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LIPID NANOCARRIER CONTAINING OLIVE OIL AND OLIVE BUTTER: AN APPROACH FOR BIOACTIVE COMPOUND DELIVERY SYSTEMS[1]

NANOCARREADOR LIPÍDICO CONTENDO MANTEIGA E AZEITE DE OLIVA: UMA ABORDAGEM PARA SISTEMAS DE LIBERAÇÃO DE COMPOSTOS BIOATIVOS

Larissa da Silva Silveira[2], Mateus Cláudio Zinhani[2], Elisandra da Silva Pena da Fonseca[2], Carolina Rapachi Fortes[2], Aline Rossato[2], Walter Paixao de Souza Filho[2], Pedro Henrique Fortes Guerim[3], Patrícia Kolling Marquezan[3], Franciane Batista Nunes[4], Roberto Christ Vianna Santos[4], Cristiano Augusto Ballus[5], Carla Andressa Almeida Farias[5], Luiz Fernando Rodrigues Júnior[2], Liana da Silva Fernandes[2] e Michele Rorato Sagrillo[2]

ABSTRACT

Olea europaea L., known as the olive tree, belongs to the family Oleaceae and is the only species of this family that produces edible fruits, olives. Olive oil, extracted from these fruits, has been widely used since antiquity due to its antioxidant, moisturizing, and soothing properties, standing out as a promising ingredient in cosmetic and therapeutic industries. However, the incorporation and cutaneous absorption of its antioxidant compounds represent a challenge, since many of them are lipophilic, unstable, and susceptible to degradation by light and oxygen, which limits their use in topical formulations. To overcome such limitations, research and industry have invested in the development of lipid-based nanocarriers, capable of increasing the solubility, stability, and bioavailability of bioactive compounds, while protecting them against hydrolysis and oxidation. This study aimed to prepare a butter containing olive oil and incorporate it, together with the oil itself, into lipid nanocarriers. Stability, biocompatibility, and antimicrobial activity analyses of the obtained systems were performed, in addition to phenolic characterization and evaluation of the antioxidant potential of the oil in its free form. The results demonstrated that olive oil has a high concentration of phenolic compounds, with expressive therapeutic potential. Both the butter and the nanocarriers developed proved to be stable and safe, with no mortality or cell damage, although the nanocarriers did not exhibit antimicrobial activity against the bacterial strains tested. It was concluded that the association between olive oil and lipid nanocarriers is promising for cosmetic applications and topical delivery, offering stability, safety, and functional potential in skin care and protection.

Keywords: *Olea europaea* L.; Sustainability; Nanobiotechnology.

1 Part of the doctoral thesis presented to the Graduate Program in Nanosciences, Universidade Franciscana - UFN, Santa Maria, RS, Brazil.

2 Graduate Program in Nanosciences, Universidade Franciscana - UFN, Santa Maria, RS, Brazil. Emails: larissa.silveira@ufn.edu.br; mateus.zinhani@ufn.edu.br; elisandrapena@ufn.edu.br; carolinarapachi@ufn.edu.br; aline.rossato@ufn.edu.br; luiz.fernando@ufn.edu.br; liana@ufn.edu.br; sagrillomr@ufn.edu.br. ORCID: https://orcid.org/0000-0003-4460-9857; https://orcid.org/0000-0003-1829-7546; https://orcid.org/0009-0007-1025-699X; https://orcid.org/0009-0001-2356-025X; https://orcid.org/0000-0001-6464-6255; https://orcid.org/0000-0002-5753-5503; https://orcid.org/0000-0003-4745-7617; https://orcid.org/0000-0001-5659-159X

3 Laboratory for Research in Oral Microbiology (LAPEMICRO) - Universidade Federal de Santa Maria, RS, Brazil. Emails: phguerim@gmail.com; patrícia.marquezan@ufsm.br. ORCID: https://orcid.org/0000-0003-1163-9451; https://orcid.org/0000-0001-5061-6039

4 Graduate Program in Pharmaceutical Sciences, Universidade Federal de Santa Maria, RS, Brazil. Emails: batistanunes-franciane@gmail.com; robertochrist@gmail.com. ORCID: https://orcid.org/0009-0002-5700-1875; https://orcid.org/0000-0002-0533-3483

5 Department of Food Science and Technology, Center of Rural Sciences, Federal University of Santa Maria (UFSM), Santa Maria, Brazil. Emails: cristiano.ballus@ufsm.br; carla.farias@acad.ufsm.br. ORCID: https://orcid.org/0000-0002-4439-1088; https://orcid.org/0000-0002-1905-2703

RESUMO

A Olea europaea L., conhecida como oliveira, pertence à família Oleaceae e é a única espécie dessa família que produz frutos comestíveis, as azeitonas. O azeite de oliva, extraído desses frutos, é amplamente utilizado desde a antiguidade devido às suas propriedades antioxidantes, hidratantes e calmantes, destacando-se como um ingrediente promissor nas indústrias cosmética e terapêutica. Entretanto, a incorporação e a absorção cutânea de seus compostos antioxidantes representam um desafio, uma vez que muitos deles são lipofílicos, instáveis e suscetíveis à degradação por luz e oxigênio, o que limita seu uso em formulações tópicas. Para contornar tais limitações, pesquisas e indústrias têm investido em no desenvolvimento de nanocarreadores lipídicos, capazes de aumentar a solubilidade, estabilidade e a biodisponibilidade dos compostos bioativos, além de protegê-los contra hidrólise e oxidação. Este estudo teve como objetivo preparar uma manteiga contento azeite de oliva e incorporá-la, juntamente com o próprio azeite, em nanocarreadores lipídicos. Foram realizadas análises de estabilidade, biocompatibilidade e atividade antimicrobiana dos sistemas obtidos, além da caracterização fenólica e avaliação do potencial antioxidante do azeite na sua forma livre. Os resultados demonstraram que o azeite apresentou alta concentração de compostos fenólicos, com expressivo potencial terapêutico. Tanto a manteiga quanto os nanocarreadores desenvolvidos demostraram ser estáveis e seguros, sem causar mortalidade ou dano celular, embora os nanocarreadores não tenham exibido atividade antimicrobiana frente às cepas testadas. Conclui-se que a associação entre azeite e manteiga de oliva em nanocarreadores lipídicos é promissora para aplicações cosméticas e terapêuticas, oferecendo estabilidade, segurança e potencial funcional no cuidado e proteção da pele.

Palavras-chave: *Olea europaea L.; Sustentabilidade; Nanobiotecnologia.*

1 INTRODUCTION

Olea europaea L., commonly known as the olive tree, is an ancient species native to the Mediterranean region. From its fruits, the olives, olive oil is extracted wich is one of the most important and valued vegetable oils worldwide, both for its culinary use and for its therapeutic potential, attributed to its diverse medicinal properties. Due to its chemical matrix rich in phenolic compounds, its products and by-products are recognized as potent antioxidant agents, offering numerous health benefits such as cardioprotective, immunoregulatory, and metabolic syndrome-preventive effects, among others (Almeida *et al*., 2021; González-Rodríguez *et al.,* 2023; Pennisi *et al.,* 2023; Pérez *et al.,* 2025).

