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Article

Development and evaluation of an emulsion containing lycopene for combating acceleration of skin aging

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Lycopene, a carotenoid and potent antioxidant is found in large quantities in tomatoes. Lycopene combats diseases, such as cardiovascular disease and different types of cancer, including prostate cancer. However, its topical use in emulsion form for the combat of skin aging is under-explored. The aim of the present study was to develop an emulsion containing lycopene extracted from salad tomatoes and evaluate its cytotoxicity, stability, rheological behavior, antioxidant activity and phytocosmetic permeation. The developed cosmetic comprised an oil phase made up of shea derivatives and was evaluated in terms of its physiochemical stability, spreadability, thermal analysis, rheological behavior, microbiological quality, cytotoxicity, antioxidant activity, cutaneous permeation and retention. The results demonstrate that this phytocosmetic is stable, exhibits satisfactory rheological behavior for a topical formula and is a promising product for combating skin aging.

Uniterms: Carotenoid/antioxidant activity. Lycopene/emulsion form/cytotoxicity. Lycopene/emulsion form/permeation. Lycopene/emulsion form/rheology. Lycopene/emulsion form/thermal analyses. Phytocosmetics. Skin aging/treatment.

Licopeno é um carotenóide com potente atividade antioxidante encontrado em grande quantidade no tomate e usado no combate a diversas doenças como doenças cardiovasculares e diferentes tipos de cânceres, incluindo o câncer de próstata. O objetivo desse trabalho foi desenvolver uma emulsão contendo extrato de licopeno obtido do tomate salada e avaliar a citotoxicidade do extrato, a estabilidade, o comportamento reológico, atividade antioxidante e permeação do fitocosmético. O cosmético foi desenvolvido utilizando fase oleosa contendo derivados de Karité e submetido à avaliação da estabilidade físico-química, espalhabilidade, análise térmica, comportamento reológico, qualidade microbiológica, citotoxicidade, atividade antioxidante e testes de permeação e retenção cutânea. Os resultados demonstraram que o fitocosmético é estável, apresenta comportamento reológico desejável para uma formulação tópica e é um produto promissor para ser utilizado no combate à aceleração do envelhecimento cutâneo.

Unitermos: Carotenóide/atividade antioxidante. Licopeno/emulsão/citotoxicidade. Licopeno/emulsão/permeação cutânea. Licopeno/emulsão/reologia. Licopeno/emulsão/análise térmica. Fitocosméticos. Envelhecimento cutâneo/tratamento.

INTRODUCTION

Lycopene is a natural carotenoid used to combat diseases, such as cardiovascular disease and different types of cancer, including prostate cancer (Shami, Moreira, 2004). It is mainly found in tomatoes but also presented in papaya, guava and watermelon (Shami, Moreira, 2004; Sentanin, Amaya, 2007). It can also be consumed with other active compounds for optimal function in humans (Kong *et al.,* 2010).

The carotenoid lycopene is a potent antioxidant with pro-vitamin A activity and it is most effective against singlet oxygen, which is potentially the most dangerous reactive oxygen species generated in the skin following exposure to sunlight (Giacomoni, 2007).

Antioxidant cosmetic formulas or phytocosmetics are commonly used to combat aging of the skin, and

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many of these products contain at least one substance with this purpose (Isaac, 1998; Giacomoni, 2007). In order to provide satisfactory anti-aging action through antioxidant activity, the formula should be stable and allow for the effective release of the active ingredient into the skin (Giacomoni, 2007).

Determination of the rheological behavior of a formula assists in the assessment of its physiochemical nature and allows for the detection of early signs of physical instability as well as quality control of the components, test formulas and final products (Barry, 1993). Cosmetic products may also undergo thermal analysis, which can be applied to the measurement of physical properties, study of chemical reactions, assessment of thermal stability, determination of the chemical composition of the materials and development of an analytical methodology (Faria, *et al*., 2002; Guillen, *et al.*, 2006). Skin permeation tests can be carried out using *in vitro* methods. The Franz-cell diffusion system allows for the assessment of permeation or retention of active compounds in a formula (Alencastre, *et al*., 2006; Franz, 1975).

The aim of the present study was to develop an emulsion containing lycopene extracted from salad tomatoes and assess its antioxidant activity for combating the acceleration of skin aging. Formula stability was determined through centrifugation assays, accelerated and preliminary tests for physicochemical stability, spreadability, and thermal analysis, and analyses of rheological behavior, microbiological quality, and cutaneous permeation and retention. The lycopene extract also was submitted to cytotoxicity testing to evaluate its safety.

