Nanoemulsions containing oil and aqueous extract of green coffee beans with antioxidant and antimicrobial activities

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Abstract
Coffee has bioactive compounds with protective effect against free radicals and antifungal and antibacterial activities that are beneficial to the health skin. This work aims to provide scientific evidence that nanoemulsions developed with oil and green coffee extract have antioxidant and antimicrobial effects. Nanoemulsions were prepared by spontaneous emulsification method using different concentrations of components (oil, extract and tensoactive) in order to optimize the preparation of formulations. The nanoemulsions presented globular morphology, negative charges, monodispersity and size around 200 nm. Two nanoemulsions, namely F7 and F16, presented characteristics of size and concentration of tensoactives more suitable for investigation regarding their antioxidant and antibacterial activities. The later assay was performed against standard strains of Staphylococcus aureus, Staphylococcus epidermidis and Escherichia coli at different nanoemulsion concentrations. The formulations proved to be inhibitors of free radicals and showed excellent antibacterial activity, demonstrating their potential for cosmetic and therapeutic applications.

Introduction

The humanity have used natural products since ancient times. According to the World Health Organization (WHO), a large proportion of the population relies heavily on traditional practitioners and medicinal plants to meet primary health care needs in many developing countries, because of its easy access and reduced costs. Indeed, herbal medicines have maintained popularity for historical and cultural reasons, also avoiding a number of adverse side effects resulting from the use of synthetic medications. Besides, in the last years many people in developed countries have begun to turn to alternative or complementary therapies, including medicinal herbs [1].

Coffee is a plant species that has been reported to be rich in antioxidant compounds, i.e., secondary metabolites able to neutralize free radicals. Studies have shown that coffee’s constituents may aid in the expression of antioxidant genes in tissues, protecting cells from the damage resulting from the oxidation of their constituents, e.g., cell membrane’s lipids and DNA, caused by reactive oxygen species [ROS] [2].

Coffeea arabica oil of green beans has been a frequent choice for both cosmetic and therapeutic formulations due to its composition in bioactive compounds with ability to protecting cells against solar radiation damages, besides its antioxidant, antimicrobial, emollient, emulsifier, anti-inflammatory, and radioprotective properties [3–6].

Although the human body presents defense systems against ROS, natural antioxidants compounds might enhance the effectiveness of the cell responses to the oxidative stress. In this sense, it is worth mentioning that coffee is a rich source of phenolic compounds able in eliminating free radicals in several tissues and organs [7]. Phenolic compounds are a class of secondary metabolites found in most plant species with ability to protect

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against damages caused by excessive solar radiation exposure, neurodegenerative and cardiac diseases, and even cancer, also showing antifungal, antibacterial, and antiviral activities [7].

In view of the above, it is important to investigate compounds with antioxidant and antimicrobial properties from natural sources [8], which might support the development of cosmetic and pharmaceutical products with improved effects due to their bioactive constituents and formulations [7]. For that, in the past years nanotechnology has been often adopted in R&D pipelines of pharmaceutical and cosmetics industries.

Nanotechnology is a science that works with the development of materials on a nanometric scale, that is, on a scale from 1 to 1000 nm, with application in many different areas [9]. This science is considered one of the greatest technological innovations that has reformulated the biological, chemical, physical, agricultural, and electronic areas, handling matter on a molecular scale [10]. Because nanotechnology covers a wide range of industrial applications, a series of nanoderivative products with therapeutic and cosmetic applications has been continuously developed and commercialized, usually with increased added-value [11].

In this context, this work aimed at developing nanoemulsions containing oil and aqueous extract of green coffee beans and further in vitro evaluating their effect regarding the inhibition of free radicals and the growth of pathogenic microorganisms to the human health. Nanoemulsions are systems with varied compositions where two non-miscible liquids (oil and water) are combined and stabilized by a specific tensoactive, in which the droplet size is in the range of nanometers (nm) [12–14].

Systems for preparing nanoemulsions use high or low energy emulsification methods. In the first case, a great amount of energy is applied to the system, determining the fragmentation of the oily (or internal) phase, thus forming droplets on a nanometric scale. High-pressure homogenizers, microfluidizers, and ultrasound are included in these methods [15].

Although high energy preparation methods are widely used in the pharmaceutical and cosmetic fields, it is necessary to apply more economically favorable and simple strategies, making the costs of the final product cheaper [15]. As an alternative to this issue, the so-called low-energy methods have been used, which are based on the energy from the constituents of the nanoemulsion and, therefore, do not use equipment that requires high energy. Spontaneous emulsification and phase inversion are examples of low-energy methods [16, 17].