According to studies conducted by Cizmarova (2022) and Garcia and collaborators (2023), clinical and epidemiological research has demonstrated that the regular inclusion of olive oil in the diet is strongly associated with the prevention of chronic non-communicable diseases, particularly cardiovascular and metabolic disorders. Furthermore, monounsaturated fatty acids, together with phenolic compounds, contribute significantly to the reduction of total serum cholesterol and LDL levels, the increase of HDL, and the improvement of insulin resistance. These properties provide cardioprotective effects and play a fundamental role in reducing the risk of metabolic syndrome, type II diabetes, and atherosclerosis.

In addition to the major health benefits that olive oil provides through therapeutic medicine, it has been increasingly attracting the interest of the pharmaceutical and cosmetic industries due to its well-known antioxidant, antimicrobial, and healing properties. Studies conducted by Rodrigues (2024) and Albini (2025).

investigated the potential of olive oil in the formulation of nutraceuticals, dermocosmetics, and nanostructured systems, and the results demonstrated its ability to deliver phenolic compounds in a more stable and effective manner.

These findings further confirmed that olive oil transcends its traditional culinary use thus establishing itself as a versatile raw material in the promotion of health and in the development of new therapeutic strategies (Furtado *et al*., 2023). Currently, the state of Rio Grande do Sul has experienced significant growth in the practice of olive cultivation. In the study conducted by Saueressig *et al.* (2019), it became evident that between 2005 and 2018, a robust productive chain was established in the state, supported by favorable soil characteristics, climatic conditions, and an institutional and organizational environment that encouraged investment in this sector.

Governmental data confirm this trend: according to the State Program for the Development of Olive Growing (Pró-Oliva), in 2017, 57,873 liters of olive oil were produced, while in 2022, production reached 448.5 thousand liters. The 2022/2023 harvest in Rio Grande do Sul registered a 29% increase compared to the previous season, rising from 448.5 thousand liters to 580.2 thousand liters, reaffirming the state as Brazil's largest olive oil producer (Pró-Oliva, 2023). Currently, 340 producers cultivate olive trees across 6,200 hectares in the state. The area in productive age (four years or older) covers approximately 4,300 hectares, with the municipalities of Encruzilhada do Sul, Pinheiro Machado, Canguçu, Caçapava do Sul, São Sepé, Cachoeira do Sul, Santana do Livramento, Bagé, São Gabriel, Viamão, and Sentinela do Sul representing the main producing regions. The most commonly cultivated olive varieties in the state are Arbequina, Koroneiki, Picual, Arbosana, and Frantoio. Among these, the Spanish variety Arbequina and the Greek variety Koroneiki stand out, being present in 96% and 90% of the olive groves, respectively (SEAPI, 2022).

Thus, considering the great functional and therapeutic potential present in the derivatives of *Olea europaea* L., and the growing need for biotechnological solutions that combine the use of bioactive compounds, sustainability, circular bioeconomy, and social entrepreneurship, the present study aims to develop a butter containing olive oil to be used alongside pure olive oil in the formulation of lipid nanocarriers, and subsequently perform biocompatibility, stability, antimicrobial activity, phenolic analysis on olive oil in its free form.

The choice of olive oil and its butter as structural components of the system not only enhances the value of a natural resource of high nutritional and bioactive quality but also aligns with a sustainability and circular economy perspective, by encouraging the full utilization of olive production and the reduction of waste. In this way, the research contributes to the advancement of innovative

strategies in controlled-release systems, promoting value addition to the olive oil production chain and strengthening sustainable practices within the agro-industrial sector.

Additionally, this research aligns with the Sustainable Development Goals (SDGs) of the 2030 Agenda. By developing a bioactive delivery system derived from a natural resource, the study contributes to SDG 3 (Good Health and Well-Being), aiming at therapeutic and cosmetic applications. The approach also supports SDG 12 (Responsible Consumption and Production) by enhancing the olive-growing production chain within a framework of sustainability and circular bioeconomy. Finally, through the use of nanobiotechnology to design innovative formulations, the research advances SDG 9 (Industry, Innovation, and Infrastructure), adding value to agricultural products (ONU, 2015).

2 METHODOLOGY

The olive oil used in this study was kindly provided by the company Olivas da Campanha Don José, a family-owned enterprise located in Caçapava do Sul. The product consists of a *blend* of different cultivars: Arbequina, Arbosana, and Koroneiki.

2.1 OLIVE OIL CHARACTERIZATION

To identify the phenolic compounds present in the olive oil, the characterization method described by Ballus and collaborators (2015) was employed.

In 15 mL Falcon tubes, 2.5 g of the sample was weighed, and 5 mL of P.A. hexane was added. The mixture was then agitated for 1 minute. Subsequently, 5 mL of methanol:water solution (60:40, v/v) was added, followed by agitation for 2 minutes. After agitation, the solution was centrifuged for 10 minutes at 3,500 rpm (2422 ×g).

After centrifugation, the upper phase (olive oil/hexane) was discarded, and the lower phase (methanol/water), containing the phenolic compounds, was carefully transferred to a 25 mL round-bottom flask and subjected to a rotary evaporator at 38 °C until complete solvent evaporation. The residue adhering to the walls of the flask was then resuspended in 500 **µ**L of a fresh methanol:water solution (50:50, v/v). Finally, the sample was filtered through a 0.22 **µ**m membrane filter (PVDF or PTFE) and stored until chromatographic analysis.

Chromatographic analyses of the phenolic extracts were performed using an Agilent 1200 *Series Rapid Resolution* LC system (RRLC; Agilent Technologies, Waldbronn, Germany), equipped with a vacuum degasser, binary pump, and autosampler. Separation was carried out on a Zorbax Eclipse Plus C18 column (150 mm × 4.6 mm i.d.; 1.8 **µ**m; Agilent Technologies, Santa Clara, USA), using acidified water with 0.25% acetic acid (eluent A) and methanol (eluent B) as the mobile phases in a linear gradient: 0 min, 5% B; 7 min, 35% B; 12 min, 45% B; 17 min, 50% B; 22 min, 60% B;

25 min, 95% B; 27 min, 5% B, followed by a 5 min conditioning cycle. The flow rate was maintained at 0.8 mL min^{-1}, the column temperature at 25 °C, and the injection volume at 10 μL.

For compound detection, the sample was analyzed using a micrOTOF™ mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with an electrospray ionization (ESI) source operating in negative mode. Interface parameters were set as follows: capillary voltage, +4 kV; drying gas temperature, 190 °C; drying gas flow, 9 L min^{-1}; nebulizer pressure, 2 bar; and end plate offset, -0.5 kV. Ion transfer parameters were set to: capillary exit voltage, -120 V; skimmer 1, -40 V; hexapole 1, -23 V; hexapole RF, 50 Vpp; and skimmer 2, -22.5 V. Spectra were acquired over the m/z range 50-1000. External calibration was performed using a sodium acetate cluster solution (5 mM) in water:isopropanol 1:1, v/v containing 0.2% acetic acid, ensuring high accuracy in molecular mass determination.

