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Development of lamellar gel phase emulsion containing baru oil (*Dipteryx alata* Vog.) as a prospective delivery system for cutaneous application

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ABSTRACT

The rational design of emulsions requires study of the main factors that influence their formation, physicochemical properties and, consequently, stability and performance. The use of vegetable oils in the pharmaceutical and cosmetic industries has recently become attractive. *Dipteryx alata* Vogel (*D. alata*) is an oleaginous species native to Brazil. The seeds of this species contain highly unsaturated oil with significant amounts of tocopherols and phytosterols, representing an important source of agents capable of combatting oxidative processes. In this work, a lamellar gel phase emulsion using oil extracted from the seeds of *D. alata* (baru) was developed. The steps involved in the development of this research were as follows: 1) development of formulations and 2) *in vitro* assays by simulating the evaporation of the final product after application to the skin and Electron paramagnetic resonance spectroscopy (EPR) of fatty acid spin labels was used to investigate the profile of interaction of the dispersed systems with stratum corneum (SC) lipids. The results indicate that the developed system shows no signs of instability during the storage period. Moreover, EPR studies indicated that *D. alata* oil and especially the developed formulation were able to increase SC lipid fluidity and extract a fatty-acid spin label from the lipid domain structures of SC, demonstrating its potential to act as a drug or skin care vehicle.

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1. Introduction

The commercial and scientific importance of emulsions to various colloidal systems is unquestionable. These systems are widely used as vehicles for the delivery of drugs and cosmetic agents to the skin due to their excellent solubilizing capacities for lipophilic and hydrophilic active ingredients and their accepted application [1–4].

The rational design of these systems requires an understanding of the major factors that influence their formation, physicochemical properties, and consequently their stability and performance. One of the most important factors is the selection of an appropriate stabilizer(s) as the surfactants [4–6].

Ethoxylated fatty alcohols (EFAs) are widely used non-ionic surfactants obtained by condensation of fatty alcohols with ethylene oxide (EO). EFAs show peculiar physicochemical behavior, as they may form lyotropic liquid crystalline phases on addition of solvent [7–11].

Lyotropic liquid crystals (LLC) are liquid crystals (LCs) – matter in a state with properties between conventional liquids and solid crystals – that play a very important role in biology. Lyotropic liquid crystalline phases are abundant in living systems such as the *stratum corneum* (SC), the outermost of the five layers of the epidermis [12].

The huge barrier function of the SC makes cutaneous absorption challenging. Skin penetration through the SC may be enabled by specific compounds called “penetration enhancers” that are recognized as modifying SC lipid fluidity [13]. Vegetable oils possess considerable levels of phytochemically active compounds, which may facilitate permeation of active molecules into the SC. These substances are currently being used to replace synthetic raw materials in cosmetic or pharmaceutical formulations [14–17].

Dipteryx alata Vogel (*D. alata*) is an oleaginous species native to the Cerrado (Brazilian Savanna), the second-largest biome of South America. Previous studies by Almeida; Silva and Ribeiro (1991) have shown that the great adaptability of *D. alata* results in high productivity and good quality fruits and seeds [18].

According to Marques et al. (2015), the nuts of *D. alata* possess an oil that is rich in mono and sesquiterpenes, phytosterols and tocopherol derivatives such as limonene, β -elemene, γ -elemene, α -caryophyllene and β -caryophyllene; β -sitosterol, stigmasterol, campesterol and cycloartenol; α and γ -tocopherol. Its chemical composition promises economic potential and should encourage the use of seeds and/or oil of *D. alata* as raw material in the food, pharmaceutical and cosmetic industries as an economical and environmentally benign approach [19–23].

Spin-labeling EPR is one of the most important spectroscopic methods to provide information about the dynamic structure of membranes and proteins [24]. Because lipid dynamics are associated with macroscopic permeability of the physical barrier of the skin, EPR spectroscopy has been widely employed to investigate the interaction of various compounds with SC membranes [25,26]. For instance, it has been shown that 1% of the terpenes L-menthol and 1,8-cineole drastically alter the lipid fluidity of SC membranes [27,28].

