

Organic cocoa extract -loaded surfactant-based systems intended to skin bioadhesion

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This study was to develop, characterize, and evaluate the physical-chemical stability, *in vitro* antioxidant activity and *in vitro* safety profile of liquid crystalline systems (LCS) and microemulsions (MEs) with and without organic cocoa (OC) extract. LCS stabilized by surfactant polyoxyethylene 20 cetyl ether, containing water and oleic acid were studied. LCS and MEs were characterized using polarized light microscopy, small angle X-ray scattering, rheology and *in vitro* bioadhesion, and were evaluated for a period of 30 days by visual aspects, centrifuge test, pH value and relative density. PLM and SAXS assays showed the presence of domains of MEs, cubic and hexagonal mesophases, varying the proportions of the components of the formulations; where in the addition of the extract did not change rheological behavior of the formulations. All of the formulations were stable in the period analyzed and presented higher bioadhesive strength. *In vitro* antioxidant activity suggests that LCS and MEs presented a high capacity to maintain the antioxidant activity of OC extract. The results showed that the incorporation of OC in LCS improved the safety profile, according to cytotoxicity assays of systems may be a promising platform to OC extract for topical application for the potential treatment of skin disorders.

Keywords: Liquid crystals/evaluation/physical-chemical stability. Microemulsions/evaluation. Surfactant-based system. Organic cocoa extract. Skin bioadhesion.

INTRODUCTION

Skin is routinely exposed to stressful environmental factors such as pollutants and UV radiation. These factors produce a large number of aggressive oxidants that damage all of the biological skin cell membranes (Silva *et al.*, 2010; Binic *et al.*, 2013; Datta *et al.*, 2011; Dupont, Gomez, Bilodeau, 2013). Accordingly, the use of skin care products and cosmetics has been utilized to mitigate the cutaneous photoaging process (Visscher, Pan, Kitzmiller, 2013) and these products are commonly composed of plant extracts (Binic *et al.*, 2013).

A great number of plants and plant extracts are studied for their antioxidative action arising from their phenolic substructures. Phenolic compounds are composed of an aromatic ring that is linked to a hydroxyl group, which donates electrons and hydrogen atoms. As a result, these compounds are exceptional antioxidants (Silva *et al.*, 2010; Binic *et al.*, 2013; Reuter, Merfort, Schempp, 2010).

Cocoa, the dried and fermented seeds derived from *Theobroma cacao*, has been consumed since 1100 B.C. by ancient civilizations such as the Mayans and Aztecs (Hurst *et al.*, 2002). Cocoa contains high amounts of the flavonoids (–)-epicatechin (EC) and (+)-catechin, and procyanidins B2 and B1. Additionally, other polyphenols such as quercetin, isoquercitrin (quercetin 3-O-glucoside), quercetin 3-O-arabinose, hyperoside (quercetin 3-O-galactoside), naringenin, luteolin, and apigenin have also been found in cocoa in minor quantities (Sánchez-Rabáneda *et al.*, 2003). While the natural amount

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of polyphenols may be high in cocoa, the manufacturing process often decreases the amount of polyphenols in the final product (as in chocolate). Accordingly, much research has focused on the health effects of polyphenol contained in cocoa extracts (Andújar *et al.*, 2011; Tomas-Barberán *et al.*, 2007; Calatayud *et al.*, 2013; Mellor *et al.*, 2010; Monagas *et al.*, 2009).

Cocoa polyphenols have a positive effect on skin structure when applied for at least 5 days. This study observed that cocoa polyphenols, at doses of 0.75% and 0.50%, improve skin tone and elasticity through the glycosaminoglycans and collagens I, III, and IV (Gasser *et al.*, 2008). In addition to demonstrating antioxidant activity higher than that of black tea and of red wine, the polyphenols also demonstrated *in vitro* inhibition of lipid peroxidation (Schinella *et al.*, 2010; Hatano *et al.*, 2002). Therefore, it can be used in topical formulations in order to prevent premature skin aging (Katz, Doughty, Ali, 2011; Williams, Tamburic, Lally, 2009) and the use of cocoa to inhibit skin aging processes is encouraging.

Organic cocoa can further enhance antioxidant activity when associated with surfactant-based systems such as liquid crystalline systems (LCS) and microemulsions (MEs). Liquid crystalline systems (LCS) are mesomorphic phases of matter in an intermediate state in the thermal transformation from a solid to a liquid. LCS have the characteristic properties of solids and liquids: they have the structural order, rigidity, and defined links as in solids, and they also have the mobility, fluidity, and disordered regions as in liquids (Chorilli *et al.*, 2011; Prestes *et al.*, 2009; Mezzenga, 2012). MEs are defined as dispersion made of water, oil, and surfactants that is an isotropic and thermodynamically stable system with dispersed in globular or interconnected size diameter varying approximately from 1 to 100 nm, usually 10 to 50 nm (Slomkowski *et al.*, 2011; McClements, 2012). LCS and MEs can increase the physical stability, solubilization, and efficacy of a series of active principles and they can be explored to drug or active compounds delivery (Chorilli *et al.*, 2011; Chen, Ma, Gui, 2014; Guo *et al.*, 2010; Lawrence, Rees, 2000). They can be formed by the association of varying concentrations of water, oil, and surfactant and changing the concentrations of surfactant can self-assemble at forms different such as microemulsions or lamellar, hexagonal, and cubic mesophase (Malmsten, 2002; Romsted, 2012).