2.2 PRODUCTION OF BUTTER FROM OLIVE OIL

The olive oil butter was produced using a technique described by Leonardi (2008), which is based on the emulsification of the lipid phase. In a beaker, 9 g of stearic acid and 7.5 g of cetyl alcohol were added and subjected to heating in a water bath (25 °C) until complete homogenization and formation of a solid lipid phase. After this step, 33.5 g of olive oil was

incorporated into the mixture under continuous and vigorous manual stirring until a fully homogeneous butter-like consistency was achieved.

To evaluate the stability of the butter, pH and macroscopic changes were monitored in aliquots stored under different climatic conditions, allowing verification of the maintenance of physicochemical properties and the emulsion's resistance to environmental variations.

2.3 PREPARATION AND CHARACTERIZATION OF SOLID LIPID NANOPARTICLES CONTAINING OLIVE OIL BUTTER AND OLIVE OIL

The lipid nanocarriers were developed based on pre-formulation tests using an Ultra Turrax® device, employing a high-speed homogenization technique to ensure the production of small, uniformly distributed particles. The final formulation consisted of olive oil butter and olive oil, combining the solid lipid phase of the butter with the liquid phase of the oil, resulting in a stable lipid matrix suitable for the encapsulation of bioactive compounds. Tween® 60 and Span® 60 surfactants were selected due to their appropriate hydrophilic lipophilic balance (HLB), which favors the formation of stable nanoemulsions compatible with the proposed lipid system. This method allowed the production of homogeneous nanocarriers with the potential to improve solubility, stability, and bioavailability of the incorporated bioactive compounds.

First, the materials were weighed into two separate beakers: one corresponding to the oil phase and the other to the aqueous phase. The aqueous phase consisted of Polysorbate 60 (Tween®) and MilliQ® water, while the oil phase was composed of a solid lipid (olive butter), a liquid lipid (olive oil), and sorbitan monostearate (Span® 60). Both beakers were then subjected to magnetic stirring for 15 minutes in a water bath at approximately 50 °C. After complete solubilization of both phases, the aqueous phase was homogenized using the Ultra Turrax® at 10,000 RPM for 5 minutes. At the end of this process, the oil phase was carefully added dropwise over the aqueous phase using a glass syringe, followed by an increase in rotation speed to 17,000 RPM, which was maintained for 40 minutes.

2.3.1 Formulation Characterization and Stability Analysis

The characterization of the nanoformulations was carried out by evaluating physical and chemical parameters, including pH, polydispersity index (PDI), particle size, and zeta potential (ZP). The stability of the formulations was monitored over a 90-day period under different storage conditions: *(i)* transparent glass container exposed to light, *(ii)* amber glass container protected from light, *(iii)* amber glass container in an oven at approximately 40 °C, and *(iv)* amber glass container stored in a refrigerator at -4 °C. Samples were periodically characterized every 15 or 30 days, allowing the monitoring of changes in physical and chemical properties over time and the assessment of the resistance of the nanoformulations to different environmental conditions.

2.3.2 Determination of Particle Diameter and Polydispersity Index (PDI)

The particle diameter and polydispersity index (PDI) of the nanoparticles were determined using dynamic light scattering (DLS) with a Zetasizer® nano-ZS (model ZEN 3600, Malvern), after diluting the dispersions in water (1:500, v/v). Results were expressed in nanometers (nm) and represent the average of three independent suspensions.

2.3.3 Zeta Potential

The zeta potential was measured using electrophoresis with the same Zetasizer® nano-ZS (model ZEN 3600, Malvern), after diluting the dispersions in 10 mM NaCl solution (1:500, v/v), which was previously filtered through a 0.45 μm membrane. Results were expressed in millivolts (mV) and correspond to the average of three independent suspension measurements.

2.3.4 pH Determination

The pH was measured using a properly calibrated potentiometer (DM-22, Digimed®), and all analyses were performed in triplicate.

2.4 IN VITRO BIOCOMPATIBILITY ANALYSIS OF LIPID CARRIERS

Peripheral blood mononuclear cells (PBMCs) derived from discarded whole blood samples from healthy adult donors were obtained from the Clinical Analysis Laboratory (LEAC-UFN) at Universidade Franciscana (experimental protocol approved by the UFN Human Research Ethics Committee, CAAE number: 44940821.3.0000.5306) with no identifiable donor information.

Blood samples were processed to isolate PBMCs using a density gradient separation procedure with Ficoll Histopaque-1077VR reagent (Sigma-Aldrich). After mixing the blood with the reagent at a 1:1 ratio (v/v), samples were centrifuged for 30 minutes at 1,500 rpm. The isolated PBMCs were plated in 96-well plates containing RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum and 1% antibiotics, at a cell concentration of 2×10^5 cells mL^{-1} per well. Cells were then exposed to different concentrations of each tested compound (30-7.5 mg/mL) according to the efficacy protocol described in this study, to assess their effects on cellular modulation through various colorimetric and fluorometric assays.

All treatments and assays were performed in triplicate to ensure statistical reliability. Hydrogen peroxide (H_2O_2, 200 μM) was used as a positive control for all tests, except for nitric oxide assays, where sodium nitroprusside (50 μM) served as the positive control.

2.4.1 MTT Reduction Assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

To evaluate cell viability and proliferation, the MTT assay was performed, following the instructions of Kang *et al*. (2010) and the method of Mosmann (1983). This colorimetric assay uses the MTT reagent, a water-soluble yellow tetrazolium salt that enters cells and is metabolized by mitochondrial enzymes in viable cells, forming insoluble violet formazan crystals that accumulate in the cytoplasm. These crystals are then solubilized with DMSO (dimethyl sulfoxide). After treatments, cells were incubated for 24 hours to assess viability and 48 hours for proliferation, in 96-well plates containing test groups, negative controls, and positive controls. Plates were maintained in an incubator at 37 °C with 5% CO_2 for 2 hours, after which 20 μL of MTT solution (0.01 mol L^{-1}, pH 7.4, 5 mg mL^{-1} in 1X phosphate-buffered saline, PBS) was added. Plates were protected from light, homogenized on a shaker, and incubated for an additional 4 hours. After incubation, the supernatant

was removed, and cells were resuspended in 200 µL of DMSO. Absorbance was measured using an Anthos 2010 plate reader at 570 nm, and the cytotoxic potential was calculated relative to the negative control (cells in culture medium).