The aim of this research was to develop a lamellar gel phase emulsion using crude oil from seeds of *D. alata* as raw material

and to investigate the interactions of the oil and formulation with the SC matrix lipid by EPR spectroscopy of fatty acid spin labels.

2. Materials and methods

2.1. Materials

Oil phase: cold-pressed oil from baru seeds (*Dipteryx alata* Vogel). Surfactants: Steareth-2 (HLB = 4.9) and Polysorbate 80 (HLB = 15.0) – Lipo do Brasil; Sorbitan oleate (HLB = 4.3) – Croda; Cetareth-5 (HLB = 9.2) and Cetareth-20 (HLB = 15.7) – Oxiteno. Aqueous phase: Purified water. Preservative: DMDM Hydantoin and Iodopropynyl Butylcarbamate (Lonza).

2.2. Methods

2.2.1. Hydrophilic-lipophilic balance determination and surfactant study

A wide range of hydrophilic-lipophilic balance (HLB) values were tested in two steps to find the HLB value for *D. alata* oil. Step 1: wide HLB range from 4.3 and 5.0 to 15.0. (interval 1.0); and step 2: narrow range with 0.25 intervals. Two surfactants from the same chemical class (oleates) were chosen: Polysorbate 80 and Sorbitan oleate. The following surfactant mixtures also were assessed to prepare emulsions: Polysorbate 80/Sorbitan oleate, Cetareth-5/Sorbitan oleate, Cetareth-20/Sorbitan oleate, Cetareth-5/Steareth-2, Polysorbate 80/Steareth-2.

2.2.2. Pseudo-ternary diagram design

The best proportion of formulation components (water/oil/surfactants) was determined using a pseudo-ternary diagram technique. The low-energy emulsification method Emulsion Inversion Point (EIP) was used to prepare all emulsions: the aqueous and oily phases were heated separately at $75 \pm 2^\circ\text{C}$, and the aqueous phase was slowly added to the oily phase under constant agitation at 600 rpm (Mechanical Agitator – IKA® RW 20 digital) until it reached room temperature ($25 \pm 2^\circ\text{C}$). The macroscopic stable formulations were examined using polarized light microscopy to detect anisotropic structures.

2.2.3. Stability study

Stability study was performed in accordance with the guidelines of the National Health Surveillance Agency [29].

Preliminary stability was evaluated after 24 h by a centrifugation test, conducted for 30 min at 3000 rpm (Excelsa Baby II-Fanem), and by thermal stress analyses wherein formulations were heated from 40 to 80°C, increasing 5°C every 30 min [29,30].

The selected emulsion was packaged in glass flasks and stored at three different temperatures ($5 \pm 2^\circ\text{C}$, $25 \pm 2^\circ\text{C}$ and $40 \pm 2^\circ\text{C}$). The following parameters were evaluated: pH, electrical conductivity, particle size, and macroscopic and microscopic characteristics. All assays were performed in triplicate at 1, 7, 15, 30 and 60 d after preparation.

2.2.4. Physicochemical analysis

Electrical conductivity, pH and particle size were evaluated at $25 \pm 2^\circ\text{C}$ using a digital conductivity meter (DM 32 – Digimed),

a digital pH meter (PG 1800 – Gehaka), and laser diffractometer (Beckman Coulter® LS 13 320), respectively. Polarized light microscopy (Microscope Olympus BX50) was used to detect the presence of anisotropic structures.

2.2.5. Microscopic behavior: water content influence on anisotropic structural changes

The formulation was evaluated using the following methods:

2.2.5.1. Phase diagram. Emulsions were prepared by Emulsion Inversion Point (EIP) methodology, and the rates of each component were calculated according to the phase diagram by maintaining the same proportion of surfactants and oil and simulating the decrease in water concentration promoted by evaporation. After 24 h, all formulations were observed by polarized light microscopy.