As such, the objectives of this study were to develop, characterize, and evaluate the physical-chemical stability, *in vitro* antioxidant activity and *in vitro* safety profile of liquid crystalline systems (LCS) with and without organic cocoa (OC) extracts.

MATERIAL AND METHODS

Material

Oleic acid, OA (Synth, Brazil); ethanol (Synth, Brazil); hexane (Synth, Brazil); Folin-Ciocalteu reagent (Sigma Aldrich, USA); polyoxyethylene 20 cetyl ether, PCE (Sigma, USA); 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical (Buchs, Switzerland); and organic cocoa seed powder - OCSP (Via Farma, Brazil) were all obtained from their specified vendors and used without further purification unless otherwise stated.

Methods

Botanical tissue characterization

A sample of OCSP was previously intumesced and discolored and it was then colored with Toluidin Blue (BT). The prepared sample was analysed with an optical microscopic, searching for lipid globules, pectic compounds, phenolics cells, starch grains and mucilage as described in the literature (Martini *et al.*, 2008a; Martini *et al.*, 2008b).

OCSP extract preparation

OCSP (1.0 g) was previously defatted with 5 mL of hexane by 20 minutes into a sonication bath. The extraction was prepared separately with hydroalcoholic solutions composed of 80 % methanol (v/v) and 70 % ethanol (v/v). Then, 10 mL of each solvent was added to the sample, and the sample was left for 20 minutes into a sonication bath. This extraction procedure was performed three times resulting a final volume of 30 mL, and it was performed in triplicate. The extracts were dried under air and in a desiccator with silica gel.

- **Determination of the phenolic content of the extracts**

The phenolic content of the extracts was determined according to the method described (Singleton, Rossi, 1965). Using the Folin-Ciocalteu reagent, the polyphenols were expressed as gallic acid and quercetin. Aliquots containing 20 µg of dried sample extracts and the standard (0.25 µg/mL, 0.50 µg/mL, 0.75 µg/mL, 1.00 µg/mL e 1.25 µg/mL) were added to 100 µL of Folin-Ciocalteu reagent with 1.58 mL of water. After 8 min, 300 µL of 20% (w/v) sodium carbonate solution were added and the mixtures were stirred. After 2 hours at room temperature, the absorbance was measured in a UV/Vis spectrophotometer (U-2001 Hitachi, Japan) at a wavelength of 765 nm. The analytical curve was constructed with the absorbance values obtained for the different concentrations of gallic acid and quercetin.

- Determination of antioxidant potential of SPOC extracts

The free radical scavenging activity was evaluated by a modified DPPH test. One hundred microliters of extract solutions (containing 10 – 60 µg/mL of dry mass) were added to 3.9 mL of DPPH (60 µM) in ethanol. After 30 minutes in the dark, the absorbance at 517 nm was measured. All measurements were taken in triplicate. The free radical scavenging activity was calculated using equation 1, where A_c is absorbance of the control and A_s is absorbance of the sample. The IC_{50} value was determined by plotting the concentration of cocoa extracts *versus* the percentage of remaining DPPH at a steady state.

$$\text{Inhibition DPPH (\%)} = \left[\frac{(A_c - A_s)}{A_c \times 100} \right]$$

Preparation of the formulations

The samples were produced by heating the mixture of the oleic acid and PCE to 45 °C. Distilled water at 40 °C (W) was then added under constant stirring, and the mixture was allowed to cool to room temperature. Binary mixtures were obtained similarly, but each mixture contained a different proportion of OA and PCE. Systems obtained with different proportions of the OA and PCE were described by pseudo-ternary phase diagrams to characterize the LCS studied. During the titration process, the formed systems and their viscosities were analyzed visually. The concentrations of each component in the formulation were calculated, and these points were used in the delimitation of different regions in the phase diagram. The transitions from the semisolid phase to the transparent liquid system (microemulsion), transparent viscous system (hexagonal, lamellar and cubic phases), cloudy system (emulsion) and separation of phases (S) were delimited. The OC extract was added to the aqueous phase of the system to prepare loaded-formulations.

Characterization of the formulations

- Polarized light microscopy (PLM)
A drop of each formulation was placed on a glass slide covered with a cover slip, and then the sample was examined under polarized light. A Motic Type 102 M Optical Microscope equipped with a digital camera was used to analyze several fields of each sample at room temperature. Photomicrographs were taken at a magnification of 200x.
- Small angle X-ray scattering (SAXS)
The nanometric structure of the phases was

investigated using SAXS. The results were collected at the Synchrotron SAXS beam line of the Brazilian Synchrotron Light Laboratory (Campinas, Brazil), equipped with an asymmetrically cut and bent Si (1 1 1) monochromator ($\lambda = 1.608 \text{ \AA}$) that yields a horizontally focused beam. A vertical position-sensitive X-ray detector and a multichannel analyzer were used to record the SAXS intensity, $I(q)$, as a function of the modulus of the scattering vector q ($q = (4\pi/\lambda)\sin(\varepsilon/2)$, and ε is the scattering angle). The parasitic scattering produced by the slits was subtracted from the total scattering intensity.