2.4.2 Dichlorofluorescein Diacetate (DCFH-DA) Assay

To assess the total reactive oxygen species (ROS) levels, the dichlorofluorescein diacetate (DCFH-DA) reagent was used, following adaptations from the literature (Esposti *et al*., 2002). This reagent is capable of crossing the cell membrane and is deacetylated by mitochondrial enzymes to form 2',7'-dichlorodihydrofluorescein, which reacts with reactive oxygen species (ROS), primarily hydrogen peroxide (H_2O_2), producing 2',7'-dichlorofluorescein, a fluorescent compound. Fluorescence was then measured using a spectrofluorimeter, with excitation at 488 nm and emission at 525 nm.

2.4.3 Fluorometric DNA Quantification Assay Using DNA-PicoGreen®

To complement the assessment of cell viability and genotoxic potential, a fluorometric assay was performed to quantify free DNA in the culture medium using the DNA-PicoGreen® reagent (Invitrogen, Life Technologies). This reagent is a fluorescent dye that binds to double-stranded DNA (dsDNA). The assay was conducted in the culture medium of treated cells to determine the presence of dsDNA released due to potential cell lysis and death. The dye was added to the samples in dark 96-well ELISA plates, followed by 5 minutes of incubation. Fluorescence was measured using a spectrofluorimeter at excitation 480 nm and emission 520 nm, according to Sagrillo *et al*. (2015).

2.4.4 Nitric Oxide Assay Protocol

The nitric oxide (NO) assay detects the presence of organic nitrite in the sample. Nitrite is identified by the formation of a pink color when the Griess reagent is added to the sample containing NO^-. The sulfanilamide component of the Griess reagent reacts with nitrite to form a diazonium salt. When the azo compound (N-1-naphthylendiamine dihydrochloride) interacts with the diazonium salt, a pink coloration develops in the sample.

For the assay, 100 µL of cell culture supernatant was pipetted into each well of a 96-well plate, followed by the addition of 100 µL of Griess reagent. The plate was incubated at room temperature for 15 minutes, and absorbance was measured at 540 nm using a spectrophotometer, according to Choi *et al*. (2012).

2.5 EVALUATION OF THE ANTIMICROBIAL ACTIVITY OF LIPID CARRIERS AND BIOACTIVE COMPOUNDS

To evaluate the antimicrobial activity of each formulation, the efficacy of nanoencapsulated oils was compared with the results of the same oils in their free form using Minimum Inhibitory Concentration (MIC) and antibiofilm assays against clinically and hospital-relevant strains.

2.5.1 Determination of Minimum Inhibitory Concentration (MIC)

Bacterial samples were cultured on Mueller-Hinton (MH) agar, and individual colonies were inoculated into 5 mL of sterile saline. The absorbance was adjusted to achieve a 0.5 McFarland standard (1×10^6 to 5×10^6 cells/mL). Subsequently, a 1:10 dilution in MH broth was performed, resulting in a final concentration of 10^4 cells/mL. For the MIC assay, the inoculum was applied to 96-well polystyrene plates. All tests were performed in triplicate. 100 µL of MH broth was added to wells 1 through 12. Then, 100 µL of the working solution was added to the first well, followed by serial dilutions across wells 2 to 12, corresponding to the logarithmic concentration curve. Next, 10 µL of the standardized bacterial inoculum was added to each well. Plates were incubated at 37 °C for 48 hours. MIC values were determined by the addition of 20 µL of 1% 2,3,5-triphenyltetrazolium chloride (TTC, Vetec®), which facilitates visualization of bacterial growth.

2.5.2 Determination of Minimum Bactericidal Concentration (MBC)

The Minimum Bactericidal Concentration (MBC) assay was performed after the MIC determination. Samples from the MIC wells were streaked onto Mueller-Hinton (MH) agar plates and incubated for 24 hours at 37 °C. The MBC was defined as the lowest concentration at which no bacterial growth was observed on the plates.

2.5.3 Determination of Antibiofilm Activity

Bacterial inocula were prepared by subculturing the species on sterile Mueller Hinton agar plates and incubating at 35 ºC for 24 hours. Colonies were suspended in sterile 0.85% saline. Fungal inocula were prepared similarly on Sabouraud Dextrose agar plates. Cell density was adjusted spectrophotometrically to 0.5 McFarland (1×10^6-5×10^6 cells/mL) at 630 nm. In sterile 96-well polystyrene plates, 180 µL of Brain Heart Infusion (BHI) broth supplemented with 1% glucose was added per well, followed by 20 µL of the microbial inoculum and 50 µL of the Solid Lipid Nanoparticle solution to evaluate antibiofilm activity. Negative controls contained only BHI with 1% glucose, and positive

controls included the inoculum in the growth medium. Plates were incubated at 35 ºC for 24 hours (bacteria) or 48 hours (fungi).

After incubation, wells were emptied and washed three times with 200 µL of sterile phosphate-buffered saline (PBS, pH 7.2) to remove non-adherent cells while preserving biofilm integrity. Biofilms were fixed with 150 µL methanol for 20 minutes, plates emptied, and air-dried upside down. Adherent biofilms were stained with 150 µL of 2% Hucker's crystal violet for 15 minutes at room temperature, rinsed with water, and air-dried. Bound dye was resolubilized with 150 µL of 95% ethanol per well, added slowly, and plates were left at room temperature for at least 30 minutes. Ethanol allows indirect quantification of cells attached to both the well bottom and walls. Biofilm formation was quantified by measuring absorbance at 570 nm (Stepanovic *et al*., 2007).

3 RESULTS AND DISCUSSION

3.1 OLIVE OIL CHARACTERIZATION

The phenolic characterization of olive oils is one of the most frequently analyzed parameters in scientific research, as it provides robust data on quality, stability, and predictive indicators directly related to the health-promoting properties of each bioactive compound (Crizel *et al*., 2020; Cizmarova *et al.,* 2022). As shown in Table 1, the evaluation of the phenolic composition of olive oil revealed a wide variety of bioactive compounds, with marked differences in their concentrations and good analytical reproducibility, as demonstrated by the coefficients of variation (CV%).

Table 1 - Phenolic Composition of Olive Oil.

Phenolic Composition	Molecular Formula	Mean (mg/kg)	Standard Deviation (mg/kg)	CV (%)
Oleacein	$C_{25}H_{32}O_{13}$	5,0000	0,400000	7,600
Oleocanthal	$C_{17}H_{20}O_6$	3,3000	0,300000	9,380
Tyrosol	$C_8H_{10}O_2$	1,9000	0,000000	5,850
Oleuropein Aglycone	$C_{19}H_{22}O_8$	1,8000	0,100000	6,060
Luteolin	$C_{15}H_{10}O_6$	1,3000	0,100000	9,780
Pinoresinol	$C_{20}H_{22}O_6$	0,9300	0,040000	4,280
Hydroxytyrosol	$C_8H_{10}O_3$	0,5200	0,040000	8,160
Apigenin	$C_{15}H_{10}O_5$	0,1900	0,010000	5,090
Vanillic Acid	$C_8H_8O_4$	0,1000	0,010000	5,580
p-Coumaric Acid	$C_9H_8O_3$	0,0420	0,003000	6,630
Vanillin	$C_8H_8O_3$	0,0160	0,002000	14,900
Protocatechuic Acid	$C_7H_6O_4$	0,0060	0,000000	3,040
Ferulic Acid	$C_{10}H_{10}O_4$	0,0047	0,000100	3,210
Caffeic Acid	$C_9H_8O_4$	0,0021	0,000200	10,060

Author: Prepared by the author.