In this work, the spontaneous emulsification method was used, where the active compound is dissolved in an organic solvent, for example, ethanol and, later, added to the oil and the lipophilic tensoactive, thus constituting the (internal) oily phase of the nanoemulsion. The aqueous phase is formed by water and hydrophilic tensoactive. After this step, the oil phase is slowly dripped onto the aqueous phase, under constant agitation, at room temperature. The tensoactives added in the formulation submitted to agitation form an interface that increases the surface tension, causing spontaneous formation of small oil droplets surrounded by water (nanodroplets) [13, 16, 18].

Nanoemulsions have unique physicochemical traits, e.g., increase the stability of the product, provide a better skin penetration of active ingredients, and a controlled release of the encapsulated components in the formulation [19]. Besides, nanoemulsions increase the efficiency of active compounds used in formulations by augmenting their solubility and also by protecting them against the degradation caused by external agents such as light, heat, humidity, and pH [20]. Because nanoemulsions provide a targeted release of bioactive compound (s), they have been increasingly used in the treatment of diseases, since they distribute the active compound(s) in an efficient and specific way [10]. Specifically in relation to the cosmetic application, nanoemulsions have been used due to some beneficial properties such as: 1—use of low concentrations of tensoactives; 2—its reduced droplet size provides uniformity in topical application; 3—improved permeation into the tissue; 4—prolonged action time on the skin’s assets [13, 21]. Despite the existence of several studies reporting the development of nanoemulsions using vegetable oils and extracts, there are still no reports regarding a nanoemulsified system using oil and green coffee extract, as proposed in this work.

**Material and methods**

**Material**

Green coffee oil and green coffee paste were kindly provided by Cooxupé - Minas Gerais, Brazil. The samples of green coffee oil were obtained by cold pressing the coffee beans (Coffeea arabica). The residual biomass of the coffee oil extraction, hereafter referred to as green coffee paste, was collected and packed in plastic bags protected from the light and moisture, following the storage at −80 °C. The chemicals used in the experiments were bought from the suppliers as follows: soy lecithin (Lipoid S75, Lipid Ingredients & Technologies, Brazil), poloxamer 188 (Kolliphor P188 micro, BASF, USA), caprylic/capric triglyceride (TCM - Miglyol 812N, PIC Química, Brazil), ethyl alcohol (Vetc, Brazil), carbon-coated copper grids (CF300 mesh-Cu, TED PELLA INC., USA), 2, 2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, USA), 2, 4, 6-tris (2-pyridyl)-s-triazine (TPTZ, Sigma-Aldrich, USA), Muller Hinton broth (CMH, Sigma-Aldrich, USA), Tween 80, resazurin (7-hydroxy-3H-
phenoxazin-3-one 10-oxide, Sigma-Aldrich, USA), and the antibiotics penicillin (TEUTO, Brazil) and ampicillin (EMS, Brazil).

**Preparation of green coffee nanoemulsions**
Firstly, the aqueous extract of green coffee beans was obtained by adding 25 ml distilled water to 2.5 g green coffee paste, following magnetic stirring for 1 h. The aqueous extract (32 mg ml\(^{-1}\) of total phenolics) was recovered by centrifugation (4000 rpm, 5 min) and stored at \(-80\,^\circ\)C. The green coffee nanoemulsions were prepared by the spontaneous emulsification method \([12]\), using different concentrations of green coffee oil, green coffee extract, soy lecithin, and the hydrophilic tensioactive poloxamer. The aqueous phase containing green coffee extract (v/v), water (ml), and poloxamer (w/v) was dripped into an oil phase containing green coffee oil (w/v), ethanol (v/v), and lecithin (w/v) until complete homogenization of the solution. Then, the organic solvent was removed under reduced pressure at 700 MPa, following by filtration of the colloidal suspensions on a cellulose support under vacuum. The final volume of the nanoemulsion was adjusted to 10 ml. For comparison purposes, blank nanoemulsions were prepared using TCM (capric/caprylic triglycerides) in replacement of the green coffee oil, under the same conditions above described. All experiments were performed in triplicate. More specific data can not be provided at this moment, because the product developed in this study is matter of patent registration and industrial secret.