MATERIAL AND METHODS

Material

Lycopene reference standard (assigned purity 90.0%) was supplied by Sigma-Aldrich. The emulsion was performed using raw materials (Shea butter, liquid shea butter, oleyl Sheabutterate, PEG-23 shea butterate and PEG 75 shea butter glycerides) donated by Ion-Química enterprise. Lycopene extract was obtained by salad tomato (adapted to Nune, Mercadante, 2004). Butylated hydroxytoluene (BHT), propylparaben, methylparaben, ethylenediamine tetraacetic acid (EDTA), propylene glycol, xanthan gum, carbomer, triethanolamine, 1,1-diphenyl-2-picrylhydrazyl (DPPH), resazurin, trypsin, chloroform, isopropanol, phosphate buffer, polysorbate 80, thioglycolate and Sabouraud agar, xylose-lysine-desoxycholate agar, bismuth sulfite agar, MacConkey agar, Vogel and Johnson agar and cetrimide agar were supplied by Galena, PharmaSpecial, Lubrizol, Sigma-Aldrich, Interlab and Synth enterprises. Vitamin C reference standard (assigned purity 99.0%) was also supplied by Sigma-Aldrich. Ethyl acetate, acetonitrile and methanol presented HPLC grade and they were supplied by Merck enterprise.

Development of an emulsion containing lycopene extract

An oil-in-water (O/W) emulsion was developed with: shea butter (1.2%), oleyl sheabutterate (0.8%), PEG-23 shea butterate (1.5%), imidazolidinyl urea (0.1%), BHT (0.02%), propylparaben (0.02%), methylparaben (0.18%), EDTA (0.02%), propylene glycol (4%), xanthan gum (0.15%), carbomer (2% dispersion) (25%), triethanolamine (q.s pH 6.0) and Aqua (water) (q.s.p. 100%). The O/W emulsion was prepared using the standard method. At 75 °C the aqueous phase was transferred into the oil phase at 75 °C and manual agitation was performed until the emulsion had cooled. The lycopene extract obtained from salad tomato (35.6 µg/mL of lycopene in the extract) (adapted from Nunes, Mercadante, 2004) was incorporated directly into the emulsion at a concentration of 0.1% using the geometric dilution method. The phytocosmetic had a final lycopene concentration of 0.58 mg in 100 g of sample.

Stability tests

Before the stability tests, 5 g of formula were submitted to three centrifuge cycles at 3000 rpm for 30 minutes/cycle at 27±2 ºC (Brasil, 2004; Idson, 1993a,b). Following Brasil (2004) and Isaac *et al.* (2008), the preparations were stored at 5±2 ºC, 45±2 ºC, –5±2 ºC, at room temperature (27±2 ºC), exposed to indirect light, and submitted to macroscopic assessment, pH, density and viscosity tests over 15 consecutive days (preliminary stability test) and over 90 days for the accelerated stability test (assays performed on days 1, 7, 14, 21, 30, 45, 60, 75 and 90). For the freezing and thawing cycles, the samples were submitted to extreme temperature conditions, alternating between 24 hours at a high temperature (45±2 ºC) and 24 hours at a low temperature (–5±2 ºC) over 12 consecutive days. The variation in the results should not exceed 10% and the assays were performed in triplicate (Brasil, 2004).

For the macroscopic assessment, color, fragrance, appearance of the emulsion and separation were assessed. The pH was determined by preparing a 10% aqueous dispersion (p/p) of the sample in recently distilled water by using a digital pH meter. The electrode was inserted directly into the aqueous dispersion (Davis, 1977). A value compatible with skin pH (5.5 to 6.5) was deemed acceptable in the stability tests and variations in the results should not exceed 10%. The assays were performed in

triplicate (Brasil, 2004). The density was determined using a mass empty pycnometer and a pycnometer containing 5 mL of the sample. Density was calculated as the difference between the masses of the full and empty pycnometers, divided by the volume of the sample (Farmacopeia, 2010). The variation in the results should not exceed 10%. The assays were performed in triplicate (Brasil, 2004).

The test for the determination of the viscosity of the emulsions submitted to the stress tests was performed using a rheological flow curve assay, with the following parameters: sheering rate ranging from 0 to 100 s^{-1} for the upward curve for 120 seconds and from 100 to 0 s^{-1} for the downward curve also for 120 seconds. The rheogram was assessed with regard to viscosity (Pa.s) in relation to the sheering rate. The test was performed in triplicate at 25±1 ºC (Cefali, *et al*., 2009a) and the variation in the results should not exceed 10% (Brasil, 2004).

Determination of the amount lycopene incorporated into the emulsion

The amount of lycopene incorporated into the emulsion was determined by dissolving 400 mg aliquots of the phytocosmetic from the five stress conditions to which it was submitted in a solvent system made up of chloroform and isopropanol (1:1, v/v) (Isaac, 1993) and transferring it to 5 mL dilution flasks for a final lycopene concentration of 0.4 µg/mL. The values were analyzed using high-performance liquid chromatography (HPLC) (Waters 1525- Binary HPLC Pump), coupled with an absorbance detector (Waters 2487). The Empower software program was used. Lycopene was identified using a reverse phase column (C_{18}) with the acetonitrile/methanol/ethyl acetate mobile phase at a proportion of 48:26:26 (v/v/v) run for 5 minutes at flow rate of 1.0 mL/min and the wavelength used was 472 nm (adapted from Nunes, Mercadante, 2004). The assays were performed in triplicate.

Determination of spreadability

Determination of the spreadability of the phytocosmetic was performed in triplicate by measuring the change in diameter of the sample in millimeters after one minute of being encompassed within two glass plates with a 200 g glass plate positioned on top. The procedure was repeated by introducing additional 200 g plates at one-minute intervals (Knorst, 1991), reaching a maximum weight of 800 g on the sample.