2.2.5.2. Evaporation test. Emulsion sample was deposited on an area limited to 5 cm² on a microscope slide, and the evaporation loss was evaluated using a balance equipped with an infrared lamp at 70 °C until > 80% of weight was lost. At 3 min intervals, images of the sample were obtained with a polarized light optical microscope.

2.2.6. EPR spectroscopy measurements

SC membranes of neonatal Wistar rats less than 24 h old were prepared as described previously [27,28]. This experiment was performed according to the Ethics Committee of the Biological Institute of Universidade Federal de Goiás – Brazil (Protocol number: 022/16).

The spin label 5-DSA, which contains a nitroxide radical moiety (doxyl) at the 5th carbon atom of the acyl chain, was purchased from Sigma Chem. Co. (St. Louis, MO). A small aliquot (1 µl) of stock solution of spin label dissolved in ethanol (5 mg/ml) was placed on a glass plate, and after solvent evaporation, the SC membrane (1.5 cm²) was hydrated with 50 µl of acetate-buffered saline (10 mM acetate, 150 mM NaCl and 1 mM EDTA, pH 5.1) at the same site where the spin label was placed and gently mixed for 10 min. The formulation and oil dispersions were spin labeled by incubating for 30 min at room temperature with an aliquot (1 µl) of the stock solution of 5-DSA. During this procedure, the samples were gently agitated. Finally, the oil and formulation, both with and without spin labeling, were applied to the SC membranes and after a 1.5 h incubation period the samples were introduced into capillary tubes, which were sealed by flame for EPR measurements.

A Bruker® ESP 300 spectrometer equipped with an ER 4102 ST resonator and operating in the X-band (9.4 GHz) was utilized in these investigations. The operational conditions of the spectrometer were as follows: microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; magnetic field scan, 100 G; sweep time of 168 sec; and detector time constant of 41 msec. All measurements were performed at room temperature, and data analysis was performed using Origin® Graphing Software.

2.2.7. Statistical analysis

All experiments were performed in triplicate. The results are expressed as the mean ± SD. All data were submitted to one-way ANOVA using GraphPad Prism 5.0 software. Tukey's Multiple

Comparison Test was used to assess differences between means, with $P < 0.05$ considered statistically significant.

3. Results and discussion

3.1. HLB determination and surfactant study

To find the HLB value for *D. alata* oil, two classical surfactants from the same chemical class (oleates) were chosen: Polysorbate 80 and Sorbitan oleate. A wide range of HLB values was tested in two steps, and the most stable formulations exhibited HLB values of 6.00 and 6.25, as shown in Fig. 1A. This analysis is an important step to define the most suitable surfactants for various systems such as water-in-oil (W/O) and oil-in-water (O/W) emulsions. This parameter was defined by Griffin (1949) and it is still widely used as an essential step to develop new formulations by determination of the correct proportion of surfactants that is able to stabilize the emulsified system [31].

After HLB determination, others surfactant pairs were investigated as described at “Material and Methods” section. As shown in Fig. 1B, the surfactant pair Cetareth-5 and Steareth-2, cetaryl alcohol with 5 molecules of EO and stearyl alcohol with 2 molecules of EO, respectively, was the surfactant combination that resulted in a stable formulation (HLB_{required} = 6.25) with anisotropic structures (liquid-crystals).

These anisotropic structures are also three-dimensional association structures that stabilize emulsions. Emulsion droplets are surrounded by this ordered structure which provides an increase in viscosity for the emulsion around the droplets, reducing instability process. Notably, the addition of fatty alcohol led to the appearance of different structural arrangements of liquid-crystal (lamellar, hexagonal and cubic) [8,32]. The presence of anisotropic structures in a well-defined maltese cross pattern (Fig. 1B) indicates the arrangement of molecules in lamellar gel phase type (LGP) [33].

3.2. Preparation of emulsions – construction of a pseudo-ternary diagram

To determine the concentration range of components (water/oil/surfactants) that leads to the formation of anisotropic structures, a pseudo-ternary phase diagram was constructed.