**Particle size and zeta potential measurements**
Dynamic light scattering (DLS) and laser-doppler anemometry (Zetasizer Nano ZS90 - Model ZEN3690 - Malvern Instruments, Worcestershire, UK) were used to determine the particle size and zeta potential, respectively, in the formulated nanoemulsions. This characterization was performed at 25 \(^\circ\)C, after dilution (10\(\times\)) of the samples in ultrapure water. The mean particle size was determined at a fixed 90\(^{\circ}\) angle, while the zeta potential was measured by transferring the samples to electrophoretic cells, followed by the application of an electrical potential ±150 mV. The zeta potential was calculated based on the mean electrophoretic mobility of the nanoparticles, according to the Smoluchowski equation \([22]\).

**Morphological evaluation**
The morphology of the nanoemulsions was investigated by transmission electron microscopy (TEM) using a Jeol equipment (model JEM-1011, Jeol Ltd, Tokyo, Japan). Aliquots of the formulations of interest were previously diluted to 10\%(v/v) in ultrapure water and subsequently deposited on carbon coated grids (200 mesh) and stained with 2\%(w/v) uranyl acetate solution.

**Determination of antioxidant activity**

**DPPH assay**
The antioxidant activity of the nanoemulsions was evaluated by the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical method \([23]\). The antioxidant activity was determined by diluting the samples in water at the desired concentrations (0.1, 0.2, 0.5, 1.0, 5.0, 10, and 12.5\%). The reaction was done after dilution (1: 100) of a DPPH stock solution (7.9 mg/2.5 ml methanol solution). As a positive control, a solution of DPPH was prepared without addition of nanoemulsion. Measurement of the reduced DPPH was performed in a UV-vis spectrophotometer (Instrutherm - UV-2000A), by recording the absorbances at 530 nm wavelength over different reaction times (1, 2, 3, 6, 9, 12, 15, and 24 h) \([24]\).

The calculation of the antioxidant activity was performed as scavenging activity of DPPH free radicals (SAFR) according to the formula:

\[
\text{SAFR} \%(\%) = \left( \frac{\text{Ac} - \text{As}}{\text{Ac}} \right) \times 100
\]

where:

- Ac = DPPH solution absorbance (control)
- As = sample absorbance

The nanoemulsions' antioxidant activity was compared to the water-soluble analog of vitamin E Trolox, using a standard curve of that carboxylic acid (0.0195 to 1.25 mM, y = 106.58\times, r\(^2\) = 0.99).

**FRAP assay**
The antioxidant activity was also evaluated by the ferric reducing antioxidant power method (FRAP) \([25]\). The FRAP reagent was prepared in a 300 mM acetate buffer, containing 10 mM TPTZ and 20 mM ferric chloride. Aliquots (45 \(\mu\)l) of samples were added to 135 \(\mu\)l ultrapure water and 1350 \(\mu\)l FRAP reagent, followed by homogenization (Vortex) and incubation at 37 °C, for 30 min. The antioxidant activity of nanoemulsions was determined by recording the absorbances at 595 nm and calculated through a FeSO_4 standard curve (50 to 1000 \(\mu\)M y = 0.0006\times, r\(^2\) = 0.99). FRAP reagent in ultrapure water was used as a blank solution.
Antibacterial activity

The technique used to determine the antibacterial activity followed the Clinical and Laboratory Standards Institute’s guidelines [26]. The assays were performed against the standard strains of *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC12228, and *Escherichia coli* ATCC 8739. Nanoemulsions and green coffee aqueous extract were prepared in different concentrations (0.1, 0.2, 0.5, 1.0, 5.0, 10, and 12.5%) in Mueller Hinton medium (MH). To perform the tests, serial dilutions of the F7 and F16 formulations of the coffee aqueous extract and the coffee oil nanoemulsions (50 to 0.37%) were added to the MH medium, followed by adding 10 μl of the inoculum suspension, equivalent to a log dilution of 105 CFU ml⁻¹. The initial inoculum was prepared using a turbidity control equivalent to a McFarland standard solution of 0.5, corresponding to a suspension containing 1 to 2 × 10⁸ CFU ml⁻¹ [26]. After 24 h incubation at 37 °C, the Minimal Inhibitory Concentration (MIC) was determined by the appearance of turbidity. In addition, absorbance (λ = 625 nm) readings on microplate reader (EL808, Bio-Tek Instruments, Inc) were taken to determine the inhibition (%) of bacterial growth. Calculation of bacterial growth inhibition (%) was performed with the aid of the following formula:

\[
\% \text{IM} = \left( 1 - \frac{\text{AC}}{\text{AO}} \right) \times 100
\]

where:
- IM = percentage of inhibition of microbial growth,
- AC = the mean absorbance of the concentrations of suspensions tested with inoculum, subtracted from the absorbance value of the same suspension concentrations without addition of the inoculum,
- AO = the mean absorbance of the microbial growth control.