- Determination of rheological behavior

The rheological analysis of the formulations were performed with a controlled-stress Carri Med CSL 100 rheometer (TA instruments, UK) with plate-plate geometry using assays of flow-dynamics. This geometry consists of two stainless steel plates that are 2 cm in diameter with a gap of 200 µm between the plates. Samples were carefully applied to the lower plate, ensuring that formulation shearing was minimized, and the samples were allowed to equilibrate for at least 3 min prior to analysis. The experiments were performed with shear rates ($\dot{\gamma}$) in the range of 0.001 s⁻¹ to 100 s⁻¹. The shear rate region used was selected based on the strength of resistance to the applied stresses. The rheological measurements were performed on both the up and down curves. The data from the shear cycle were fitted to a power-law model using Rheology Solutions Software (version Data V1.1.7, TA Instruments).

- *In vitro* bioadhesion assays

In this study, pig ear skin was obtained from a local slaughterhouse. The cutaneous bioadhesive strength was evaluated in terms of the force required to separate the formulations of pig ear skin. The maximum force of detachment was analyzed using a Stable Micro Systems TA-XTplus texturometer (Surrey, England). The formulations were warmed in a thermostatic bath at 32 °C for the bioadhesive analyses. These assays were performed under the following conditions: a strength (trigger force) of 0.002 N for viscous samples, a strength of 0.01 N semi-solid samples, and a strength of 0N for liquid samples. All assays used a contact time of 60 s.

- Preliminary stability studies

The formulations were evaluated for a period of 24 hours by visual inspection, centrifuge testing, pH values and relative densities and 1 month to visual aspect of formulations. For visual evaluation, the samples were visually observed for changes such as color, phase

separation, and homogeneity. The evaluation of the stability to centrifugation was performed using 5 grams of each test sample. The samples were centrifuged at 3000 rpm for 30 min. The pH measurement was performed using a pH meter, using 5% (p/v) samples diluted in distilled water. The relative density (RD) was determined using a pycnometer. The relative density was calculated by the methodology described by Brazilian Pharmacopea (Anvisa, 2010). All formulations were kept at room temperature ($25^{\circ}\text{C} \pm 2^{\circ}\text{C}$) and without exposure to light.

- Determination of the antioxidant potential of the LCS

To determine the antioxidant activity of the formulations, one gram of the extract formulations was solubilized in 1 mL of 70% ethanol. One hundred microliters of each solution was used in the DPPH assay. A formulation without extract was used as the blank solution.

Cell culture

In vitro testing for the cytotoxicity analysis of the formulations was performed using J-774 mouse macrophages as a template. Cells were seeded in the bottom of the microplates (Nucleon, 96 wells, $2.5\text{--}10.0 \times 10^5$ cells/well) with different doses of the formulations and free OC extract (18.6, 10, 5 and 1 μM) or control for 48 hours. The cells were washed with PBS after removing the compounds, and the cell viability was assessed by a colorimetry formazan assay (MTT). The method of 3 [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) is a simple, reliable, and reproducible colorimetric method for measuring mitochondrial metabolic reduction of yellow tetrazolium salt to insoluble formazan crystals in an aqueous solution of viable cells. Cells and MTT (0.4 mg/mL) were incubated at 37°C for 3 h. Subsequently, the supernatant

was removed and formazan crystals were dissolved in DMSO (180 μL). The plates were agitated for 10 min, and the optical density was measured using a multiwell spectrophotometer operating at 560 nm. Concentrations were measured in triplicate using six additional controls (cells in medium). Cell viability was calculated using the following equation 2:

$$\text{Cell viability (\%)} = \frac{OD_s}{OD_c} \times 100$$

where OD_s and OD_c are the samples and control optical density, respectively.

Statistical analysis

The data were analysed using the mean and standard deviation and were compared by analysis of variance (ANOVA). The Tukey post-hoc test was used to obtain a unidirectional comparison and to evaluate significant differences between samples, where values of $p < 0.05$ were considered to be statistically significant. Origin[®] 7.0 SRO (OriginLab, Northampton, MA) was used for data analysis.

RESULTS AND DISCUSSION

Botanical tissue characterization

Because the seeds were composed of a fine powder, certain structural features present in OC seeds described by the literature could not be observed (Elwers *et al.*, 2010). Phenolic compounds were observed in the OSCP sample, taking the forms of grains of starch and mucilage (Figure 1). This finding suggests that the sample is composed of OC and corroborate with supplier report analysis.

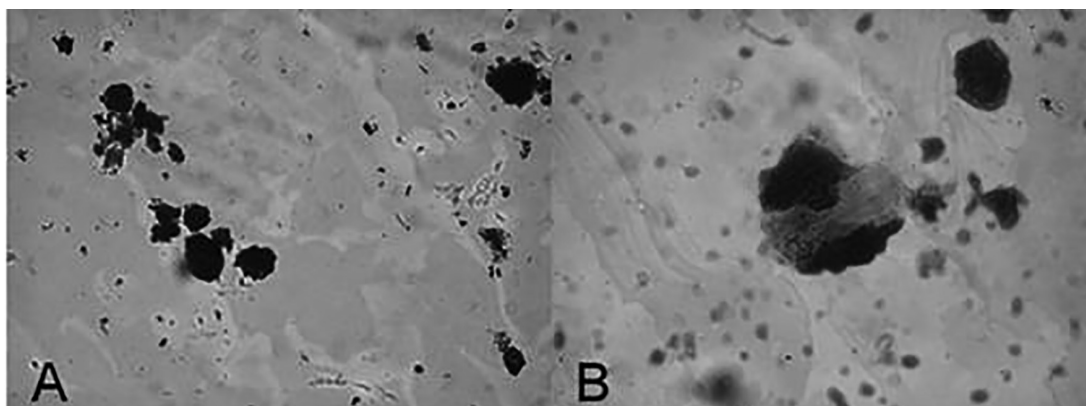


FIGURE 1 - A sample of powder of divided seeds viewed using an optical microscope at 400x. Phenolic compounds (A) and mucilages (B) are present.

