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Formação, caracterização e atividade biológica de sistemas lipídicos contendo óleo essencial

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Formação, caracterização e atividade biológica de sistemas lipídicos contendo óleo essencial

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Faculdade de Ciências Farmacêuticas de Ribeirão Preto/USP para obtenção do Título de Doutor em Ciências

Área de Concentração: Medicamentos e Cosméticos.

Orientador: Prof. Dr. Wanderley Pereira Oliveira

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FOLHA DE APROVAÇÃO

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Dedico esse trabalho aos meus pais Ana e Robertinho
que desde cedo me ensinaram a sonhar
e me mostraram com maestria como lutar
para que cada sonho se realize.
Obrigada por lutarem comigo ontem, hoje e sempre.

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Aos meus queridos amigos Patrícia e Fernando, por me acolherem com tanto carinho e amor em Portugal, e à minha "família" de Portugal, Fábio, Ana, Clarinha e Thalita: obrigada por tornarem meus dias em Braga ainda mais especiais.

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“tudo é ousado para quem a nada se atreve”

Fernando Pessoa

RESUMO

BALDIM, I. **Formação, caracterização e atividade biológica de sistemas lipídicos contendo óleo essencial**. 2022. 260f. Tese (Doutorado). Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2022.

Óleos essenciais (OEs) têm despertado grande interesse nos últimos anos, principalmente devido a sua ampla gama de atividades biológicas, como antifúngicos, antibacterianos, inseticidas, antivirais, antioxidantes, entre outras. Seu uso como ingrediente ativo traz uma série de benefícios, e talvez a principal vantagem esteja relacionada à sua capacidade de combater a resistência muitas vezes apresentada pelos microrganismos contra os antibióticos e antifúngicos disponíveis no mercado. Contudo, sua alta volatilidade e sensibilidade restringem seu uso e limitam sua incorporação a produtos mais elaborados. A microencapsulação e nanoencapsulação dos OEs surgem como estratégias viáveis e eficientes à proteção e modulação da liberação de seus compostos bioativos, promovendo o aumento da estabilidade físico-química, proteção contra fatores ambientais, redução da volatilidade, aumento da solubilidade, biodisponibilidade e atividade biológica, redução da toxicidade, entre outros benefícios. Os sistemas lipídicos são uma estratégia especialmente promissora para incorporação de OEs, pois são biocompatíveis, apresentam alta capacidade de carga, baixo custo, baixa toxicidade e são capazes de encapsular tanto substâncias lipofílicas quanto hidrofílicas. Dentre esses sistemas, temos os lipossomas, que são vesículas biocompatíveis, biodegradáveis e com potencial aplicação farmacêutica, e os carreadores lipídicos nanoestruturados (NLC), nanopartículas capazes de carregar moléculas quimicamente diferentes e fornecer um perfil de liberação ajustável, podendo ser produzidas em larga escala e sem a necessidade de solventes orgânicos. A influência do sistema de encapsulação, componentes lipídicos, tipo de emulsificante e outros excipientes deve ser avaliada para otimizar a estabilidade do sistema, a retenção de compostos bioativos e melhorar sua atividade biológica. Nesta tese se investigou várias rotas tecnológicas envolvidas na obtenção de sistemas lipídicos micro ou nanoestruturados para estabilizar e modular a liberação dos óleos essenciais alecrim-pimenta (*Lippia sidoides*) e cravo (*Syzygium aromaticum*), enfatizando-se processos de preparação, propriedades físico-químicas, estabilidade e atividade biológica frente a microrganismos multirresistentes de importância clínica. As principais variáveis de formulação e de processo foram analisadas, sendo definidas condições ótimas de processamento para sistemas a base de lipossomas encapsulando óleo essencial complexado em β-ciclodextrina e para os NLCs. Ensaios de atividade antifúngica frente a diferentes microrganismos foram realizados para os NLCs, que se mostraram eficientes no combate do fungo multirresistente *Candida auris*, que tem se disseminado globalmente principalmente em ambientes hospitalares, para o qual as opções de tratamento são muito reduzidas ou inexistentes.

Palavras-chave: óleos essenciais, encapsulação, carreadores lipídicos nanoestruturados, lipossomas, *Lippia sidoides*, microrganismos multirresistentes

ABSTRACT

BALDIM, I. **Formation, characterization, and biological activity of lipid systems loaded by essential oil**. 2022. 260f. Thesis (Doctoral). Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo, Ribeirão Preto, 2022.