Fig. 2 shows that five formulations (21, 27, 28, 35 and 36) from the pseudo-ternary diagram remained stable after centrifugation tests, and three of them present anisotropy. Nevertheless, only one formulation (composed of 10.0% oil, 10.0% surfactants and 80.0% water – number 36) showed clear, uniform anisotropic structures.

3.3. Stability study

After preliminary stability tests [29,30] the formulation 36, composed of 10:10:80 *D. alata* oil:surfactants:purified water, was selected to be stored at different temperatures (5 ± 2 , 25 ± 2 and 40 ± 2 °C) and to proceed with further stability tests.

Stability study carried out up to 60 d and was monitored by physicochemical analysis Table 1 shows the results of: pH,

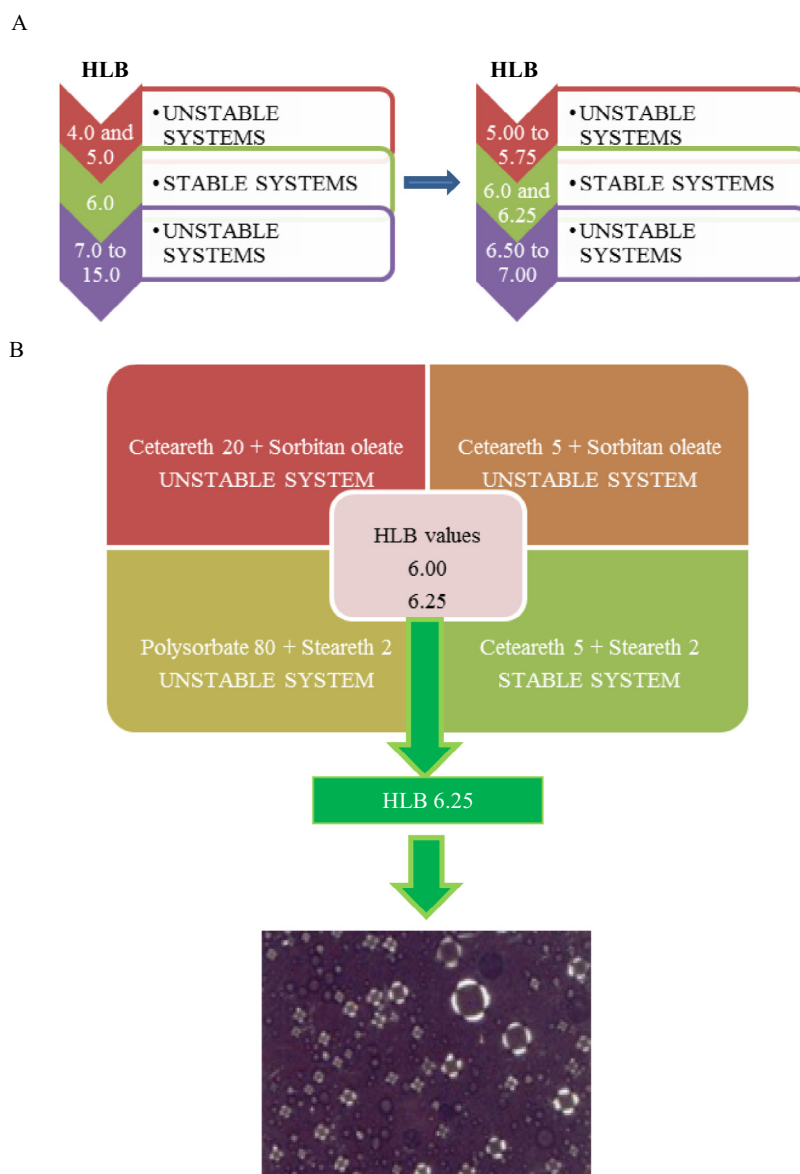


Fig. 1 – Results of surfactant study. Legend: (A) Results of the hydrophilic-lipophilic balance (HLB) value determination. It was tested in a wide range of values. (B) Others surfactant pairs were assessed to prepare emulsions.