Extract characterization

Determination of total phenolic

Least-squares linear regression of the analytical data obtained for gallic acid ($y = 61.2x + 0,0457$; $R^2 = 0.9982$) and quercetin ($y = 118.0x + 0.0825$; $R^2 = 0.9980$) was used to determine the total phenolic content. The total gallic acid content obtained from the 80% methanol and 70% ethanol extractions was 1.40 mg/g and 1.96 mg/g, respectively. The total quercetin content obtained from the 80% methanol and 70% ethanol extractions was 480 $\mu\text{g/g}$ and 870 $\mu\text{g/g}$, respectively. According to these results, the ethanol extractions yielded greater amounts of polyphenols were expressed as gallic acid and quercetin. This outcome is important, as the toxicity of methanol restricts methanol-based botanical extractions in pharmaceutical processes.

Determination of antioxidant potential of OC extract

The IC_{50} values for the 80 % methanol and 70 % ethanol extractions were 43.5 and 29.2 $\mu\text{g/mL}$, respectively. Therefore, the ethanol extract exhibits a higher antioxidant activity than does the methanol extract. These results are in agreement with that obtained in the determination of total phenolics content, in which higher concentrations of phenolics result in a higher antioxidant activity. Then, the 70% ethanol extraction was used to prepare the subsequent formulations.

Preparation of the formulations

Figure 2 presents the ternary phase diagram of the system with different proportions and type of components resulted in distinct systems. The phase diagram shows the characteristics of points classified as phase-separated (SF), microemulsions (ME), as a cloudy system (EM), or as a liquid crystalline system (LCS). The regions known as EM, like emulsions, were observed in the upper vertex of diagram, where there was low water concentration (0-25%) and high surfactant concentration (100-70%); in the bottom vortex of diagram, there are found system like EM, where the water concentrations was higher (70-100%) and oily phase was used in different range of concentrations (10-80%). LCS was found in the left region and center of the diagram with a oil concentration (0-50%) and surfactant concentration (30-70%). PS occurred with a low water concentration (10-40%) and surfactant concentration (~20%) and water concentration (~10%) and surfactant concentration (50-70%).

In this diagram is possible to evaluate the regions of ME can generate liquid crystalline systems where

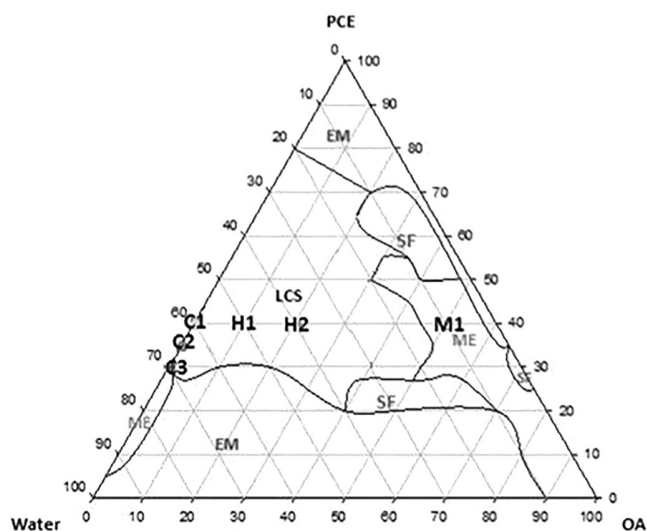


FIGURE 2 - Phase diagram of W, OA, and PCE indicating areas where the formulation will exist as fully phase-separated (SF), as a microemulsion (ME), as a cloudy system (EM), or as a liquid crystalline system (LCS).

the water in the system is increased. This phenomena, known as transition mesophase, is also possible to be noted within regions of LCS, where the increase of water in the system, led a formation of more complex structures (hexagonal to cubic mesophases). This change from a less viscous phase into a hexagonal phase or cubic structure can be explained by the critical packing parameter (cpp) (Mezzenga, 2012; Malmsten, 2002). The cpp relates the shape of the molecule to properties that influence the curvature of the polar-nonpolar interface and consequently the type of aggregate formed. Therefore, the cpp is a useful parameter for predicting the mesophase that is preferably formed by an amphiphile and can be calculated via Equation 3 (Israelachvili, 1994).

$$cpp = \frac{v_s}{a_0 \times l_c}$$

where cpp represents the critical packing parameter, v_s is the hydrophobic chain volume, a_0 is the polar head group area, and l_c is the chain length. The increase in the water system leads to an increase in hydration of the polar head of the surfactant, increasing its curvature and consequently increase the value of v_s . Lamellar mesophase the angle of curvature is zero and increased surfactant curvature angle and it tends to generate self-assembly systems, such as hexagonal and cubic liquid crystals (Garti, Libster, Aserin, 2014).