Among the compounds identified in the sample, the most prominent were oleacein (5.0 mg/kg) and oleocanthal (3.3 mg/kg), both belonging to the secoiridoid class and derivatives of oleuropein. Studies by Omar (2010) and Cicerale *et al*. (2012) emphasize the importance of these compounds as anti-inflammatory and antioxidant agents, with neuroprotective and cardioprotective effects, highlighting not only the nutritional but also the therapeutic value of olive oil.

Crizel *et al*. (2022) reported similar findings, where the major compounds were oleocanthal and oleacein in olive oil samples cultivated in Pinheiro Machado, Rio Grande do Sul. They noted that olive maturation, as well as the latitude and climate of the cultivation area, directly influence the levels of these bioactives. Three other components present at significant concentrations were tyrosol and oleuropein aglycone. Several studies describe these compounds as promoters of oxidative stability in olive oils and contributors to cardioprotection in in vivo studies (Visioli *et al*., 2002; Servili *et al.,* 2004). Within the flavonoid group, significant amounts of luteolin and apigenin were observed, which are recognized for their ability to neutralize free radicals and modulate inflammatory pathways (Obied *et al*., 2005).

Another active compound identified was hydroxytyrosol, which results from the hydrolysis of oleuropein during olive maturation, processing, or storage of the oil. This compound has been extensively studied due to its high antioxidant capacity and beneficial effects in preventing the oxidation of low-density lipoproteins (LDL), a risk factor for cardiovascular diseases (Manna *et al*., 1997; EFSA, 2011).

Phenolic acids were detected at lower concentrations. Vanillic acid (0.10 mg/kg), p-coumaric acid (0.042 mg/kg), and ferulic acid (0.0047 mg/kg) were the most abundant in this group. Despite their low concentrations, these acids contribute significantly to the antioxidant activity of olive oil (Medina *et al*., 2006). Caffeic acid showed the lowest content, at only 0.0021 mg/kg. The data reflect a phenolic profile consistent with high-quality olive oils, dominated by oleuropein-derived phenols, simple phenolic alcohols, and flavonoids. It is noteworthy that olive oil phenolic composition can vary widely due to agronomic and technological factors, including olive cultivar, fruit ripeness, climatic conditions, and extraction and storage methods (Bendini *et al.,* 2007; Servili *et al*., 2009).

3.2 PRODUCTION OF BUTTER FROM OLIVE OIL

A butter product was prepared using olive oil, resulting in a solid, homogeneous material with a light and uniform color. This finding is consistent with the study by Fernandes (2013), who also prepared butter from olive oil. To assess the stability of the butter based on physical parameters, aliquots of the product were separated and stored under different conditions: a climatic chamber at ±40 °C (oven), exposed to light at room temperature (light), protected from light at room temperature (dark), and in a refrigerator at -4 °C. The samples were maintained under these conditions for a period of 120 days.

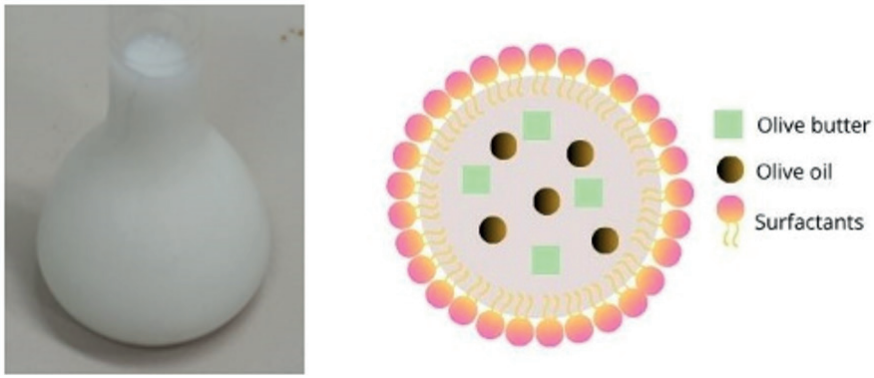
During the first 30 days, the only sample that exhibited changes was the one stored in the climatic chamber. This sample showed a darker coloration and a consistency that was no longer normal, becoming more liquid. The other samples showed no alterations in color or phase separation. After 60 days, the samples stored in the refrigerator and at room temperature, both in light and dark conditions, remained unchanged, without sedimentation or phase separation. The sample in the climatic chamber, however, became even more liquid than before, although its color was slightly lighter compared to the previous evaluation.

After 120 days, the only samples that remained unchanged were those stored in the refrigerator and at room temperature protected from light. The sample in the climatic chamber, which had been altering since the first 30 days, was now completely liquid with a very pale color. The samples stored at room temperature under light exposure remained solid but had turned completely white, indicating degradation of components. Thus, the best conditions for maintaining the stability of the butter without causing changes are refrigeration, which preserves its components, and room temperature storage protected from light, suggesting the use of amber packaging.

3.3 PREPARATION AND CHARACTERIZATION OF NANOFORMULATIONS

The lipid nanocarriers were prepared using an Ultra Turrax® device via the high-speed homogenization technique. They exhibited a milky appearance with a white-blue coloration and low viscosity (Figure 1). The formulation showed no signs of macroscopic instability, such as clumping, phase separation, or color changes, throughout the entire testing period.

Figure 1 - Solid Lipid Nanocarrier and schematic of its constituent distribution.



Source: Prepared by the author.

Confirming the presence of nanometric particles in the formulation, the Tyndall effect can be observed a parameter frequently used for the qualitative evaluation of nanometric formulations. This effect is characterized by the scattering of light as it passes through a colloidal or nanoparticulate suspension. Observation of the Tyndall effect indicates the presence of particles of sufficient size to scatter light, typically in the range of 1 to 500 nm.

This phenomenon can be easily demonstrated by directing a light beam through the sample, allowing the assessment of the homogeneity of the dispersion and colloidal stability, where particles are large enough to scatter light but small enough to remain suspended. Moreover, analyzing this effect also serves as a preliminary indication that the particles remain individualized and have not aggregated, which is essential for maintaining the functional properties and efficacy of nanocarriers in pharmaceutical, cosmetic, and food applications (Suar, 2025; Suryavanshi *et al*., 2025).

3.3.1 Formulation Characterization and Stability Analysis

The stability of the formulations was evaluated over 60 days under different storage conditions: transparent glass container exposed to light, amber glass container protected from light, amber glass container in a climatic chamber at ±40 °C, and amber glass container in a refrigerator at -4 °C. The characterization of the nanoformulations was performed using parameters such as pH, polydispersity index (PDI), particle size, zeta potential (ZP), and macroscopic analysis for sedimentation and phase separation.

