carried out using ethanol/ultrapure water Milli Q mixture (gradient flow), supplemented with 0.1 M H_3PO_4 (pH = 3.7), at a flow rate of 0.7 mL/min and detection at 220 nm. DMC (retention time = 9.4 min) was quantified from a linear calibration curve (R = 0.998) built in the concentration range of 10–50 µg/mL.

2.4. Sunscreen Formulations

Three formulations (Table 1) were prepared using the o/w emulsification technique as previously described by Souza et al. (2017a, 2017b). Oil phase ingredients (C12-15 alkyl benzoate, caprylic/capric triglyceride, cetearyl alcohol/dicetyl phosphate/ceteth-10 phosphate, butylated hydroxytoluene, and phenoxyethanol/methylparaben/ethylparaben/butylparaben/propylparaben/isobutylparaben) and aqueous phase ingredients (water, disodium ethylenediaminetetraacetic acid, butylene glycol, and glycerin) were heated separately at 70 °C. Oil phase was added to aqueous phase under continuous stirring (600 rpm, 10 min; Heidolph magnetic stirrer, Fisaton, SP, Brazil), followed by