Characterization of the formulations

PLM

The formulations were analyzed using MLP, in which structures such were identified as lamellar mesophase and hexagonal arrays (Figure 3). The system cubic and microemulsions are showed as dark field in PLM, because these systems are anisotropic. MEs are transparent and fluid systems whereas cubic phases are transparent and a rigid system, like a gel (Shah, Sadhale, Chilukuri, 2001).

Six points from the phase diagram were selected to incorporate the OC. Namely, these points consisted of three cubic phases (C1, C2, C3), two hexagonal phases (H1, H2) and one microemulsion (M1). Table I shows the percentages of formulation components for each selected point.

TABLE I - Percentages of components of the formulations

Formulation	Oleic Acid (%)	PCE (%)	Water (%)
C1	0.0	40.0	60.0
C2	0.0	35.0	65.0
C3	0.0	30.0	70.0
H1	10.0	40.0	50.0
H2	20.0	40.0	40.0
M1	50.0	40.0	10.0

The concentration of OC to be incorporated in the formulation was determined according the IC_{50} of the extract analyzed. Ultimately, 0.6% of the OC extract was incorporated in the aqueous phase of the system. The formulations containing the extract are named as C1E, C2E, C3E, H1E, H2E and M1E.

Small angle X-ray scattering (SAXS)

The values of the scattering vector q , shown in

Table II, correspond to peaks q_1 , q_2 , q_3 , q_4 , which are equal to 1:1,73:2:2,64. This result indicates that the sample has a liquid crystal hexagonal structure. The liquid crystal cubic phase correlated values are 1,41:1,73:2,82:3 (Beaucage *et al.*, 1995). The cubic phase is an isotropic, thermodynamically stable, and highly viscous formulation with a bicontinuous structure (Hyde *et al.*, 1984). Because of its amphiphilic character, the cubic phase is suitable for accommodating both hydrophilic, hydrophobic, and amphiphilic substances. In systems of microemulsions, SAXS curves exhibit a broad band or peak, associated with low 3D spatial correlation (Yariv *et al.*, 2010).

The results confirm the information of the analysis of PLM. It was also observed that the incorporation of extract in the formulations did not alter the type of structure observed.

Determination of rheological behavior

Due to the high viscosity of the formulations C1, C1E, C2, C2E, C3 and C3E, it was not possible to analyze the rheological behavior of these formulations. Figure 4 shows the variation in shear stress as a function of shear rate for formulations H1 and H2. The curves indicate that the formulations behave as non-Newtonian systems because the curves exhibit a linear relationship between tension and shear rates.

The formulation H1 exhibited pseudoplastic and thixotropic fluid characteristics because of the decrease in viscosity. Such behavior can be attributed to the disorganization of the internal system with the applied shear. These results reveal the presence of a structured network formed by the interaction between the components of the formulation. This network is gradually broken with increasing shear rate, resulting in a reduction of the internal resistance of the system, and ultimately decreasing the viscosity. This rheological parameter indicates that sample H1 can show good spreadability

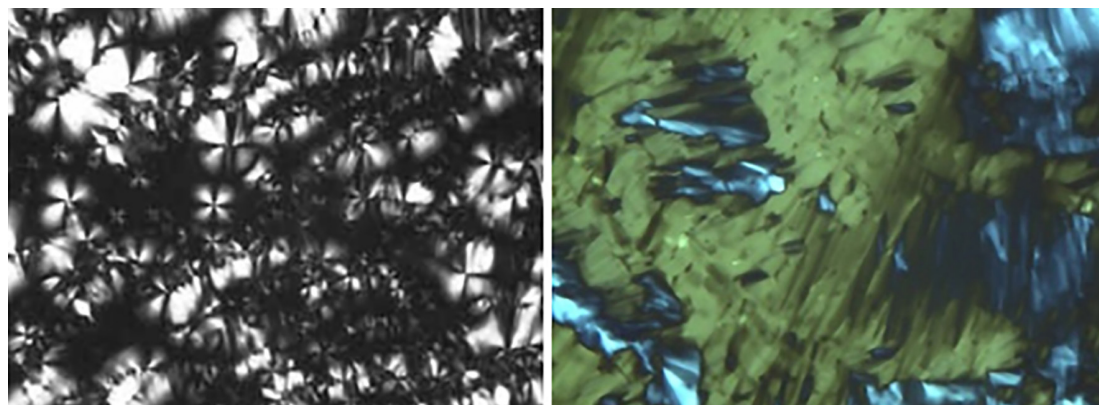


FIGURE 3 - Polarized light micrography of LCS: lamellar phase (left) and hexagonal phase (right). Magnification of 400 x.

TABLE II - Ratio of the interplanar distances and lattice parameter d (nm) for the formulations obtained by SAXS

Formulation	$q_{\max 1}$	$q_{\max 2}$	$q_{\max 3}$	d_1/d_2	d_1/d_3	d (nm)	Structure
C1	0.91	1.29	1.58	1.41	1.73	6.90	Cubic
C1E	0.90	1.27	1.56	1.41	1.73	6.97	Cubic
C2	1.03	1.47	1.79	1.42	1.73	6.09	Cubic
C2E	0.89	1.26	1.54	1.41	1.73	7.05	Cubic
C3	0.90	1.28	1.57	1.40	1.72	6.97	Cubic
C3E	0.89	1.27	1.56	1.39	1.72	7.05	Cubic
H1	0.97	1.68	1.94	1.73	2.00	6.47	Hexagonal
H1E	0.96	1.67	1.94	1.73	2.02	6.54	Hexagonal
H2	0.95	1.65	1.91	1.73	2.01	6.61	Hexagonal
H2E	1.02	1.77	2.04	1.73	2.00	6.15	Hexagonal
M1	0.85	-	-	-	-	7.38	Microemulsion
M1E	0.86	-	-	-	-	7.33	Microemulsion

during application in the skin (Alam, Aramaki, 2009; Ribeiro, Morais, Eccleston, 2004; Zhang, Liu, 2013; Isaac *et al.*, 2012). It can be correlated with the spreadability and with how easy formulation flow from package (Niraula *et al.*, 2004).