Essential oils (EOs) have aroused great interest in recent years, mainly due to their wide range of biological activities, such as antifungal, antibacterial, insecticide, antiviral, antioxidant, among others. Its use as an active ingredient brings countless benefits, and maybe the main advantage is related to its ability to fight the resistance often presented by microorganisms against antibiotics and antifungals available on the market. However, the high volatility and sensitivity of these compounds restrict their use, limiting the incorporation into more elaborate products. Microencapsulation and nanoencapsulation of EOs emerge as viable and efficient strategies to protect and modulate the release of their bioactive compounds, promoting increased physical-chemical stability, protection against environmental factors, reduced volatility, increased solubility, bioavailability and biological activity, reduction of toxicity, among other benefits. Lipid systems are an especially promising strategy for EO incorporation, as they are biocompatible, present high loading capacity, low cost, low toxicity, and can encapsulate both lipophilic and hydrophilic substances. Among these systems, the liposomes are biocompatible, biodegradable vesicles with a potential pharmaceutical application; and nanostructured lipid carriers (NLC) are nanoparticles able to carry chemically different molecules, providing an adjustable release profile, which can be produced on a large scale, without the need for organic solvents. The influence of the encapsulation system, lipid components, type of emulsifier, and other excipients must be evaluated to optimize system stability, retention of bioactive compounds, and improve their biological activity. This thesis aimed to investigate the technological routes involved in obtaining micro or nanostructured lipid systems to stabilize and modulate the release of rosemary-pepper (*Lippia sidoides*) and clove (*Syzygium aromaticum*) essential oils were investigated, emphasizing the preparation processes, physicochemical properties, stability, and biological activity against multidrug-resistant microorganisms of clinical importance. The main formulation and process variables were analyzed, defining optimal processing conditions for liposome-based systems encapsulating essential oil complexed in β-cyclodextrin and for NLCs. Assays of antifungal activity against different microorganisms were performed for NLCs, which proved to be efficient in combating the multidrug-resistant fungus Candida auris, which has spread globally, mainly in hospital environments, with very limited or non-existent treatment options.

Keywords: essential oils, encapsulation, NLC, liposomes, *Lippia sidoides*, multidrug-resistant microorganisms

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LISTA DE ABREVIATURAS E SIGLAS

EO	Essential oil
OE	Óleo essencial
CD	Cyclodextrin
HPH	High-pressure homogenization
PDI	Polydispersity index
Xp	Moisture content
Aw	Water activity
ρa	Densidade aparente
ρc	Densidade de compactação
RH	Razão de Hausner
IC	Índice de Carr
SLN	Solid lipid nanoparticle
NLC	Nanostructured lipid carrier
DSC	Differential scanning calorimetry
TGA	Thermogravimetric analysis
MIC	Minimum inhibitory concentration
MFC	Minimum fungicidal concentration
ANOVA	Analysis of variance
RENISUS	Relação Nacional de Plantas Medicinais de Interesse ao SUS

SUMÁRIO

CHAPTER 1. INTRODUCTION

The growing consumer demand for more natural, sustainable, and environmentally friendly products has changed the way of consuming in the world. The industry has responded to this demand by trying to identify natural alternatives to functional synthetic ingredients in products from the most varied fields, including pharmaceuticals, food, cosmetics, and personal care products (CARVALHO, ESTEVINHO e SANTOS, 2016; MCCLEMENTS e GUMUS, 2016; OZTURK e MCCLEMENTS, 2016).

In a prominent position in the list of natural functional actives are essential oils (EOs). These natural substances also have countless biological activities and an indisputable potential for applicability in products that support a more natural claim. Among the biological activities stands out the ability to combat microorganisms resistant to antibiotics present in the market (BALDIM *et al.*, 2022; FEYAERTS *et al.*, 2018; RAI *et al.*, 2017). Nevertheless, it can be taken in account that these compounds have limitations, such as: chemical complexity, high volatility, susceptibility to degradation/oxidation, insolubility in aqueous systems, and low bioavailability, limiting their use in more elaborate products (BALDIM *et al.*, 2020).