electrical conductivity, particle size and polarized light microscopy images of the formulation 36. The results have shown that the emulsion remained stable during the entire 60 d testing period. No significant variation in pH, electrical conductivity (except for formule 36 stored at 40 ± 2 °C) or particle size was registered, and the polarized light microscopy images demonstrated the great structural stability of the LCs. Considering that extreme storage conditions (40 ± 2 °C) commonly alter the physicochemical properties of commercial products, these results suggest good quality of the emulsified system [29].

The pH of the emulsions remained stable over the entire study period for all conditions tested. pH values reflects the quality of a product because it suggests the maintenance of efficacy and safety. Our results suggest that the developed system has good quality.

In this work, no changes in electrical conductivity at 25 ± 2 or 4 ± 2 °C storage conditions were observed, but a statistically

significant increase (6842 ± 139 to 9266 ± 246 $\mu\text{S}/\text{cm}$) occurred at 40 ± 2 °C. Masmoudi et al. (2005) found a progressive increase in electrical conductivity several months before emulsion destabilization. Similar results were found in this research under extreme storage conditions [34].

The droplet size of the formulation remained constant over 60 d indicating the formulation stability. Droplet size measurements are a good indicator of formulation stability. Notably, there is not a fast droplet size increase, which would indicate the system stability [35].

Polarized light microscopy was used to characterize the structures of LCs. The results indicated the presence of anisotropic structures in a well-defined maltese cross pattern which indicates typical LCs structures with lamellar arrangement. The presence of these structures helps to stabilize the emulsions and may impart benefits as a skin delivery system. Research on lipid organization in human SC has shown the presence of

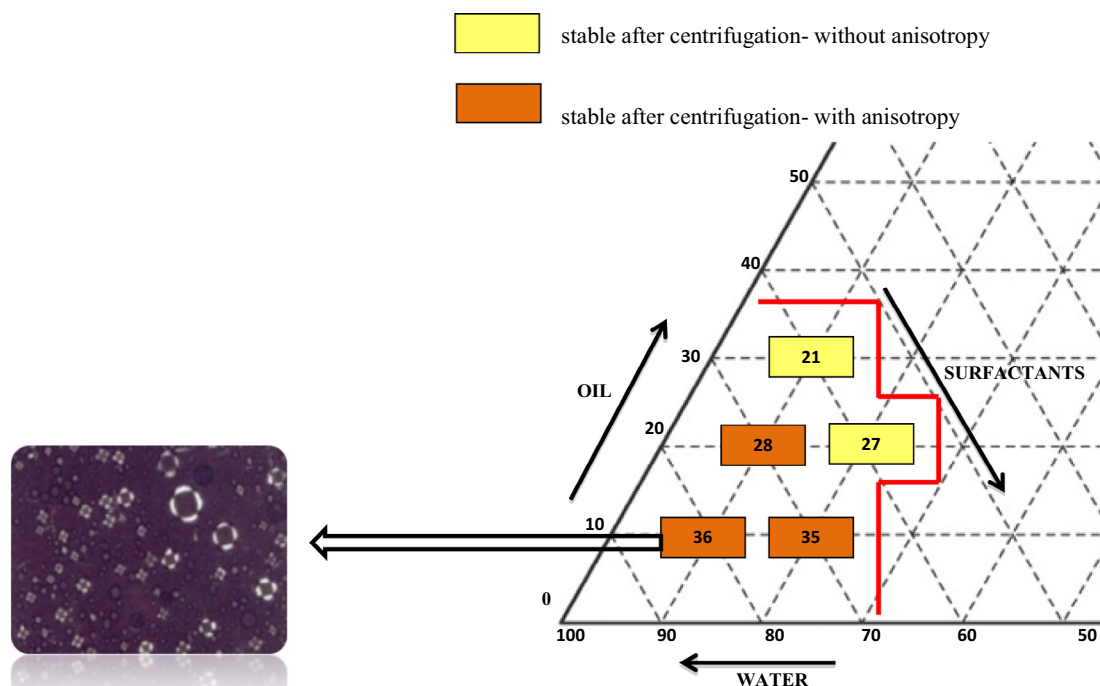


Fig. 2 – Pseudo-ternary diagram for baru oil.