For the H2, the opposite phenomenon was observed. This formulation exhibited characteristics of dilatant and reopetics fluids because of the increase in viscosity with increasing shear stress. This behaviour can be explained by filling the space between the particles with increasing shear stress: the sorting of particles promotes an increase in volume, causing an increase in melt strength, i.e., the viscosity (Carvalho *et al.*, 2010a).

After the extracts were added to formulations H1E and H2E, no alteration was observed in the rheological behaviour of formulations. Thus, the addition of the extract did not influence the rheological behaviour of the formulations.

In vitro bioadhesion assays

Bioadhesion is used to describe the adhesion of synthetic or biological macromolecules to biological tissues. Table III shows the results from the bioadhesion tests. The force obtained by the graph represents the maximum force observed for the detachment of the skin sample of the formulation, while the area is the work of bioadhesion. From these results, it can be determined that between all formulations, the formulation of the cubic phase had greater bioadhesive strength than that of the hexagonal phase, which in turn had greater strength than did the microemulsion. The addition of the

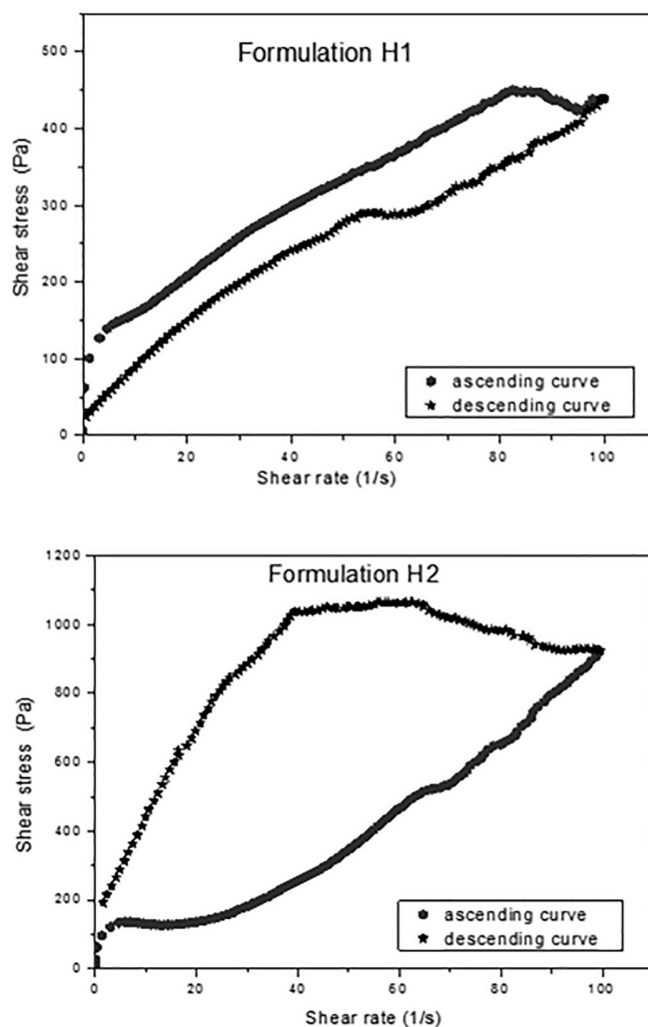
**FIGURE 4** - Flow curve of formulations H1 and H2.

TABLE III - Bioadhesion parameters (strength and area), pH and density values of the formulations.

Formulation	Strength (N)	Area (N.sec)	pH	Density (g/cm ³)
C1	0.567 ± 0.043	0.674±0.062	4.97	1.020
C1E	0.562 ± 0.041	0.669±0.052	4.35	1.090
C2	0.378± 0.022	0.966± 0.212	4.79	1.024
C2E	0.375± 0.015	0.959± 0.201	4.35	1.039
C3	0.346 ± 0.034	0.699± 0.139	4,60	1.015
C3E	0.347 ± 0.029	0.701± 0.187	4.40	1.023
H1	0.076±0.014	0.158±0.043	4.16	0.985
H1E	0.071±0.011	0.155±0.022	4.16	0.103
H2	0.035±0.001	0.175±0.012	4.23	0.981
H2E	0.032±0.017	0.169±0.015	4.15	0.995
M1	0.005±0.012	0.0143±0.001	3.95	0.973
M1E	0.004±0.121	0.0139±0.003	4.01	0.985

extract did not significantly alter bioadhesiveness of the formulations.

Nielsen, Schubert and Hansen (1998) has studied bioadhesion by tensiometric measurements, and the results showed that LCS phases have the greatest mucoadhesion, followed by lamellar phases and cubic phases. The mechanism of bioadhesion is unspecific and most likely involves dehydration of the mucosa (Nielsen, Schubert, Hansen, 1998).