As shown in Table 2, the lipid nanocarrier composed of butter and olive oil (NCL-MA) exhibited a monodisperse particle distribution with a mean PDI of 0.302, which is within the ideal range (<0.4) according to the literature (Machado *et al*., 2019). The mean particle size was approximately 90.79 nm, and the zeta potential value was -10.82 mV.

Table 2 - Observed Parameters in the Characterization of Lipid Nanocarriers.

PDI	Size (nm)	Pz (mV)	pH
0,302	90,79	-10,82	4-5

Author: Prepared by the author.

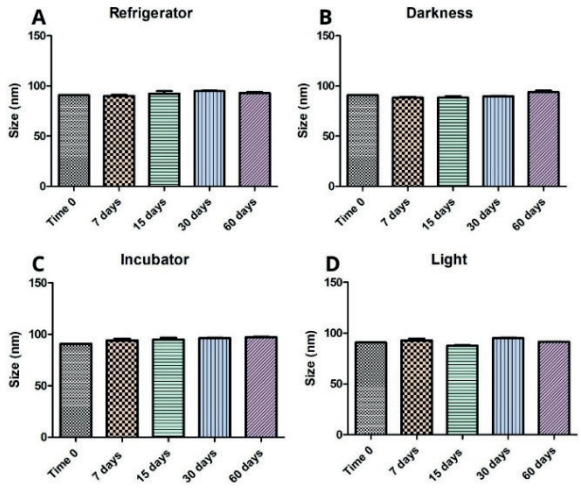
The evaluation of nanoparticle size and distribution represents a key criterion in assessing formulation quality, while zeta potential and pH serve as indicators of particle stability. Studies have been conducted to investigate the main factors influencing the size of nanostructures, highlighting that formulation composition and preparation method are significant determinants in this context. In addition to being a fundamental parameter for stability analysis, determining and monitoring variations in particle size distribution serve as indicators of potential aggregation and sedimentation tendencies (Pedrozo, 2023; Coelho, 2024).

3.3.1.1 Determination of Particle Size

As shown in Figure 2, the nanoparticle size in formulations stored under light exposure, protected from light, in the oven, and in the refrigerator followed a consistent pattern, with no significant changes in size compared to time 0, when the mean size was 90.79 nm.

The analysis of nanoparticle stability under different storage conditions highlights that factors such as light, temperature, and environment are crucial for preserving their properties. Studies have shown that light exposure can induce photochemical reactions that lead to nanoparticle aggregation or degradation, as demonstrated by Zhang et al. (2018) in gold nanoparticles. Temperature also plays a significant role, as nanoparticles stored at elevated temperatures exhibit higher rates of aggregation, as observed by Liu et al. (2019). Furthermore, Chen et al. (2020) indicated that nanoparticles in solutions with controlled pH maintain their characteristics for longer periods. These findings support the conclusion that light protection and temperature control are essential for nanoparticle stability, corroborating the results that show the maintenance of an average particle size of 90.79 nm over time under various storage conditions.

Figure 2 - Stability analysis results of nanoparticle size over 60 days under different storage conditions: amber glass container in the refrigerator at -4 °C (Fig. 2-A), amber glass container protected from light (Fig. 2-B), amber glass container in a climatic chamber at ±40 °C (Fig. 2-C), and transparent glass container exposed to light (Fig. 2-D).



The results were expressed as a percentage of the positive control (100%). Data are presented as mean ± standard deviation (SD). Analyses were performed using one-way ANOVA followed by Dunnett's post hoc test. Values with $p < 0.05$ were considered statistically significant.

According to studies conducted by Reatgui (2021), formulation composition is one of the most influential factors affecting nanoparticle size. We suggest that the dimensions observed in this nanocarrier are related to the low melting point of butter, which is approximately 32 °C. Research indicates that the size of lipid nanoparticles tends to correlate with their melting point: a higher melting point results in larger nanoparticles, and vice versa.

Furthermore, it is important to note that nanoparticles with diameters ranging from 20 to 200 nm are unable to penetrate the stratum corneum of the skin, preventing systemic absorption and consequently reducing the risk of toxicity to organisms.

Understanding the relationship between composition and nanoparticle size is crucial for the development of safer and more effective drug delivery systems. The fact that nanoparticles between

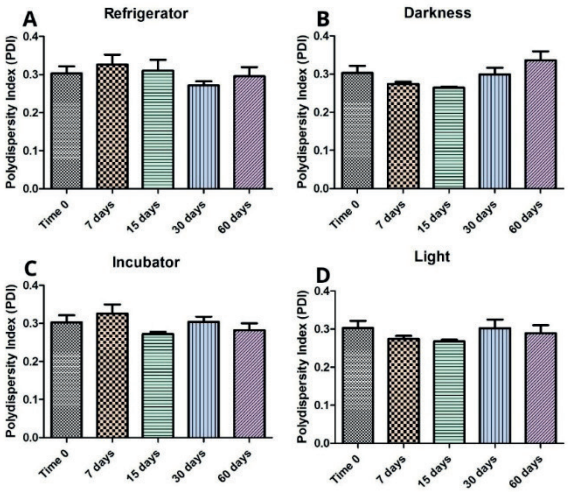
20 and 200 nm cannot cross the skin barrier significantly contributes to the safety of these particles in biomedical applications, minimizing the risk of adverse effects. This information is essential for the formulation of nanocarriers aimed at localized therapies, while ensuring an appropriate safety profile and enhancing our understanding of the interactions between formulation parameters and their effects on nanoparticle properties, thereby optimizing their clinical applications (De Andrade *et al*., 2024).

3.3.1.2 Polydispersity Index (PDI)

As illustrated in Figure 3, the polydispersity index (PDI) of the analyzed samples remained stable throughout the study period, with values close to 0.302, as observed at time 0. This consistency in PDI is a positive indication, suggesting that the nanoparticle size distribution did not undergo significant changes during the stability analysis.

According to studies by Bernardi *et al.* (2011) and Machado *et al*. (2019), a PDI below 0.4 is considered ideal for formulations, as this parameter is fundamental for evaluating nanoparticle stability and uniformity. A PDI below this threshold indicates that the samples are predominantly monodisperse, meaning that most nanoparticles have similar sizes, resulting in a unimodal distribution. This property is desirable because monodisperse nanoparticles tend to exhibit more predictable behavior in biological and industrial applications, such as controlled drug release, where uniformity in size distribution can influence treatment efficiency and efficacy. Furthermore, maintaining the PDI at levels around 0.302 over time suggests that interactions between the particles and the dispersion medium did not result in aggregation or sedimentation, factors that could compromise formulation stability. Therefore, PDI analysis not only reinforces the quality of the initial formulation but also provides evidence of its robustness under storage and handling conditions, which is crucial for the development of effective pharmaceutical and nanotechnological products (Filippov *et al*., 2023; Mehta *et al*., 2023; Shi *et al.,* 2024). In summary, the observed PDI stability supports the viability of the formulation for future applications, highlighting the importance of monitoring this parameter during the development and evaluation of nanoparticle-based products.