In this case, MIC was considered the concentration that presented a percentage of inhibition higher than 80% [26]. After the absorbance readings, 50 μl resazurin developer (100 μg ml⁻¹) were added and the MIC was visually confirmed by the change of the reaction medium’s color from pink to blue. The presence of blue color in the wells means absence of bacterial growth and the opposite for pink color. MIC was considered the last blue color in the wells prior to the pink one. In all assays, two formulations containing the tensoactives were tested, at the same concentrations, and considered as negative controls, as well as Tween 80 (1%, v/v). Additionally, the antibiotics penicillin and ampicillin (500 mg ml⁻¹) were used as positive controls.

Statistical analysis

Statistical analyzes were performed using univariate techniques, e.g., one-way ANOVA (p < 0.05) and Tukey’s test. The statistical software Graph-Pad Prism 6 (GraphPad Software Inc., San Diego, CA, USA) was used for the analysis of the data set. All experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD).

Results

In this study, green coffee nanoemulsions were successfully developed by the spontaneous emulsification method. The spontaneous emulsification mechanism allows one obtaining nanoemulsions by mixing an organic phase, usually an oil, a lipophilic tensoactive, a water-miscible solvent (acetone or ethanol, for instance), and an aqueous phase containing a hydrophilic tensoactive [12].

Nanoemulsions loaded with oil and green coffee aqueous extract showed a milky appearance and a slightly yellow coloration, while nanoemulsions without the bioactive compounds presented a white color (figure 1).

In order to optimize the conditions of preparation of the nanoemulsions by the technique of spontaneous emulsification, 18 formulations were prepared using different concentrations of oil and green coffee aqueous extract, tensoactives, and organic solvents (data not shown). Taking into account the ongoing process of intellectual property aiming at an issue of a patent, specific details of the composition of each formulation investigated cannot be furnished at this moment.

In general, the formulations showed to be homogeneous, without precipitation or phase separation, except for the F18 formulation which presented the highest particle size, eventually resulting from the coalescence among nanoparticles. The results regarding the mean particle size, polydispersity index, and zeta potential found in the green coffee nanoemulsions are shown in table 1.

A wide range of particle sizes was detected among the formulations, varying from 182.2 to 707.1 nm, with an average of 266.8 nm. In some formulations (F1, F2 and F4) the mean particle size recorded showed to be a direct function of the nanoemulsion composition, more specifically in respect to the tensoactive concentration. Regarding the Pdi, formulations with values up to 0.25 were monodisperse. In turn, the zeta potential found...
was > −30 mV in all the formulations tested, a positive trait since high zeta potential values in module are fundamental to confer physicochemical stability to nanoemulsions.

After determining the nanoemulsions' physicochemical properties, it was decided to continue the experiments with the F7 and F16 formulations for further in vitro analysis of antibacterial and antioxidant activities, because their traits, i.e., nanoparticle size and concentration of tensoactives compounds, seemed to be more suitable for pharmaceutical and cosmetic applications.

F7 and F16 nanoemulsions showed a nanoparticle size distribution as depicted in figure 2. The nanoparticle average size was around 200 nm, corroborating with the data found by transmission electron microscopy (TEM—figure 3).

The results revealed nanoparticles with spherical shape and an average size of approximately 200 nm, similar to that determined by the dynamic light scattering technique (figure 2).
Green coffee nanoemulsions F7 and F16 were tested over time (1 to 24 h) at different concentrations (0.1, 0.2, 0.5, 1.0, 5.0, 10 and 12.5%, v/v) to determine their potential antioxidant activity either by DPPH and FRAP methods.

Figure 4 shows the results of the antioxidant activity of the F7 and F16 green coffee nanoemulsions determined by the DPPH assay. F7 nanoemulsion (10% v/v, 3 h) had a 90.46% ability to sequester the DPPH radical, while F16 (5% v/v, 2 h) showed an activity of 70%. Despite the lower antioxidant activity, F16 nanoemulsion showed a similar profile in sequestering DPPH radical over time in respect to F7 nanoemulsion. It is possible to note an increasing release of bioactive compound(s) over time until 10% concentration, as for higher values (12.5%) the antioxidant activity begins to decrease \((p < 0.001, \text{Tukey’s test})\), eventually because the active compound(s) in the nanoemulsion are being depleted. It can be observed that the antioxidant activity reached a maximum at 10% nanoemulsion and probably it would be futile to proceed with higher concentrations of formulation. Thus, the maximum antioxidant efficiency of green coffee nanoemulsion was detected at 10% concentration thereof (figure 4).