The micro and nanoencapsulation of EOs have received significant attention in the pharmaceutical and food sectors, being a promising strategy to overcome the limitations of using these substances *in natura* form. This approach allows for example to modify physicochemical properties, promote reduced volatility, increased solubility, stability, protection against environmental factors, bioavailability, and biological activity. This technique consists of the inclusion of the active agent in carrier systems, which may consist of carbohydrates, gums, proteins, lipids or other natural or synthetic polymeric materials. These systems are able to release their content under specific conditions through a controlled release, which results in greater effectiveness of the actives, allowing the use of an optimal dosage, which improves the cost-efficiency of the product (BALDIM, SOUZA e OLIVEIRA, 2021).

In the last two decades, encapsulation in lipid systems has been highlighted, due to several advantages, such as low toxicity, ease of scalability, low production cost and the possibility of encapsulating hydrophilic and lipophilic compounds. The dehydration of these systems can generate dry dispersions with high encapsulation efficiency, solubility, and greater stability against liquid forms, which can be redispersed when necessary or used in the development of topical and/or oral products (BOUREZG *et al.*, 2012; VARSHOSAZ, ESKANDARI e TABBAKHIAN, 2012).

Recent publications show the use of micro and nanoencapsulation for protection and release of various compounds of plant origin such as quercetin (VIJAYAKUMAR *et al.*, 2017), lutein (LIU *et al.*, 2014), β-carotene (SALMINEN *et al.*, 2016), essential oils (BALDIM *et al.*, 2019), among others. Factors such as composition of the EO used, type of lipid, type and concentration of surfactants, emulsification process (high or low energy), and dehydration/cooling conditions influence the characteristics of the particles obtained, however this information are scarce in the literature. Therefore, conducting systematic studies to determine the mechanisms involved on particle formation that influence the achievement of a product with desired properties (stability, high encapsulation efficiency, solubility, and biological activity) is essential for the development of new strategies to encapsulate these bioactives.

Lippia sidoides, popularly known as pepper rosemary, is a small tree native to northeastern Brazil. It is an important medicinal plant due to its wide range of biological applications described over time not only in folk medicine, but also in several scientific works published in the literature. Its main biological activities include insecticidal, fungicidal, bactericidal, antileishmanial, larvicidal, acaricidal and anti-inflammatory activities (BALDIM *et al.*, 2019). Its EO is extracted from the leaves and is rich in thymol, the compound usually related to its biological activity. This species is also listed in the list of medicinal plants with the potential to generate products of interest to the Brazilian Public Health System – RENISUS (BRASIL, 2009).

Given the above, the present study presents as a contribution the development of nanostructured lipid systems loaded with essential oil as an innovative strategy to protect and modulate their release profile, aiming to improve their antimicrobial potential against multidrug-resistant microorganisms.

I adopted a structure based on the journal papers written for this thesis, to provide a clear understanding of all stages of this work. Consequently, chapters can be read independently, and some overlap may be found between them. Thus, in addition to the Introduction Chapter (Chapter 1) described here, this thesis is structured in 11 chapters. Chapter 2 presents a summary of the main objectives of this thesis. Chapter 3 presents a comprehensive review on encapsulation of essential oils in lipid nanosystems. Chapter 4 provides an overview of preformulation studies to obtain proliposomes loaded by essential oil. Chapter 5 describes an innovative system to encapsulate essential oils, making it possible to encapsulate them both in the hydrophilic and lipophilic portions: the drug-in-cyclodextrin-in-liposomes system. Chapter 6 provide a comprehensive review on lipid nanoparticles (SLN and NLC) for skin

administration. Chapter 7 provide an experimental design for the development of NLC loaded by *L. sidoides* EO, ranging from pre-formulation studies, through characterization and antimicrobial activity. Chapter 8 evaluates the toxicity and antifungal potential of NLC containing *L. sidoides* EO against a multidrug-resistant fungus of clinical interest: *Candida auris*. Chapter 9 brings an experimental design to describe the parameters involved in the drying of NLC loaded by *L. sidoides* EO, and how they influence the characteristics of the final product. Finally, Chapter 10 describes the final considerations of this thesis and Chapter 11 the conclusions.

CHAPTER 2. OBJECTIVES

The present study aims to study different technological routes involved in the production of nanostructured lipid systems loaded by essential oil and evaluate its potential as an antimicrobial agent in combating multidrug resistant pathogens. For this purpose, the following specific objectives are required:

1. Design and develop pre-formulation studies.
2. Optimize the production of different lipid nanosystems loaded mostly with *Lippia sidoides* essential oil
3. Characterize the physicochemical properties and evaluate the stability.
4. Evaluate both the toxicity profile and antimicrobial activity of lipid nanosystems against multidrug-resistant microorganisms of clinical interest.
5. Analyze the drying profile of these nanosystems.