Figure 3 - Stability analysis results of the polydispersity index (PDI) over 60 days under different storage conditions: amber glass container in the refrigerator at -4 °C (Fig. 3-A), amber glass container protected from light (Fig. 3-B), amber glass container in a climatic chamber at ±40 °C (Fig. 3-C), and transparent glass container exposed to light (Fig. 3-D).



The results were expressed as a percentage of the positive control (100%). Data are presented as mean ± standard deviation (SD). Analyses were performed using one-way ANOVA followed by Dunnett's post hoc test. Values with $p < 0.05$ were considered statistically significant.

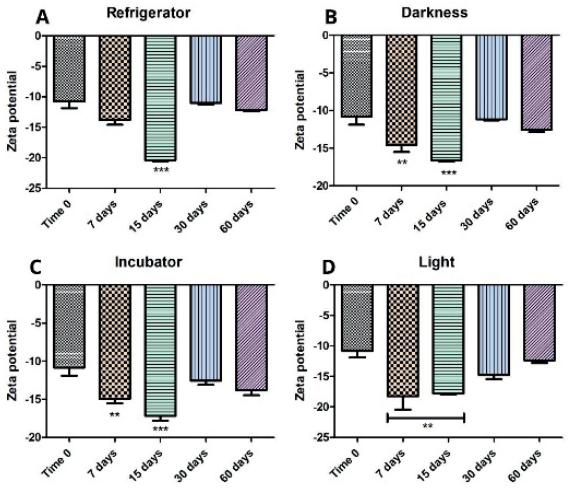
3.3.1.3 Zeta Potential

As shown in Figure 4, significant changes in zeta potential values were detected under all storage conditions, with an increase observed around day 15, followed by stabilization from day 30 onward.

Nanostructured lipid carriers, or solid lipid nanoparticles, exhibit negative zeta potentials due to the chemical composition of their matrix, which contains oleic acid. In the present study, the formulation showed an initial zeta potential value of -10.82 mV. According to the literature, zeta potential values equal to or greater than ±30 mV are considered ideal.

As illustrated in Figure 4, some zeta potential measurements of the nanoparticles produced in this study showed significant variations ($p > 0.05$) after the storage period.

Figure 4 - Stability analysis results of zeta potential over 60 days under different storage conditions: amber glass container in the refrigerator at -4 °C (Fig. 4-A), amber glass container protected from light (Fig. 4-B), amber glass container in a climatic chamber at ±40 °C (Fig. 4-C), and transparent glass container exposed to light (Fig. 4-D).



The results were expressed as a percentage of the positive control (100%). Data are presented as mean ± standard deviation (SD). Analyses were performed using one-way ANOVA followed by Dunnett's post hoc test. Values with $p < 0.05$ were considered statistically significant.

For formulation 2, the main observed change was an increase in zeta potential across all storage environments, followed by a slight decrease after 30 days. These fluctuations in the analyzed parameters may be related to the molecular reorganization and adaptation within the formulation (Schaffazick *et al*., 2003; Marques, 2023).

The literature also reports that low zeta potential values can be associated with stable formulations. A possible explanation is the use of Tween and Span surfactants, where Tween provides steric stabilization through interactions between its hydrophilic groups and water, creating a protective aqueous barrier around the particles. In agreement with these studies, the zeta potential observed in this study ranged from -10 to -20 mV, generating a negative electric field around the nanoparticles, which is not considered critical for aggregation. Therefore, low zeta potential values in these formulations do not necessarily indicate poor stability (Marques, 2023).

Another parameter analyzed during the stability assessment of the lipid nanocarrier was the pH values, which remained stable over 60 days, ranging between 4.0 and 5.0 at 0, 7, 15, 30, and 60 days. This stability has practical implications for topical applications. A pH within this range is compatible with the so-called "acid mantle" of the skin and supports stratum corneum homeostasis, as well as the activity of skin maturation enzymes and natural antimicrobial defense; acidic products tend to be better tolerated and can even help normalize skin pH under conditions of alkalinization (Lukic *et al.,* 2021).

The maintenance of pH suggests good physicochemical stability of the system and indicates that the buffer system and/or surfactants did not cause undesirable deviations in acidity during storage,

reducing the risk of hydrolytic degradation of the components. Furthermore, in lipid nanocarriers, pH can influence both the crystalline structure of the lipid and the release of encapsulated compounds; stable pH within the acidic range observed in this study is likely to minimize changes induced by lipid recrystallization or hydrolysis, which frequently compromise long-term efficacy (Lukic *et al*., 2021; Khan *et al*., 2023).

Thus, considering topical cosmetic/dermatological applications, a formulation with a pH between 4 and 5 is compatible with lipophilic ingredients derived from olive oil and may promote tolerability (reduced irritation) and maintenance of the skin barrier, while potentially contributing to the beneficial functional effects reported for olive oil-based preparations, such as hydration and erythema reduction (Santoyo *et al.,* 2025).

3.4 IN VITRO BIOCOMPATIBILITY ANALYSIS OF LIPID CARRIERS

Cell viability of the formulations was assessed using the MTT reduction assay. Total reactive oxygen species (ROS) levels were quantified using the dichlorofluorescein diacetate (DCFH-DA) assay. To determine nitrite production indices in cells subjected to different treatments, a nitric oxide assay was performed, targeting this extremely short-lived free radical in biological systems. Endogenous nitric oxide production by nitric oxide synthase (NOS) plays a crucial role in vascular homeostasis, neurotransmission, and host defense mechanisms (Bryan & Grisham, 2007). A fluorimetric DNA quantification assay was employed to detect double-strand DNA damage in the supernatant of treated samples.

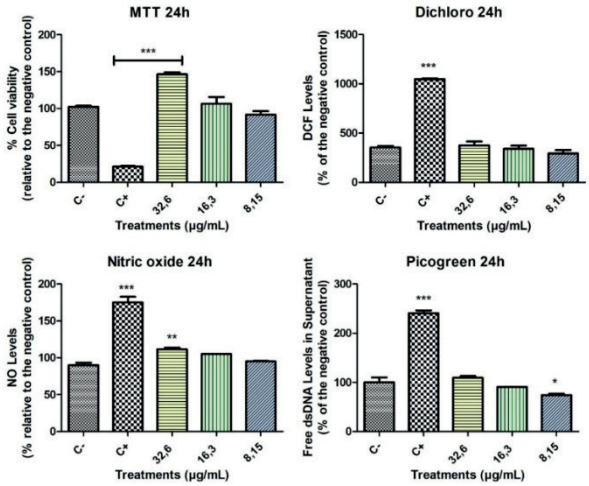
For all tests, H_2O_2 was used as a positive control, while sodium nitroprusside served as the positive control for nitric oxide. Sodium nitroprusside is widely used in nitric oxide assays because it acts as a chemical donor of NO, spontaneously releasing nitric oxide in aqueous solution, leading to detectable increases in nitrite/nitrate in fluorimetric or colorimetric assays. Its predictable and rapid action allows validation of method sensitivity and performance, enabling relative comparison between treatments (Wang *et al*., 2002). All tests were performed in triplicate over a 24-hour period.