By the way, the F7 nanoemulsion demonstrates greater antioxidant potential when compared to F16 one. In addition, our results show higher EC50 values (2.20 mg chlorogenic acid/ml nanoemulsion) for the nanoemulsion F7 than for F16 (1.60 mg chlorogenic acid/ml nanoemulsion). Importantly, high levels of chlorogenic acid (CGA) were detected in the aqueous extract (1121 \(\mu g\) ml\(^{-1}\) nanoemulsion), which is the major...
compound in the green coffee nanoemulsions. Besides, other antioxidant compounds were also found in the aqueous extract as, for instance, the alkaloids trigonelline, the second most abundant compound in the samples (740.81 μgm l⁻¹), and caffeine (347.45 μgm l⁻¹), as well as caffeic acid (28.23 μgm l⁻¹). The presence of these metabolites in the investigated nanoemulsions is relevant, since CGA and its derivatives have been reported to present a series of benefits to human health, including the inhibition of cellular oxidative stress [2, 27].

A gradual release of the actives contained in F7 and F16 nanoemulsions can be visualized in curves that increase the potential to sequester DPPH radical activity over time in contact with the samples (figure 5). In addition, it can be observed that the F7 nanoemulsion has an active release up to 15 h of reaction, while for F16 this release occurs up to 24 h (figure 5). The samples differed statistically at virtually all points over time, except for the lowest nanoemulsion concentrations (0.1 to 0.5%).

F7 and F16 green coffee nanoemulsions presented increased antioxidant activity (figure 6) up to 10% concentration (v/v), where the highest values (p < 0.001) were found, reaching 100% and 97.47% for the F16 and F7 formulations, respectively. In fact, a concentration dependent behavior of both nanoemulsions was observed until the concentration of 10%, followed by a significant decrease (p < 0.001) of the total antioxidant activity at 12.5%, as also demonstrated by the DPPH method.

In a second approach in this study, and due to the claimed antimicrobial effect of green coffee biomass [28], colloidal suspensions of extract and oil green coffee were prepared and evaluated at different concentrations (0.37, 0.75, 1, 5, 3.0, 6.0, 12, 25 and 50%) against standard strains of Staphylococcus aureus ATCC 25923, Staphylococcus epidermidis ATCC 12228, and Escherichia coli ATCC 8739.

F16 nanoemulsion showed to be quite more effective than F7 one in inhibiting bacterial growth (figure 7) following a 24 h-exposure time. In fact, the former nanoemulsion was able to inhibit S. aureus and S. epidermidis growth by ≥25% at 0.75% concentration, as F7 nanoemulsion reached similar effect only at ≥6%. In their highest concentrations (25 and 50%) both nanoemulsions were able to completely inhibit the growth of those Gram-positive bacteria. Importantly, E. coli revealed to be less sensitive to treatment with the nanoemulsions investigated, requiring higher concentrations, i.e. ≥12% (F16%–58.79% growth inhibition) and 50% (F7%–82.80% growth inhibition) after 24 h of incubation (figure 7).
Minimum inhibitory concentration (MIC) was established as the concentration that provides percentual values of bacterial growth inhibition equal or greater than 80%, according to the Clinical and Laboratory Standards Institute Manual [26]. Thus, that variable was determined for the three bacterial strains of interest, being the MIC values found for the F7 and F16 nanoemulsions shown in table 2.

In addition to the data presented in figure 7, inhibition of bacterial growth was also visually confirmed by the colorimetric method using the rezasurin developer. Rezasurin is a dye used in cell viability assays that indicates the proliferation of living organisms by reducing rezasurin (purple staining) into resorufin (pink staining) in mitochondria of viable cells. In figure 8, the blue color in the wells represents absence of bacterial growth and the pink one the opposite. It can be emphasized that the MIC values found for the investigated treatments was confirmed by the colorimetric method and coincide with the concentrations presented in table 2. Besides, no antibacterial activity was detected in the negative control (figure 8, column 6), which contains only the tensoactives and solvents at the same concentrations of the F7 and F16 formulations. This result suggests that the growth inhibitory potential against microorganisms was directly related to the bioactive compounds of the nanoemulsions. Finally, by comparing the effect of the positive controls (ampicillin and penicillin, 500 mg ml\(^{-1}\) - figure 8, column 12) to the nanoemulsions investigated, one can note that the latter were as effective as those antibiotics in inhibiting the growth of the microorganisms in question.