Determination of rheological behavior

The rheological behavior was assessed using a rheometer HAAKE, sensor cone/plate (C35/2º Ti) and the data were analyzed with the Rheowin 3.5 program. The complete rheological behavior was determined using flow curve assays with sheering rates ranging from 0 to 100 Pa/s for the upward slope for 120 seconds and from 100 to 0 Pa/s for the downward slope for 120 seconds. The sheering tension at which the sample exhibited limited flow (underwent deformation) was determined through the flow limit tension ramp test with a tension range of 0 to 10 Pa for 120 seconds (Isaac *et al*., 2013a). Tension and frequency scanning assays were performed to analyze the dynamic viscosity (η) and stocking module (G) of the sample. For the tension scanning test, a sheering tension range of 0 to 100 Pa and frequency of 1 Hz were used. The frequency scanning test was performed using a frequency range of 0.01 to 100 Hz at a tension of 1 Pa. Thus, the elastic module (G') and viscous module (G") were determined (Chiari, *et al.*, 2009; Isaac *et al.*, 2013b). The fluency and relaxation assay was performed to determine the viscoelasticity of the sample using a sheering tension of 1 Pa for 300 seconds for fluency and 300 seconds for relaxation (Cefali, *et al.,* 2009a; Isaac *et al.*, 2013b). All assays were performed in triplicate, at 25±0.5 ºC using approximately 1 g of sample for each test.

Thermal analysis

The phytocosmetic underwent differential scanning calorimetry (DSC) and thermogravimetry (TG) analysis using the TA-4000 thermal analysis system in triplicate. The mass of the sample was 7.4mg, and an aluminum straw with a perforated cap was used for the DSC analysis. The heating rate was 10 °C/min in a N_2 atmosphere, using a temperature range of 30 to 600±1°C. The TG analysis required a platinum straw, sample mass of 6.31mg and 10 ºC/min heating rate in a N_2 atmosphere, with a temperature range of 30 to 900±1°C (Faria, *et al.,* 2002; Guillen, *et al.,* 2006; Ribeiro, Morais, Eccleston, 2004).

Assessment of in vitro cytotoxic activity

The *in vitro* cytotoxicity test was performed using aliquots of the extract containing lycopene. J774 macrophages and CCL-60 fibroblasts were added to determine the cytotoxicity index or viability. The concentration of the extract ranged from 200 to 1.56 µg/mL. The method consisted of collecting macrophages by scraping and fibroblasts by trypsinization (0.25% trypsin and 0.53 mM EDTA solution). The cells were counted using Turk's stain, adjusted to a concentration of 1 x 10^5 cells/mL of culture medium. The cells in each suspension were incubated on different 96-well microplates at 37±2 °C in a 5% CO_2 atmosphere for 72 h (Ohno, Miyajima, Sunouchi, 1998; Takahashi *et al.,*

2008). Fifteen milligrams of aqueous resazurin solution (0.1 mg/mL) was then added, and the microplates were incubated for 3 h at 37±2 °C in a 5% CO_2 atmosphere. Twenty milligrams of DMSO/medium solution (1:5; v/v) without cells in a sequence of microplate wells were used as the negative control. A solution containing only live cells with no extract was used as the positive control. The results were determinated visually by differentiating between the colors blue (absence of live cells) and pink (presence of live cells) (O'brien *et al.,* 2000) as well as with the aid of a fluorescence reader (Spectra Fluor Plus – Tecan) with 530 and 590 nm filters, using the Magellin program for the analysis. The assays were performed in triplicate.

Microbiological control

One gram of sample was diluted in 9ml of phosphate buffer solution, pH 7.2. One mL of this solution was pipetted, diluted and added to 20 mL of thioglycolate agar for bacteria and Sabouraud for yeasts in Petri dishes, which were subsequently incubated at 35±2 °C for 24 hours and at 25±2 °C for seven days for the examination of bacteria and fungi growth, respectively. For the study of *Salmonella* sp, *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*, the diluted samples were spread into xylose-lysine-desoxycholate agar, bismuth sulfite agar, MacConkey agar, Vogel and Johnson agar and dishes containing cetrimide agar. The dishes were incubated at 35±2 ºC for 24 hours and the characteristics of the colonies were assessed (Farmacopéia, 2010; Pinto, Kaneko, Ohara, 2003; USP 36, 2013). The assays were performed in triplicate.

Skin permeation

For the release test, necessary to determine the release of lycopene from the emulsion (Alencastre *et al.,* 2006; Sasson, 2006), synthetic cellulose membranes (Millipore, 0,45 μm) were placed on the diffusion cells in contact with the receiving medium (7 mL of phosphate buffer, pH 7.2 and containing 2 mM of polysorbate 80). Two-hundred milligrams of the sample was placed on the membranes. For the skin permeation test, pig ear skin was obtained from the Olho D'agua fridge in Ipua city in São Paulo state. The whole ears including hair were cleaned and dermatomized immediately following acquisition. The skins were kept at a temperature of -5±2 °C and used after 24 h. The dermatomized pig ear skin was placed on the diffusion cells with the dermis in contact with the same receiving medium. Two hundred milligrams of the sample was placed on the stratum corneum. These experiments were conducted at 37±2 ºC and the receiving solution was constantly agitated at 300 rotations per minute (rpm). The receiving medium was collected after 2, 4 and 8 hours, and the concentration of lycopene was determined through HPLC, using the same conditions as described previously.