As shown in Figure 5, none of the treatments at the tested concentrations reduced cell viability compared to the positive control after 24 hours of culture; in fact, the highest concentration even promoted a slight degree of cell proliferation. This finding indicates that olive oil derivatives, even when nanoencapsulated, do not exert cytotoxic effects on mononuclear cells such as lymphocytes and monocytes, corroborating studies by Fraga (2023), which demonstrated the cytoprotective effects of *Olea europaea* L. derivatives, attributed to the high polyphenol content in their chemical matrix. Studies by Gorzynik-Debicka *et al*. (2018) emphasize that phenolic compounds derived from vegetables, fruits, and herbs play a crucial role in regulating epigenetic modifications. These compounds not only protect cells but also promote the proliferative activity of healthy cells, demonstrating significant beneficial effects in maintaining cellular health and preventing disease.

These results suggest that bioactive compounds present in olive, particularly in their derived forms, may offer a safe option for applications where cellular protection and regeneration are desired. The absence of cytotoxicity highlights the compatibility of olive oil compounds with immune system cells, which is particularly relevant in research contexts concerning health and immune response (Fraga, 2023). Quantification of reactive oxygen species (DCFH-DA) showed a marked increase only in the positive control with H_2O_2, confirming the method's sensitivity, while treatments maintained ROS levels close to the negative control. This result indicates the absence of oxidative stress induced by the formulations, supporting reports that phenolic and lipid compounds in olive possess antioxidant properties and can modulate cellular redox responses (Mazuco, 2023).

According to Luca *et al*. (2024), *Olea europaea* L. leaf extract, due to its chemical matrix rich in phenolic compounds, is capable of significantly reducing ROS generation levels, promoting normal and adequate cell growth.

Figure 5 - Results of the 24-hour MTT, DCF-DA, nitric oxide, and PicoGreen assays for the Lipid Nanocarriers**.**



The results were expressed as a percentage of the positive control (100%). Data are presented as mean ± standard deviation (SD). Analyses were performed using one-way ANOVA followed by Dunnett's post hoc test. Values with $p < 0.05$ were considered statistically significant.

Similarly, the nitric oxide assay indicated a slight increase in NO levels only at the highest concentration, without reaching cytotoxic values, which may reflect a moderate physiological activation of NO synthesis related to protective and cellular signaling effects (Bryan & Grisham, 2007). Storniolo *et al*. (2014) reported experimental conditions with high levels of ROS in which phenolic compounds from olive oil, such as hydroxytyrosol, were able to reverse these parameters.

Finally, the PicoGreen assay results showed low levels of free DNA in the supernatant for the lowest concentration formulation, suggesting the absence of significant genetic damage after 24 hours of treatment, further supporting the safety of the developed nanostructures. The lack of significant DNA damage observed aligns with results obtained by Liu *et al.* (2002), who analyzed the protective properties of

seven phenolic compounds against hydrogen peroxide-induced DNA damage in human peripheral blood lymphocyte cell lines. One of the compounds capable of protecting the cells was vanillin, a compound present in the olive oil used for synthesizing the nanocarriers tested in this study. In a more recent study, Potapovich *et al.* (2022) confirmed that phenolic compounds are capable of reducing DNA breaks, as demonstrated in tests conducted with human HaCaT cells exposed to ultraviolet radiation.

3.5 EVALUATION OF THE ANTIMICROBIAL AND ANTIBIOFILM ACTIVITY OF LIPID CARRIERS AND OLIVE OIL

The antimicrobial and antibiofilm potential of the lipid nanocarriers and olive oil was evaluated against various bacterial species, including both Gram-positive and Gram-negative strains: Enterococcus faecalis, Enterococcus faecium, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella enteritidis, and Staphylococcus aureus. However, no antimicrobial effect was observed against the tested bacteria, which prevented the continuation of the antibiofilm assays.

A possible explanation for the lack of antimicrobial activity of the lipid nanocarriers against these strains may result from a combination of physicochemical and microbiological limitations. Although phenolic compounds and acids present in olive oil, as characterized in this study, have been reported as potent antimicrobials in free systems such as the study by Rodriguez *et al*. (2021), which highlighted antimicrobial activity against Staphylococcus aureus and Enterococcus incorporation of olive oil into a secondary lipid matrix may restrict its availability to reach the bacterial cell. The literature also reports that phenols can remain strongly retained or exhibit limited diffusion when in contact with a solid lipid phase, reducing the penetration of nanocarriers into the bacterial membrane. Additionally, bacterial resistance mechanisms, such as efflux pumps and permeability barriers especially in Gram-negative bactéria further hinder the permeation of phenolic compounds (Sharma *et al*., 2025).

Encapsulation does not always maintain or enhance antimicrobial activity; depending on the system, it may decrease the effective interaction between the active compound and the microbial target. Another possible explanation is the low content of the most antimicrobial-active compounds in the formulation (Tadic *et al*., 2012).

4 CONCLUSIONS

The present study allowed for the characterization and evaluation of the potential applications of olive oil and its derived formulations, highlighting their chemical, physical, and biological properties. Phenolic analysis revealed the presence of bioactive compounds with nutritional and therapeutic relevance, particularly oleacein and oleocanthal, which are recognized for their antioxidant, anti-inflammatory, and cardioprotective activities. The antioxidant activity of the olive oil was moderate,

reinforcing that its therapeutic potential is not limited to free radical neutralization but also relies on the synergistic effects of the various compounds present in its matrix.

The production of butter from olive oil proved feasible, demonstrating stability under appropriate storage conditions, particularly when refrigerated or kept at room temperature protected from light. Lipid nanocarriers derived from the olive oil butter exhibited physical and colloidal stability, with monodispersed nanoparticles, consistent PDI, and zeta potential within acceptable ranges, suggesting low aggregation tendency and compatibility for future applications.

In vitro biocompatibility assessment revealed no cytotoxicity, oxidative stress, or significant genetic damage, indicating that the lipid nanocarriers are safe for mononuclear cells. However, antimicrobial activity tests showed no efficacy against the evaluated bacterial strains, possibly due to limited diffusion of phenolic compounds within the lipid matrix or low concentrations of specific antimicrobial-active compounds.

In summary, the results indicate that olive oil and its lipid-based formulations possess high antioxidant potential and biocompatibility, making them promising for nutritional, pharmaceutical, and cosmetic applications, while antimicrobial functionality may require formulation adjustments or increased concentrations of bioactive compounds.

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