As shown in (table 2), MIC values of 12% and 25% were found for F16 and F7 nanoemulsion, respectively, against S. aureus.

**Discussion**

There are several protocols reported for the development of nanoemulsions and although high-energy methods have been widely used in pharmaceutical and cosmetic industries, it seems to be always relevant to adopt more economically favorable and simple strategies for obtaining nanoemulsions, reducing the costs of the final
As an alternative to this question, the so-called low-energy methods have been used, taking advantage of the energy coming from the constituents of the nanoemulsion and, therefore, do not require high-demanding energy equipment \[28, 30\].

Due to their nanometric size, the nanoemulsions developed in this work might potentially penetrate through the skin layers and, consequently, exert their effects on the dermis’s deeper layers \[21, 31, 32\]. Nanoemulsions are more effective in penetrating and/or permeating the skin when compared to other systems (e.g. polymeric nanoparticles) because they are more flexible and do not contain a polymer with affinity for the stratum corneum \[33\].

TEM analysis was performed aiming at obtaining high resolution images of the oil particles dispersed in the green coffee nanoemulsions. This provides a qualitative size of nanoparticle dimensions and their distribution \[12, 13\].

Green coffee beans are reported for their antioxidant potential, due to their composition in secondary metabolites, such as phenolic compounds, able to inhibit the generation of free radicals that might damage DNA \[34\] and consequently promote cell aging \[35\]. In addition, coffee beans’ phenolics have also been described to improve the skin integrity when used topically \[36\], supporting the research and development of coffee-based products for the pharmaceutical and cosmetics industries \[28\].

The oxidation-reduction (redox) properties of phenolic compounds, such as flavonoids and phenolic acids, for example, help neutralize/stabilize free radicals, as they donate electrons to reactive species of oxygen and nitrogen (e.g.) and, therefore, prevent the development of chain oxidation reactions. The hydroxyl groups linked to the aromatic ring(s) of phenolic compounds, present both in the green coffee aqueous extracts and oil investigated, are responsible for electron donation, which in turn enhances the antioxidant efficacy as herein described. The double bond presents in the molecules favors the stabilization of the free radical by resonance, providing the electron pairing \[37\].

![Figure 6. Total antioxidant activity of F7 (a) and F16 (b) nanoemulsions over time, determined by the FRAP method. Concentrations (%) of nanoemulsions: 0.1; 0.2; 0.5; 1.0; 5.0; 10; and 12.5. Data were analyzed by one-way ANOVA, followed by the Tukey’s test (** p < 0.001). Data are represented as mean ± standard deviation (SD) of three independent replicates, performed in triplicates.](image-url)
F16 and F7 nanoemulsions revealed differences in their antioxidant activities, a finding associated to the particle size found, i.e., the former nanoemulsion having a particle size ∼60 nm larger than the later one. One can infer that this is related to the surface area of the nanoparticles, since F7 and its smaller size has a larger surface area in comparison to F16, allowing a more intensive contact with its surrounding medium and chemicals, directly reflecting the observed values of its antioxidant activity. This is accordance with previous reports [32], which state that the size of the nanoparticles in a solution increases the surface area of the nanoemulsions systems per unit mass.

In a study carried out by our research group, the antioxidant activity of the green coffee oil was determined, being related to the presence of phenolic compounds in this matrix. Interestingly, samples of green coffee oil (250 mg ml⁻¹) showed 85% antioxidant activity [30], as F7 nanoemulsion even in a quite lower concentration of coffee oil (1 mg ml⁻¹) was much more effective at sequestering the DPPH radical. This quite superior result is probably related to the nanoencapsulation of the green coffee oil’s active compounds and also due to the antioxidant synergistic effect with secondary metabolites of the aqueous extract of the green coffee paste.

In a comparative study, Agostinho (2017) formulated nanoemulsions containing green coffee oil or urucum oil and determined their antioxidant potential. Green coffee oil nanoemulsion (70%) revealed a superior antioxidant activity in respect to the urucum one (45%), when measured against the DPPH free radical at the same concentration [38, 39]. In another approach, taking into account the extracts of green coffee beans, Cheong et al (2013), measured the EC 50 of samples finding values of 9.96 and 8.23 mg of gallic acid/g samples for extracts of roasted and green coffee beans, respectively [29]. EC50 expresses the concentration required for

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**Figure 7.** Microbial growth inhibition (%) caused by F7 (a) and F16 (b) nanoemulsions after 24 h of incubation with Staphylococcus aureus (ATCC 25923), Staphylococcus epidermidis (ATCC12228), and Escherichia coli (ATCC 8739). p < 0.0001 ****, p < 0.001 ***, p < 0.01 **, according to the one-way ANOVA followed by the Tukey’s test. Data are represented as mean ± standard deviation (SD) of three independent replicates.