At the end of experiment, the skin samples were removed from the diffusion cells, the excess formula was removed with distilled water and the samples were dried with absorbent paper. The specimens were fixed on a flat surface and the retention area for the removal of the stratum corneum was delimited. The stratum corneum was removed using the tape stripping method, which consists of 12 strippings using adhesive tape (3M®). The first stripping removed the excess formula on the skin and was discarded. The remaining 11 strippings were transferred to test tubes containing 4 mL of methanol and shaken on an agitator for one minute. The tubes were then sonicated for 15 minutes in an ultrasound bath. The supernatant was analyzed using HPLC. The skin samples without the stratum corneum were cut with scissors and transferred to conical tubes containing 4 mL of methanol, and then sonicated for 30 minutes. The final solution was analyzed for the amount of lycopene retained in the epidermis and dermis using HPLC. All the experiments were carried out in quadruplicate using the base emulsion as the blank control.

In vitro *antioxidant activity of lycopene incorporated into the phytocosmetic*

Different concentrations of the sample were placed in test tubes, followed by the addition of 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution (concentration: 0.004%). The assay was performed with the phytocosmetic (0.5 g) dissolved in 5 mL of solvent solution composed of chloroform and isopropanol (1:1) (Isaac, 1998). Absorbance was measured using a methanol and DPPH solution as the negative control and methanol and emulsion without lycopene as empty controls at 531 nm using UV/VIS spectrophotometry. In the presence of lycopene, the absorbance intensity at 531 nm diminished and the percentage of inhibition (%inhibition) was calculated using Equation I (adapted from Cuendet, Hostettmann, Potterat, 1997). The experiment was performed in triplicate.

Equation I: %inhibition = ((A Max – A Test)/Amax). 100

A Max is the absorbance of the free radical in the absence of the sample, and A Test is the absorbance of the radical in the presence of the sample.

Statistical analysis

Statistical analysis was performed using Student's

t-test (P<0.05) for independent variables and the program used was the Microsoft Excel program, version 2000.

RESULTS AND DISCUSSION

Development of phytocosmetic

Since thickening agents provide a high degree of stability to O/W emulsions, the concentrations of shea-based emollients and emulsifiers used were low, resulting in a less-oily emulsion a non-greasy feel after being applied.

The base emulsion obtained had an even, odorless, white, and shiny appearance and was chosen for the subsequent tests. After being submitted to three centrifuge cycles , the base emulsion exhibited stability, as determined by the lack of separation of the phases even 24 hours after preparation, and was therefore chosen for the development of the study.

The process for the preparation of the phytocosmetic was carried out slowly, with care taken to avoid the loss of extract. The final formula had an even, odorless, and shiny appearance and yellowish coloration owing to the presence of the extract.

Stability test

During the preliminary stability tests no alterations in the appearance were detected in the phytocosmetic. Coloration, however, changed when the emulsion was submitted to the oven, light and the freeze/thaw cycle. This change in color may have occurred in the temperature range of –5 ºC to 45 ºC.

The phytocosmetic had a stable pH with a mean value of 5.85±0.21, which is compatible with that of skin. Under all conditions, the daily measurements of pH of the phytocosmetic had a standard deviation value of less than 0.21, which represents a very small variation (less than 0.02) (P<0.05) and demonstrates stability of the formula. The maintenance of density is an important parameter that influences the stability of a formula. The phytocosmetic demonstrated stability with a measured density value of 1.0037±0.14 g/cm^3, and under all conditions, the daily measurements of density had a standard deviation value of less than 0.01% (P<0.05).

Viscosity is one of the physiochemical parameters assessed during the stability test of cosmetic products and involves the study of flow properties of a liquid (Braseq, 2014; Brasil, 2004). In the present study, the viscosity values increased significantly when the phytocosmetic was exposed to a temperature of 45±2 °C. The viscosity ranged from 5394.3±0.22 Pa.s at ambient temperature to 6647.6±0.36 Pa.s (standard deviation equal to 20.44%) (P>0.05) and to 6382.0±0.31 Pa.s (standard deviation equal to 15.47%) (P>0.05) during the freeze/thaw cycle. The increase in temperature led to the evaporation of water, thereby increasing the viscosity. Thus, the phytocosmetic should not be stored at a high temperature. The viscosity values presented were obtained when the phytocosmetic was submitted to the lowest shearing rate (0.005±5 s^{-1}) and the least shearing tension (23±5 Pa) in order to determine the viscosity of the emulsion at near resting conditions. Similar to a material with non-Newtonian behavior, the viscosity of the sample diminished with an increase in applied tension.

The consistency (K_H) and flow behavior (n_H) values were obtained using the Herschel-Bulkley rheological model, which was the most suitable model for styding viscosity curves, with a coefficient of variation of 0.99. The consistency of the emulsion increased when it was submitted to stress conditions and throughout the evaluation period, leading to an increased viscosity, which was pronounced at 45 ºC. The results of the flow behavior revealed values of less than 1, thereby classifying the sample as having non-Newtonian and pseudoplastic behavior, and the stress conditions did not alter this behavior.