Gonçalez, Correa and Chorilli (2013) developed an LCS and studied the bioadhesion of hexagonal phases on porcine skin, and the results showed that bioadhesion depended on the water phase in the systems. Carvalho *et al.* (2013) developed an LC precursors system, and the mucoadhesion was evaluated. The results suggest that the adhesive force is not influenced by the mesophase type (Carvalho *et al.*, 2010b). However, the adhesive forces presented by lamellar phases (0.304–0.262 N) are similar to well-known mucoadhesive polymers to skin (Carvalho *et al.*, 2012) such as carbomers and polycabophyls, which exhibit great bio/mucoadhesive properties (Carvalho *et al.*, 2012; Bruschi *et al.*, 2007; Peppas, Sahlin, 1996).

Studies explained that the force of bioadhesion is correlated with a rank order of resistance measurements, such as the index of viscosity (*k*) of semisolids matrixes (Carvalho *et al.*, 2012; Carvalho *et al.*, 2013). Another study studied by Tamburic and Craig found a correlation between rheological and textural results, that is, an increase in the interaction between a highly viscous sample and mucus (Tamburic, Craig, 1997).

Various theories exist to explain at least some of the experimental observations made during the bioadhesion process. LCS are bioadhesive systems, because they leads

a dehydration of skin. These findings show that the water present in the mucus layer can interact with liquid crystal system to adhesion process (Souza *et al.*, 2014; Ganem-Quintanar, Quintanar-Guerrero, Buri, 2000). Mesophase formed in LCS can influence this process, where lamellar systems exhibit mucoadhesive properties higher than hexagonal mesophase (Lee, Young, Kellaway, 2001).

Preliminary stability studies

No phase separation or color changes were observed over a period of one month. The formulations were evaluated 24 hours after their preparation. Stability evaluation to centrifugation test found that the formulations kept the same visual appearance and phase separation was not observed. The determination of pH was performed 24 h after preparation and the results are shown in Table III. The pH values were 3.95 to 4.97 to the formulations and the addition of cocoa extract led to little change in the pH of the formulations. The skin surface pH is on average 4.7 (Lambers *et al.*, 2006) and the use of these formulations under the skin should not influence or change the pH of the skin. The maintenance of the correct pH of the skin plays a role in controlling the presence of resident skin microflora as well as supporting important physiological processes like the formation of an optimal structure of the lipid barrier and stratum corneum homeostasis. (Parra, Paye, 2003)

All formulations had densities near to density of water ($d = 1.0 \text{ g/cm}^3$). The cubic phase formulations had a higher density than did the other formulations, and the microemulsion had the lowest density. The incorporation of OC extracts lead an increase of density the formulations.

In vitro antioxidant activity of the formulations.

The formulations with or without the addition of OC extract were evaluated for antioxidant activity during a period of 21 days. The results obtained are shown in Figure 5.

It can be seen that in the period of 21 days, there was a decrease in antioxidant power. The samples C1, C2, C3, H1, H2 and M1 with cocoa were not significantly different in relation to the extract ($p > 0.05$).

Regarding the formulations, the initial antioxidant activity was lower when compared to the extract, but by day 7, inhibition of DPPH of the formulations C2 and C3 with the extract was observed. This result is most likely due to a greater stabilization of the formulation. The same outcome was not observed for C1, and the difference between these formulations is the concentration of the surfactant. C1 has a higher concentration of surfactant than does C2 and C3. In the cubic phase formulations, it can be observed that the formulation prevented the extract from being oxidized, maintaining the antioxidant activity similar to the extract, and in some cases the antioxidant activity was slightly higher when compared to that of the extract.

Numerous studies have demonstrated the high capacity of several plant polyphenolic antioxidants to

protect living organisms from alterations produced by reactive oxygen species (ROS), including skin damage caused by exposure to ultraviolet (UV) radiation (Reuter, Merfort, Schempp, 2010; González *et al.*, 2013; Adhami *et al.*, 2008; Saija *et al.*, 1998). ROS load has been implicated in several pathological states including photoaging of the skin, leading to abnormal matrix degradation and accumulation of non-functional matrix components (Pillai, Oresajo, Hayward, 2005).

For formulations H1, H2 and M1, inhibition by DPPH was not higher than that observed in the extract. These formulations exhibit an oily phase, unlike C1, C2 and C3, which have only components of the aqueous phase and surfactant. The presence of an oil phase, which was composed of oleic acid, may have interfered with antioxidant activity because unsaturated oils tend to oxidize. In this case, the OC extract added may have oxidized to protect the formulation from oxidation.

In vitro safety profile

An *in vitro* cytotoxicity study of the formulations using J-774 mouse macrophages as the cellular model was performed. The data are expressed in percentage of cellular viability, according to Figure 6.

The assay results of cellular viability indicated that the OCE free and the formulations were unable to kill normal cell macrophages: all of them exhibited cellular viability greater than 94%. The use of higher concentration of fatty acids, such as OA, induce apoptosis and necrosis using J-774 mouse macrophages (Martins de Lima *et al.*, 2006). None of the formulations without the drug showed toxic activity (C1, C2, C3, H1, H2 and M1), nor did they show toxic activity when the extract was incorporated in the formulations (C1E, C2E, C3E, H1E, H2E and M1E). No have differences between mean of groups ($p < 0.05$).