CHAPTER 3. ENCAPSULATION OF ESSENTIAL OILS IN LIPID-BASED NANOSYSTEMS

An update of this chapter was published as:

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3.1 GENERAL ASPECTS

The growing interest of consumers towards "green products" has increased the use of plant-derived products. This trend has led the industry to look towards novel and safer approaches to replace the existing synthetic active substances. EOs have a high potential of application in several industrial sectors, namely pharmaceutical, food, cosmetic, health, agriculture, and livestock. The type of application depends on EOs' inherent biological activities (e.g. bactericidal, virucidal, fungicidal, antiparasitic, insecticidal, analgesic, sedative, anti-inflammatory, spasmolytic, and local anaesthetic). Some EOs and their isolated bioactive compounds (e.g., lemongrass, peppermint, rosemary, chamomile, basil, clove, thyme, lavender, mustard, rose, menthol, linalool, carvacrol, limonene, citral, p-cymene, thymol, linalool) are already listed as Generally Recognized As Safe (GRAS) by the U.S. Code of Federal Regulations [1].

EOs are complex mixtures of various volatile hydrophobic molecules produced by the plant's secondary metabolism. These volatile liquids exhibit high solubility in lipids and organic solvents and generally a lower density than water [2]. They are synthesized by different organs of the plant, such as flowers (jasmine, rose, violet, and lavender), flower buds (clove), leaves (eucalyptus, sage), fruits (anise), branches, bark (cinnamon), seeds (cardamom), wood (sandalwood), and roots (ginger). The term "essential oil" comes from Paracelsus von Hohenheim's theory (1493–1541), a physician and alchemist. They named the effective constituents of the plant as *Quinta essentia* or *Quintessence* to separate the "*essential*" part from the "*nonessential*" [3]. To date, nearly 3000 different essential oils have been described; around 300 are commercially employed by the flavour and fragrance market [4].

Typically, EOs have about 20 to 60 constituents, but this number can reach more than 100 isolated substances at quite different concentrations. The EOs' constituents include different cyclic and acyclic hydrocarbons compounds and their oxygenated, nitrogen or sulfur derivatives, coming from different biosynthesis pathways. The main EOs' classes of compounds are derived from three biosynthetic pathways: the mevalonate pathway leading to sesquiterpenes, the methyl-erythritol-pathway leading to mono- and diterpenes, and the shikimic acid pathway en route to phenylpropenes, and oxygenated compounds (e.g., alcohols, esters, ethers, aldehydes, ketones, lactones, phenolic ethers, and others). Generally, the principal constituent is usually associated with the EOs' biological properties [2].

The most representative class of EOs constituents are the terpenes, aromatic, and short-chain aliphatic hydrocarbon derivatives. They are formed by a combination of various 5-carbon-base (C_5) (isoprene) units. The extension of the chain allows a high diversity of structures. The simplest terpenes are monoterpenes, containing two isoprene units (C_{10}) and constitute around 90% of the EOs [2]. Sesquiterpenes have three isoprene units (C_{15}) and diterpenes, four (C_{20}). The increase in the number of carbons (and consequently in the molecular weight) reduces the compound's volatility, requiring more energy to be released from plant parts by steam distillation. Due to their low volatility, diterpenes are present in small quantities in many EOs obtained by steam distillation. Heavier terpenes (as tri- and higher (such as sterols or carotenoids) are only present in the nonvolatile fractions of plants, for example, resins or gums [4].

Phenylpropanoids (ethers), composed of an aromatic ring with a three-carbon side chain (C_6C_3 skeleton), can also occur, but less frequently than the terpenes. However, when present, they occur in appreciable proportions, such as in the clove EO, where the eugenol concentration can reach 70 to 90% [5]. Ethers are often less aggressive to the skin than phenols [5].

Although less common, sulfur- or nitrogen-containing organic compounds may also be present in the EOs of some plant families, such as Alliaceae, Rutaceae, and Brassicaceae [5]. Sulfur confers the pungent and characteristic aroma and taste of garlic, while isothiocyanates are the common constituents of mustard oils [6]. Table 3.1 represents the chemical classification, structure, and properties of various EOs' constituents.