The results of the preliminary stability test revealed that the incorporation of the lycopene extract changed the color of the emulsion and its viscosity was affected by exposure to high temperatures. The sample also underwent a change in color when exposed to indirect light. It is therefore important that the phytocosmetic be stored away from light and heat.

During the accelerated stability test, neither the appearance nor the aroma of the phytocosmetic altered. However, in the preliminary test, the product had an intense yellow coloration. The pH of the phytocosmetic remained stable (5.90±0.32), with a standard deviation <0.10 (P<0.05). Based on the results presented thus far, the phytocosmetic exhibited considerable stability, and the presence of the extract did not significantly alter the pH of the formula.

The phytocosmetic exhibited stability with regard to density (1.0035±0.16 g/cm^3) with little variation observed between evaluation days and with standard deviation values of less than 0.10% (P<0.05).

As in the preliminary test, the viscosity of the phytocosmetic during the accelerated stability test increased significantly when exposed to 45±2 °C from 5477.0±0.35 Pa.s to 7396.3±0.22 Pa.s (standard deviation equal to 28.64%) (P>0.05), as the increase in

temperature led to the evaporation of the water, thereby increasing the viscosity. The viscosity values presented during the accelerated stability test were obtained when the phytocosmetic was submitted to the lowest shearing rate (0.0052±5 s^{-1}) and least shearing tension (22±5 Pa) in order to determine the viscosity of the emulsion at near resting conditions. As a material with non-Newtonian behavior, the viscosity diminished with the increase in applied tension. The consistency (K_H) and flow behavior (n_H) values had achieved a coefficient of variation of 0.99, as observed in the preliminary test.

The results of the accelerated stability test revealed that the incorporation of the lycopene extract changed the color of the emulsion and its viscosity was affected by exposure to high temperatures, as observed in the preliminary test. The sample also underwent a change in color when exposed to indirect light. It is therefore important that the phytocosmetic be stored away from light and heat.

Determination of lycopene content in emulsion

The lycopene concentration in the emulsion was 0.59±0.22 µg/mL, but when exposed to indirect light and 45±2 ºC, the lycopene concentration diminished significantly to 0.43±0.14 µg/mL and 0.39±0.18 µg/mL, respectively, with a variation of >10% (P>0.05) after day 90 (Figure 1). Lycopene contents were found to be similar (P<0.05) in the other conditions. Lycopene may undergo oxidation because of exposure to light and heat, and the phytocosmetic should be stored away from light and heat in order to avoid degradation.

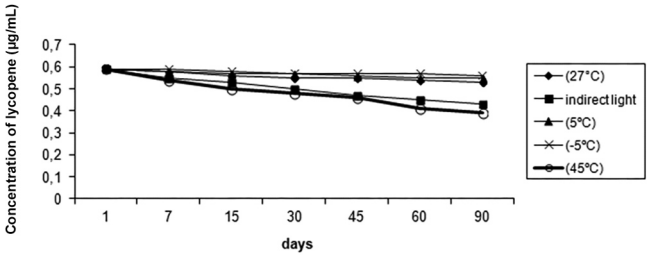

FIGURE 1 - Determination of lycopene content in emulsion submitted to stress conditions on 90 days.

Determination of spreadabilty

Spreadability was assessed by submitting the phytocosmetic to successive increments of weight and distension was determined by measuring the resulting diameter of the sample. The area of the sample was 2521.94±0.33 mm^2 when it was submitted to the first 200 g of weight, and this value increased with increasing weight, achieving a maximum area of 4819.17±0.28 mm^2. The spreadability values (r=0.99935) showed linearity, as the increase in weight or tension applied to the sample was directly proportional to its spreadability. The analytical curve obtained presented in Equation II, with an R^2 value of 0.9987.

Equation II: y = 757.31x + 1788.5

This study was also performed with the base emulsion, which had an area of 2642.01±0.32 mm^2 when submitted to 200 g, with an increase in area accompanying the increase in weight, reaching a maximal area of 4860.27±0.45 mm^2 (Cefali *et al*., 2009a). These values are similar to those obtained with the phytocosmetic, with no significant differences (P<0.05), indicating that the extract did not affect the spreadability of the emulsion.

Determination of rheological behavior

The rheological behavior analysis determines whether a sample exhibits Newtonian or non-Newtonian behavior, whether the fluid is plastic, pseudoplastic, or dilatant, whether it exhibits thixotropy and whether it has viscoelastic behavior.