lamellar phases that are crucial for the skin barrier. Therefore, formulations such as those developed in this work can contribute to a wider variety of drugs and cosmetic actives that can be applied dermally and transdermally [36,37]

3.4. Optical behavior: influence of the proportion of volatile components on microscopic structural changes

The aim of this test was to study the optical behavior of the emulsion due to structural changes during evaporation of volatile components, simulating *in vitro* evaporation after application to the skin.

As observed in Fig. 3, during evaporation tests (Fig. 3B) and in the other formulations developed by phase diagram (Fig. 3A), the arrangement of anisotropic structures changes in accordance with water content. These tests give insight into the structures that would appear after evaporation. At the beginning of evaporation tests, anisotropic structures are well defined. As the evaporation rate increases, they become less defined and ultimately amorphous. This phase diagram approach shows that the presence of vegetable oil is intimately related to the arrangement of anisotropic structures and that water content influences the arrangement of anisotropic structures, as also found in evaporation tests. Our results suggest that both methods (phase diagram and evaporation) adequately predict the behavior of emulsions after application to the skin.

3.5. EPR spectroscopy: effect of oil and formulation on SC lipid dynamics

The interaction of *D. alata* oil and the emulsified system with SC was investigated using EPR spectroscopy with a spin-probe analog of stearic acid 5-DSA. Fig. 4 shows the EPR spectra of

Table 1 – Stability tests: physicochemical properties and polarized light microscopy images during 60 d.

Time (d)	1	60
4 ± 2°C		
pH	6.2 ± 0.1	6.0 ± 0.2
Electrical conductivity (μS/cm)	7353 ± 201	6965 ± 467
Particle size (μm)	11.8 ± 0.5	12.4 ± 0.9
Polarized light microscopy images		
25 ± 2°C		
pH	6.2 ± 0.1	6.1 ± 0.1
Electrical conductivity (μS/cm)	7371 ± 429	7111 ± 102
Particle size (μm)	12.0 ± 0.6	12.5 ± 0.9
Polarized light microscopy images		
40 ± 2°C		
pH	6.1 ± 0.1	6.1 ± 0.2
Electrical conductivity (μS/cm)	6842 ± 139 ^a	9266 ± 246 ^a
Particle size (μm)	11.8 ± 0.4	11.9 ± 0.8
Polarized light microscopy images		

Note: Results are expressed as the mean ± SD (n = 3).

^a Statistically significant, P > 0.05.