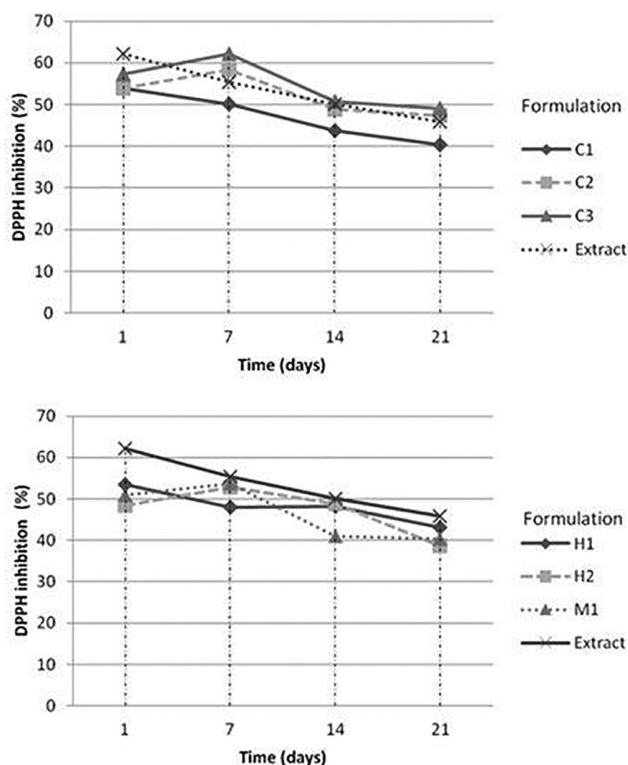


FIGURE 5 - Percentage inhibition of DPPH radical of formulations for a period of 21 days to the formulations.

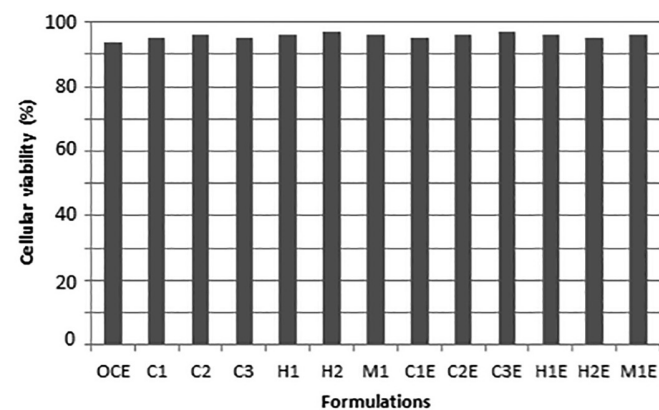


FIGURE 6 - Cellular viability (%) to OCE free, OCE-unloaded and -loaded formulations.

The same study was performed by others authors using J-774 mouse macrophages as the cellular model. The results of cellular viability for formulations of liquid crystals and nanostructured lipid carriers were greater than 92% (Gonçalez, Correa, Chorilli, 2013; Oliveira *et al.*, 2014; Kolenyak-Santos *et al.*, 2015; Oyafuso *et al.*, 2015).

CONCLUSION

The results of bioadhesion studies showed a bioadhesive potential of cubic phase than hexagonal mesophases. For drug delivery purposes, the term bioadhesion implies attachment of a drug carrier system to a specific biological location (Smart, 2005). Topical drug delivery has become a very attractive alternative to subcutaneous delivery as the skin has the largest area. It provides good compliance of patients and controls release characteristics of drugs, and avoids drug degradation from the gastric intestinal tract or first-pass liver effect. The skin can also provide a painless interface for systemic administration (Prausnitz, Mitragotri, Langer, 2004; Thomas, Finnin, 2004), besides the new methods of bioadhesive system applied in skin delivery system, which can prolong the contact time greatly by adhesion effect and doesn't produce discomfort (Zhu *et al.*, 2013). However, the advantages of using bioadhesive systems as drug carriers are the prolongation of drug residence time at the absorption site (Smart, 2005; Carvalho *et al.*, 2010b).

In vitro safety profile showed that this formulation is not cytotoxic and *in vitro* antioxidant activity suggested that LCS presented a higher capacity to maintain the antioxidant activity of OC extracts. Novel drug delivery systems, such as nanotechnological, provide several advantages over traditional forms including the enhancement of solubility, bioavailability, protection from toxicity, enhancement of pharmacological activity, enhancement of stability (Ajazuddin, 2010) and controlled release of biomarkers (Devi, Jain, Valli, 2010; Summerlin *et al.*, 2015). Antioxidant capacity can provide protection from endogenous and exogenous oxidative stresses by scavenging free radicals. Topical antioxidants are available in multivariate combinations through over-the-counter skin care products that are aimed at preventing the clinical signs of photoaging (Bogdan Allemann, Baumann, 2008). The incorporation these antioxidant agents into different types of nanomaterials used in cosmetic formulations to obtain the best effect of antioxidants applied onto the skin has been reported in many studies (Vinardell, Mitjans, 2015)

In summary, it was possible to develop a system with adhesive properties, besides maintaining the antioxidant

properties of the extract incorporated in them, with good biocompatibility. Therefore, it is feasible to conclude that these systems can be used for optimization of drug delivery systems on the skin for the treatment of skin aging.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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