The flow curve revealed that the phytocosmetic exhibited non-Newtonian characteristics, since the sample underwent deformation when submitted to the shearing tension test and did not exhibit constant viscosity. The emulsion was also characterized as thixotropic, as the sample underwent a reduction in viscosity with shearing and had a hysteresis area of 658.7±0.11 Pa/s (Figure 2). Thixotropy is related to the deformation of a sample when submitted to tension, with greater thixotropy indicating greater deformation of the sample and greater spreadability of the product when applied to the skin. After application, a thixotropic emulsion recovers its viscosity, thereby

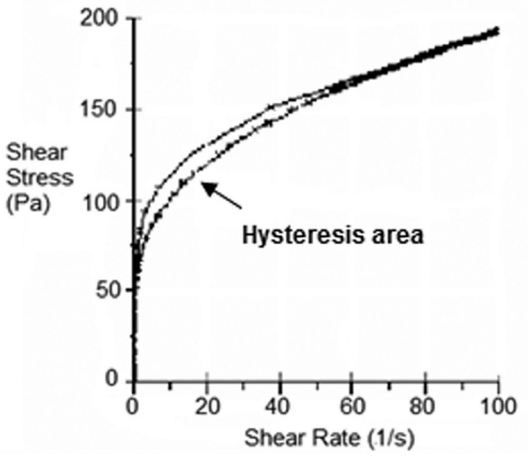

FIGURE 2 - Flow curve of emulsion with lycopene extract.

avoiding dripping. Moreover, a thixotropic product tends to have a longer shelf life, as it exhibits constant viscosity during storage, which hinders the separation of the components of the formula (Martin, 1993).

The flow limit assay is a test that determines the tension necessary for a sample to begin to flow. The phytocosmetic exhibited deformation when the shearing tension was 0.04919±0.52 Pa after 5.91±0.11 seconds from the onset of the test, thereby demonstrating that the product deforms rapidly after being submitted to tension (Figure 3) (Isaac *et al.,* 2013a).

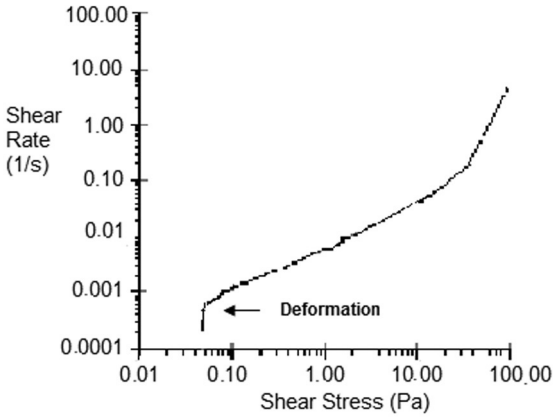

FIGURE 3 - Yield stress curve of emulsion with lycopene extract.

The elastic and viscous behavior of the sample was also assessed to determine whether the sample was more viscous or elastic and to what extent it recovers its elasticity after the shearing tension ceases (Ribeiro, Morais, Eccleston, 2004). The tension scanning test was performed prior to the viscoelasticity tests in order to determine the shearing tension values at which the sample did not undergo deformation within a range of linearity. In the range from 0 to 5 Pa, the sample did not undergo deformation, as the G', G" and viscosity (η) values remained linear in this shearing tension range (Figure 4). This range of values was then used in the frequency scanning and fluency/relaxation tests.

Studies carried out by Chiari *et al.* (2009) and Zambon *et al.* (2009) demonstrated that emulsions with the thickener carbomer (2% dispersion) at a concentration higher of greater than 20% exhibited linearity in G', G" and viscosity with values of about 1Pa, which are similar to those observed for the phytocosmetic analyzed in the present study.

Frequency scanning determines the G' (storage module) and G" (loss module) of a formula. The phytocosmetic had a higher G' than G'' value and viscosity diminished with the increase in shearing tension

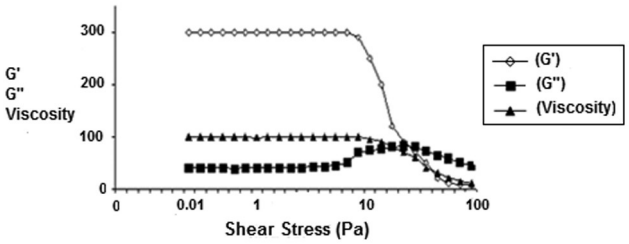

FIGURE 4 - The tension scanning test of emulsion with lycopene extract. Plots of G' (◊), G" (■) and viscosity (▲) with stress.

(Figure 5), which is the typical behavior of viscoelastic samples. When a viscoelastic material exhibits a storage or elastic module greater than the viscous or loss module, the tension energy is temporarily stored during the test and may be recovered later. This generally occurs in O/W emulsion systems. The test also revealed that the frequency range in which the G' and G" cross, shown in Figure 5, which is known as the crossover range, had two points, demonstrating that there was no change in the behavior of the formula during the shearing test.

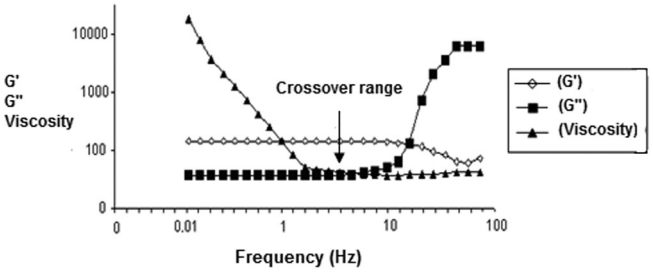

FIGURE 5 - The frequency scanning test of emulsion with lycopene extract. Plots of G' (◊), G" (■) and viscosity (▲) with frequency.