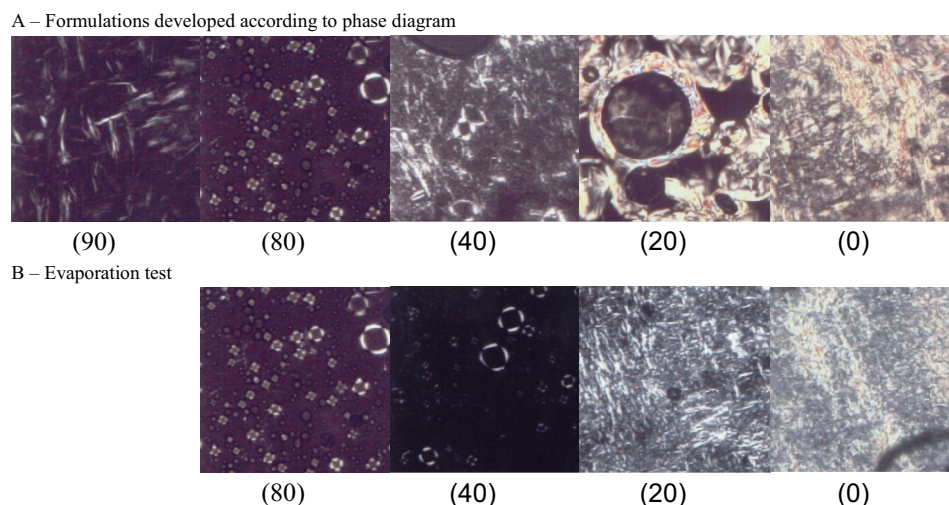


Fig. 3 – Structural changes in emulsions – the influence of water content. Note: The figure shows the microphotographs of formulations obtained from evaporation test (B) and according to phase diagram (A). The number in parentheses shows the water content.

5-DSA incorporated into SC membranes, *D. alata* oil or the formulation. The spectra of 5-DSA incorporated into the lipid domain of SC showed a broad line shape and high values of $2A_{//}$ (65.6 G) consistent with the low fluidity of these membranes; $2A_{//}$ is a spectral parameter measured as indicated in Fig. 4C and widely used to monitor lipid fluidity [38]. *D. alata* oil showed high molecular mobility (high fluidity), as evidenced by the narrow lines in the EPR spectrum (Fig. 4B). For this reason, it was not possible to measure $2A_{//}$. The formulation

showed lower molecular mobility than *D. alata* oil (Fig. 4C). This could be attributed to the presence of a lamellar gel phase, which confers an organized arrangement to the emulsified system when compared with the *D. alata* oil. However, formulation fluidity was significantly higher than the SC lipids, as evidenced by the value of the $2A_{//}$ parameter (55.4 G), which was approximately 10 G lower than SC.

The penetration capacity of *D. alata* oil and of the formulation into lipid domains of SC can be understood from the

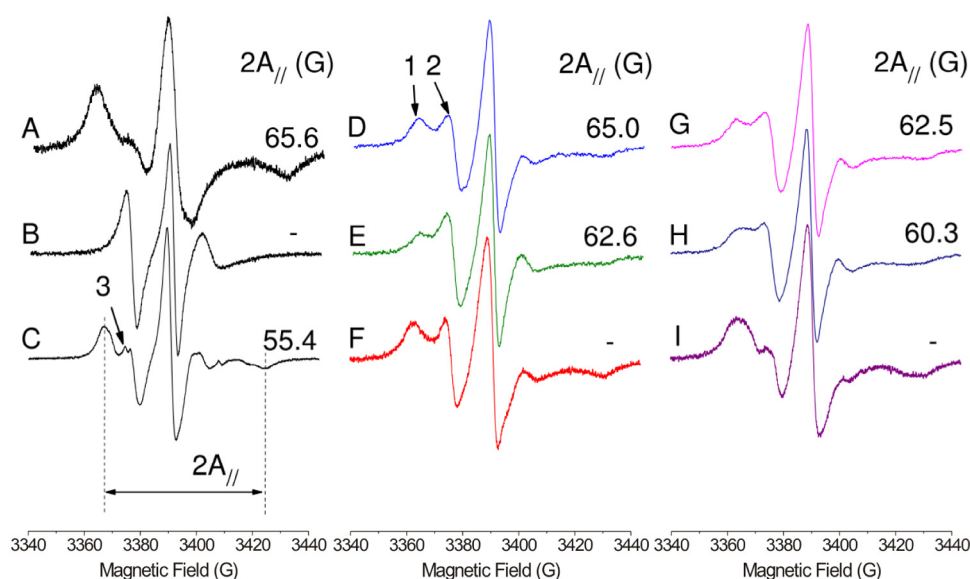


Fig. 4 – Experimental EPR spectra at 25 °C of spin label 5-DSA incorporated into SC (A), *D. alata* oil (B), formulation (C), SC and after treated with *D. alata* oil (D), *D. alata* oil and after added to SC (E), SC and after treated with the formulation (G), formulation and added to SC (H). Spectra (F) and (I) are the sums of spectra (A) + (B) and (A) + (C), respectively. The values of the outer hyperfine splitting parameter ($2A_{//}$), which is measured directly in the EPR spectra, are indicated. The Y-axis of the EPR spectra was omitted from the figure; in each EPR spectrum the Y-axis represents the intensity of the EPR signal (a.u.). The total scan range of the magnetic field was 100 G.