**Table 2.** Minimum inhibitory concentration (MIC) of F7 and F16 nanoemulsions tested against *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC12228), and *Escherichia coli* (ATCC 8739).

| Strains                  | F7 nanoemulsion | F16 nanoemulsion |
|--------------------------|-----------------|------------------|
| *Staphylococcus aureus* (ATCC 25923) | 25%             | 12%              |
| *Staphylococcus epidermidis* (ATCC12228) | 12%             | 12%              |
| *Escherichia coli* (ATCC 8739) | 50%             | 50%              |
the action of a given drug to induce 50% of its maximum effect, following the exposure time of an certain organism to the bioactive compound [40]. Our results show higher EC50 values (2.20 mg chlorogenic acid/ml nanoemulsion) for the nanoemulsion F7 than for F16 one (1.60 mg chlorogenic acid/ml nanoemulsion). In both cases, the investigated nanoemulsions revealed a quite superior performance to eliminate free radicals using smaller concentrations than those above reported. Besides, this is an additional evidence of the increased efficiency of nanoemulsions over antioxidant potential due to the small size of nanoparticles, as well as the synergistic action between the nanoemulsions’ constituents, resulting in a higher antioxidant potential comparatively to the green coffee extracts alone.

Controlled release findings of bioactive compound(s) by nanoemulsion systems is an important trait from the point of view of a cosmetic/pharmaceutical product, since it would be necessary to apply the product less often. In the case of the nanoemulsions in study, it is possible speculate that their application would be necessary only once a day, since the gradual release of antioxidant compounds lasted 24 h as shown in figure 5. Thus, it is suggested that the nanoemulsions investigated present an antioxidant effect for prolonged periods due to the nanoscale sizes of their constituents, being considered a promising product for the cosmetic and pharmaceutical industry.

It is known that the cell aging process is directly related to the oxidative stress, because as the body ages more free radicals are generated and excessively accumulated, thus accelerating aging. For this reason, the search for antioxidant compounds able to inhibit or block the action of free radical molecules has been increased [2].

Regarding antimicrobial activity, in the MIC values, F16 nanoemulsion revealed to be more toxic to S. aureus than F7 one, as similar results were found for the other two bacterial strains. The lower inhibition values for both

![Figure 8. Visual representation of the MIC for the F7 and F16 nanoemulsions tested against Staphylococcus aureus (ATCC 25923), using the resazurin revelation method. The blue color in the wells represents absence of bacterial growth as the pink one the opposite. Legend: 1 to 5 - Broth + suspensions of nanoparticles + inoculum; 6 - Broth + vehicle + inoculum; 7 - Broth + inoculum; 8 - Broth; 9 to 11 - Broth + suspensions of nanoparticles; 12 - Broth + inoculum + antibiotic (penicillin and ampicillin). Nanoemulsion concentrations (%): 0.37; 0.75; 1.5; 3; 6; 12; 25; and 50.](image-url)
nanoemulsions against \textit{E. coli} was expected due to the high structural complexity of the cell wall found in Gram-negative bacteria\cite{38}. It can be speculated that the antibacterial activity of the tested formulations is related to their green coffee oil content, where F16 has a higher amount than F7 and, consequently, was more effective against the studied bacterial strains.

Regarding the adoption of nanoemulsion containing natural products as an alternative approach to antibiotics to treat bacterial infections, the findings of this study are in accordance with the investigations of Dolgachev \textit{et al} (2016) who demonstrated that nanoemulsions of refined soybean oil reduced \textit{Pseudomonas aeruginosa} and \textit{S. aureus} infections, with decreased bacterial counts relative to the negative control \cite{41}. This is due to the fact that nanoemulsions increase the penetration of lipophilic bioactives molecules through bacterial membranes, causing an extravasation of the cytoplasm, which in turn promotes microbial lysis \cite{32}.