The last test performed was the fluency/relaxation text, which determines the viscoelasticity of a sample. Viscoelastic behavior is characterized by deformation when a sample is submitted to a given tension and the recovery of its elastic structure when the tension ceases, thereby facilitating spreading during topical application. This behavior was observed in the phytocosmetic analyzed since the sample resisted the tension required for flow and recovered its elasticity when the shearing tension ceased (Figure 6).

The complete rheological behavior of the base emulsion was assessed in the same way as that of the phytocosmetic (Cefali, *et al.,* 2009b) and similar results were achieved. Thus, the presence of the lycopene extract did not alter the rheological behavior of the formula.

Thermal analysis

Thermal analysis assays were carried out, as

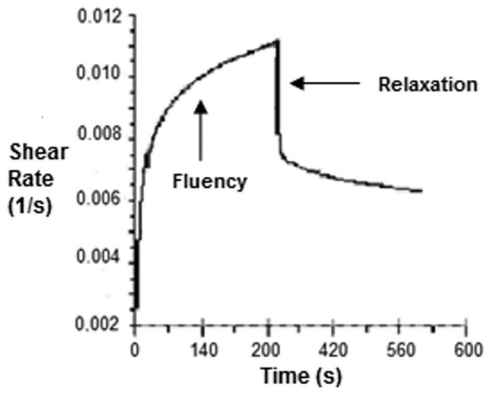

FIGURE 6 - The fluency/relaxation test of emulsion with lycopene.

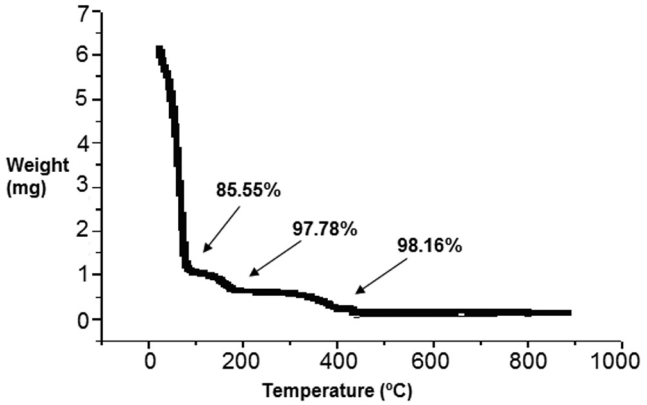

FIGURE 8 - Thermogravimetry (TG) analysis of emulsion with lycopene.

calorimetric methods, allowing for measurements of alterations in the chemical and/or physical properties of a sample as a function of temperature. DSC and TG were performed. The former is a thermal analysis method that records the heat energy flow associated with transitions in materials as a function of temperature and the latter measures the variation in mass during the heating of a sample (Casimiro, *et al.,* 2005).

DSC revealed an endothermic peak at 109±0.25 ºC, which is attributed to the fusion and evaporation of the components of the formula (Figure 7). When submitted to TG, the phytocosmetic underwent thermal decomposition in three steps – the first at 95.5±0.15 ºC, the second at 536±0.12 ºC and the third at 804±0.09 ºC, with sample losses of 85.55%, 97.78% and 98.16% respectively (Figure 8).

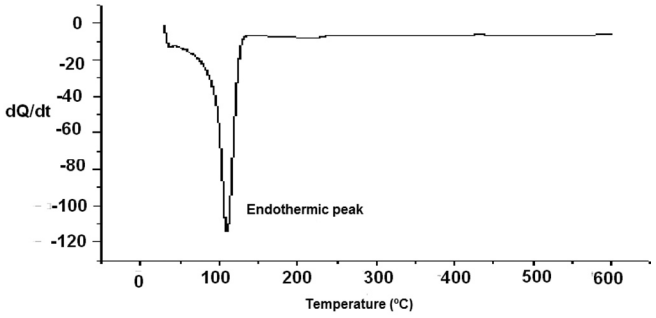

FIGURE 7 - Differential scanning calorimetry (DSC) of emulsion with lycopene.

Thus, the thermal analysis revealed that both the lycopene and the other components of the emulsion were stable up to a temperature of 109 ºC. The preparation of this product and other emulsions should be performed at temperatures below 100 ºC in order to avoid evaporation and the consequent degradation of the compounds.

According to Almeida, *et al.* (2010), the TG/DTG and DSC analyses are important tools used for characterizing the thermal profile of materials, such as vitamins and creams, and can be used for routine analysis in quality control laboratories in cosmetic industries.

Microbiological control

Microbiological control is of extreme importance when developing cosmetics. In the phytocosmetic, there was no growth of specific pathogenic microorganisms, such as *Salmonella* sp*, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus* (Farmacopeia, 2010; Pinto, Kaneko, Ohara, 2003; USP 36, 2013). The total count of these microorganisms was less than 10 CFU of bacteria, fungi and yeasts in each gram of the sample (Table I), thereby demonstrating the absence of pathogenic microorganisms (Farmacopeia, 2010; Usp 36, 2013).