spectra shown in Fig. 4D to 4I. The application of *D. alata* oil to the spin-labeled SC membranes significantly reduced the parameter $2A_{//}$ from 65.6 to 65.0 G (Fig. 4A and 4D), corresponding to a small increase in SC membrane fluidity; this finding indicates that *D. alata* oil is able to penetrate the SC membranes. However, two spectral components are present in the EPR spectrum (indicated in Fig. 4D by the numbers 1 and 2). Component 1 corresponds to the spectrum of 5-DSA in the SC membranes altered by the presence of oil, and component 2 corresponds to the spectrum of the spin probe transferred from SC membranes to the oil. This result suggests that *D. alata* oil acts as a fluidizer and fatty-acid extractor. Both effects on the SC intercellular membranes are associated with increased SC permeability [39]. To examine the oil's ability to extract SC lipids, spin-labeled *D. alata* oil was applied to the non-spin-labeled SC and the resulting EPR spectrum is shown in Fig. 4E. This spectrum is similar to that shown in Fig. 4D, but the *D. alata* oil considerably increased the SC lipid dynamics, as indicated by the value of $2A_{//}$ (62.6 G). In this case, the second spectral component was more intense than the first, indicating that the major fraction of spin probes remained in the *D. alata* oil and that only a small fraction migrated to the SC lipid domains. The normalized sum of the spectra of Fig. 4A and 4B resulted in the spectrum shown in Fig. 4F, which is slightly different from the spectrum of Fig. 4C, denoting a weak interaction between *D. alata* oil and SC lipids for a 1.5 h incubation period. In the formulation case, the sum of the spectra 4A and 4C resulted in the spectrum 4I, which is very different from the spectrum 4G, indicating that the spectrum 4G of the spin-labeled SC and treated with the formulation is not the result of spin probes in two distinct microenvironments (SC and formulation) but show an interaction between formulation and SC lipids, supporting the interpretation that the formulation can overcome the SC barrier.

Fig. 4C shows the appearance of a third component (number 3) from the spin labels dissolved in the aqueous phase of the formulation. Similarly to the oil effect on SC, the formulation was able to penetrate SC membranes, reducing the $2A_{//}$ parameter by 3.1 G (Fig. 4G) compared with the spectrum 4A. This reduction was approximately five times greater than that caused by *D. alata* oil. The formulation increased the intensity of the peak relative to the second component of the spectrum, suggesting greater formulation-SC interaction. When the spin-labeled formulation was applied to SC membranes, a drastic increase in fluidity was observed, indicated by a decrease of 5.3 G in the $2A_{//}$ parameter of the spectral component, corresponding to the SC lipid domains.

An increase in the fluidity of the lipid SC is directly linked to increased capacity in skin permeation ability [13,39,40]. Thus, these results and others support the idea that the developed lamellar gel phase emulsion can be used as a prospective delivery system for cutaneous application or as a potential product for skin care.

4. Conclusions

The results of this research suggest that the developed system may be a good alternative skin-care delivery system, particularly

because the use of vegetable oil follows a global trend of using renewable resources for sustainable development in cosmetics. Moreover, the lamellar gel phase confers many functional advantages to the system, such as increased stability and structural similarity to SC membranes. The EPR spectra indicated that the *D. alata* oil causes a small increase in fluidity in the lipid matrix of SC and it was able to extract a fatty-acid spin label from the lipid domain structures of SC. The developed formulation containing the *D. alata* oil showed strong interaction with the SC lipids, demonstrating a greater capacity to fluidize and extract the spin label present in the SC lipid matrix. These results corroborate the suitability of this prospective delivery system for several model drugs and cosmetic actives.

Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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