In addition, green coffee nanoemulsions may have acted as inhibitors of the synthesis of peptidoglycans and heteropolysaccharides that make up the cell wall of some bacterial species. Thus, nanoemulsions can eventually inhibit cell wall synthesis in gram-positive bacteria such as \textit{S. aureus} and \textit{S. epidermidis}, followed by autolysis and cell death \cite{42}. These results prove to be of great value, because in Brazil there are countless cases of aggravating and deaths caused by \textit{S. aureus} and \textit{S. epidermidis} in hospital environments, which are considered resistant to some antibiotics currently used in clinics. This emphasizes the findings of this study, where alternative treatments using natural products would be promising not only for the treatment of lesions and problems related to the skin, but also as a viable strategy to mitigate the problem of microbial resistance to antibiotics \cite{43}.

In addition, it has been reported that nanoemulsions may aid in the control of biofilms due to the targeting of particles that may adhere and cause rupture of microbial cells’ membranes. They can also help controlling antibiotic-resistant strains by making use of compounds of plant origin as here in reported \cite{44, 45}. Therefore, topical formulations, such as the nanoemulsions developed in this paper, can be an excellent therapeutic alternative, as they minimize the side effects caused by systemic treatments.

Fatty acids such as linoleic, oleic and palmitoleic have been reported to accelerate the healing of wounds. In addition, they are considered to have antimicrobial activity in the treatment of cutaneous lesions, being effective against \textit{S. aureus} and \textit{S. epidermidis} \cite{46}. Oleic acid has shown anti-inflammatory properties and can be used to treat skin wounds, inhibiting the production of reactive oxygen species \cite{47}.

Nanoemulsion systems containing vegetable oils have gained increasing interest in medicine by being biocompatible and increasing the availability of bioactive compounds. These traits aid their antimicrobial potential eventually associated to as promoters of cell membrane rupture of Gram-positive and Gram-negative microorganisms and fungi \cite{45}. Besides, the use of nanoemulsions as antimicrobial agents has increased because of their interaction with the lipids constituents of microbial membranes. This is because nanoemulsions seem to be able to fuse with pathogens’ cell membrane, gradually releasing their active compounds and causing cell lysis and death \cite{45}. In this sense, the antibacterial activity of the nanoemulsions demonstrated in this study were superior and much more significant than those reported by Wagemaker \textit{et al} (2012) who also tested formulations containing green coffee oil, without finding satisfactory results \cite{48, 49}. The results of this work contrast the lack of antibacterial activity found by those authors and emphasize the probable synergistic effect between the oil constituents and green coffee aqueous extract in the formulations tested.

The findings here in described are thought to be relevant to the human health, because, for instance, \textit{S. epidermidis} is a well known pathogenic bacterium belonging to the skin’s microbiota and strongly related to acne problems \cite{49}. In its turn, \textit{S. aureus} is considered the predominant bacterium species in episodes of both cutaneous and systemic infections. Finally, \textit{E. coli} has been reported to cause a number of serious enteric infections that can result in high death rates, especially in children \cite{50}. This scenario underscores the importance of the formulations here in developed for cosmetic and therapeutic purposes.

It is possible to infer that nanotechnology has aided in the development of new systems able to overcome some physicochemical limitations observed in certain formulations, such as the low solubility of oils in aqueous medium, incorporating them in a stable form, and thus being able to exploit the potential of their assets and also improving their transportation through biological membranes, leading to an increased biological action due to their very small size \cite{32, 44}. Thus, the green coffee nanoemulsions here in investigated showed to be potent antioxidants, as well as antimicrobial agents, being rather more effective against \textit{S. aureus} and \textit{S. epidermidis} in comparison to \textit{E. coli}, demonstrating their potential of application not only for cosmetics products, but also in the medical and pharmaceutical areas. However, further investigations should be performed with preclinical studies to confirm these findings.

**Conclusions**

In summary, it can be concluded that green coffee nanoemulsions were successfully prepared by the spontaneous emulsification method, having a spherical shape and sizes considered suitable for topical

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applications. Their antioxidant activity was demonstrated with excellent scavenging activity against the DPPH free stable radical and also through the FRAP method (between 90 and 100%, respectively). F7 and F16 nanoemulsions also proved to be very effective against S. aureus and S. epidermidis, reaching bacterial growth inhibition values between 85 and 100%. In addition, at higher concentrations of nanoemulsions (50%) they were able to inhibit the growth of E. coli by 88.79%. Therefore, the nanoemulsions developed in this work can be considered as promising antioxidant and antimicrobial agents for applications in the cosmetic and pharmaceutical industries.

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Declaration of competing interest

The authors state no conflict of interest. In addition, the authors declare the availability of the data for publication on Nano Express.

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