Assessment of in vitro cytotoxic activity

For the determination of cytotoxicity of the lycopene extract, the resazurin stain was used to identify the existence of live cells (pink color) and dead cells (blue color). All wells containing the extract exhibited a pink color, demonstrating the presence of live cells. Thus, the extract did not exhibit cytotoxicity toward the macrophage and fibroblast lineages analyzed, and the highest concentration tested (200 µg/mL) did not achieve 50% on the cytotoxicity index.

Permeation test

Following the release test, the receiving liquid was collected and analyzed by HPLC for the determination and quantification of lycopene released from the formula. The presence of lycopene was not identified at any of the times in which aliquots were collected (2, 4 and 8 h). Some substances may be impeded from being

TABLE I - Microbiological control of phytocosmetic

Microorganisms	Recommendation (Farmacopéia, 2010)	Results*
Aerobic bacteria	10^3 a 5x10^3 CFU/g	Not exceed to 10 CFU/g
Fungi and yeasts	10^3 a 5x10^3 CFU/g	Not exceed to 10 CFU/g
Pseudomonas aeruginosa	Absence	Absence
Salmonella sp	Absence	Absence
Staphylococcus aureus	Absence	Absence
Escherichia coli	Absence	Absence

* Media among tree readings (P<0.05).

released from a formula due to the incompatibility or low solubility of the receiving liquid (buffer, pH 7.2). However, positive results were achieved with regard to the skin retention tests that demonstrate that the lycopene was released, as the buffer solution solubilized the active ingredient when in contact with the pig skin. Synthetic membranes are used in the release test because it is possible to detect only the release of the active ingredient rather than its retention, which would occur using any type of skin, human or animal (Alencastre *et al.,* 2006; Sato, Sugibayashi, Morimoto*,* 1991). Thus, although the release test gave negative results, tests of skin permeation and retention in the stratum corneum and epidermis/dermis were performed.

Pig ear skin is largely used in permeation and retention tests because it simulates human skin (Sato, Sugibayashi, Morimoto, 1991), and Klang, *et.al.* (2012) confirmed that the porcine ear is an excellent *in vitro* model for tape stripping experiments compared to *in vivo* testing on human skin, using different topical formulations.

According to Schueller, Romanowski (2000), emulsion is the most frequently used pharmaceutical topical release system, since it allows quickand convenient transport of several substances. The type of emulsion [oil-in-water (O/W) or water-in-oil (W/O)] should be considered, since it may influence the release, permeation, and retention of active substances in the skin. Alencastre *et al.* (2006) found that the W/O emulsion provided enhanced penetration of vitamin E in both the free and encapsulated forms in pig's skin. Paleco *et al*. (2014) investigated the potential use of microneedles and their combination with lipid microparticles (LMs) loaded with quercetin, as a system to improve flavonoid permeability through the skin, using the *in vitro* release and permeation tests with pig ears and concluded that the proposed combination of microneedles with LMs could be an effective delivery strategy for topical administration of quercetin.

In the present study, aliquots of the receiving solution and supernatant from the tests of retention in the stratum corneum and epidermis/dermis were collected automatically after 2, 4 and 8h and analyzed using HPLC. The lycopene incorporated into the O/W emulsion did not permeate the pig skin, but was retained in the epidermis/dermis after 4 (0.0131±0.32µg/ml) and 8h (0.0373±0.41µg/ml). This result is desirable, since a large number of oxidative reactions caused by UV radiation occur in the viable epidermis and dermis, and it is in this region that lycopene could remove free radicals and reduce the formation of wrinkles.

The present study demonstrates that the frequent application of this phytocosmetic may increase the concentration of lycopene in the skin and provide desired anti-aging effect. The contribution of this *in vitro* permeation study to the assessment of pharmacokinetic properties of the formula and pharmacotechnical development is clear, since it is practical, fast, and inexpensive. However, caution must be exercised when extrapolating the results to *in vivo* situations, since the *in vitro* methodology constitutes a preliminary selection of formulae prior to carrying out clinical tests (Sato, Sugibayashi, Morimoto, 1991).

In vitro *antioxidant activity of lycopene in cosmetic*

In the antioxidant activity test, the phytocosmetic exhibited free radicals removal, as the absorbance was 0.755±0.005 for DPPH in contact with the emulsion alone (without lycopene) and 0.607±0.02 for DPPH in contact with the phytocosmetic, corresponding to 19.51% inhibition.

The phytocosmetic-induced inhibition of free radicals was not significant, but demonstrates the *in vitro* action of lycopene incorporated in the cosmetic formula. With daily use of the phytocosmetic, the lycopene content may increase if it is retained in the skin and may reach a sufficient concentration to inhibit or avoid the action of free radicals. Thus, the phytocosmetic developed is a promising formula to combat of aging of the skin. No data were found in the literature on the *in vitro* antioxidant

activity of substances incorporated into emulsions that could be compared with the results of the present study.

CONCLUSION

The present study demonstrates that the phytocosmetic is stable, exhibits satisfactory rheological behavior for a topical formula, and that the lycopene extract is no citotoxic. The phytocosmetic has antioxidant activity and allows for the retention of the active ingredient in the skin. Thus, the phytocosmetic is a promising product for combating the aging of the skin.

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