3.1.1 Extraction of Essential Oils

In general, the content of essential oils in fresh aromatic plants is low (about 1%), varying according to the species and the plant's organ where it accumulates. For this reason, they are rare and highly valued substances. EOs can be obtained by different extraction methods,

classified as conventional/classical or advanced/innovative methods [7]. Examples of conventional methods are hydrodistillation, vapour-hydrodistillation, steam distillation, organic solvent extraction, and cold pressing. More efficient extraction processes have been studied recently to reduce energy consumption and increase the extraction yield, such as supercritical fluid extraction, ultrasound-assisted extraction, and microwave-assisted extraction. The selection of an extraction method depends on the plant characteristics, part of the plant used, and EOs properties. The extraction method used has a strong influence on the EOs' chemical profile [3]. Due to their density often lower than that of water and hydrophobic nature, EOs can be easily separated from the aqueous phase by decantation. However, some constituents of EOs have high water solubility or may present instabilities in contact with steam and heat, requiring specific extraction methods [7].

Indeed, the most traditional and commonly used method for EOs extraction is the water steam drag and its variants [8]:

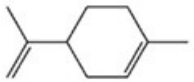
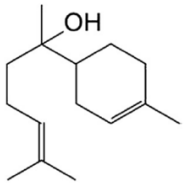
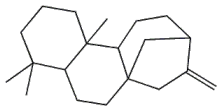
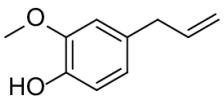
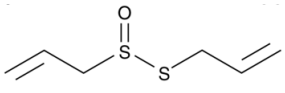
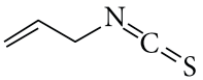
1. Hydrodistillation, well represented by the Clevenger apparatus, which consists of immersing the herbal material content directly in the boiling water and recovering the EO by condensation;
2. Vapour-hydrodistillation, in which the herbal material is placed on a perforated plate system positioned above the boiling water;
3. Steam distillation is a method similar to vapour distillation, but the vapour is generated from an external source.

Hydrodistillation is a suitable alternative for the extraction of petals and flowers, as it avoids compaction and chomping of the material. However, the long extraction time required can degrade some thermosensitive compounds, changing the EOs chemical profiles. These drawbacks may be minimized or even avoided both in vapour-hydrodistillation and steam distillation [7].

Organic solvent extraction and cold pressing are other traditional and well-established methods. The first method consists of macerating the plant material in an organic solvent, removed after the EO extraction. This technique is suitable for the extraction of thermosensitive compounds. On the other hand, the use of organic solvents, mainly with higher boiling points, may compromise the EO's quality and safety. Cold pressing is the method of choice to extract OE from the outer waxy layer of citrus peels. The pressing process breaks the oil sacs, releasing the EO and yielding an oil/water mixture, further separated by pressurized centrifugation [8].

Innovative approaches arise as alternatives to reduce extraction time, energy consumption, and organic solvent use.

TABLE 3.1. Important essential oils constituents, their molecular structure, properties and examples.

Class of compound	Subclass	Substance	Structure	Properties	Example of plant	Part used
Terpenes	Monoterpene (C_{10})	D-limonene		Promotes skin penetration and diffusivity of drugs [14]; excellent flavoring agents and provide effective gastroprotection [124]	*Citrus aurantium*	Fruit peel
	Sesquiterpene (C_{15})	α-bisabolol		Anti-inflammatory, antinociceptive-like action [5, 125]; skin penetration enhancer [14]; antiglioma activity [43]	*Chamomilla recutita* (L.) Rauschert	Flower
	Diterpene (C_{20})	Phyllocladene		Antibacterial activity [126]	*Araucaria angustifolia*	Leaves
Aromatic compounds	Phenylpropanoids	Eugenol		Antimicrobial activity due to its ability to permeate the bacterial cell membrane and interact with proteins [6]; inhibit *Escherichia coli* biofilm formation [127]; improve the stability of nanoemulsion delivery systems [128]; insecticide and herbicide [129]; anti-inflammatory effects [39]	*Eugenia caryophyllata (Syzygium aromaticum* L. Myrtaceae*)*	Buds and leaves
Other EOs constituents	Sulfur- and nitrogen-containing compounds	Allicin		Antibacterial (Gram-negative and Gram-positive), antifungal, antiparasitic, and antiviral properties [6]	*Allium sativum* L.	Bulb
		Allyl isothiocyanate		Antimicrobial activity [6]	*Brassica juncea*	Seeds

Supercritical fluid extraction (SFE) has also attracted significant interest, especially in recent years. The SFE principle is similar to maceration and percolation. However, the extracting fluid (gas) must be kept in conditions above its critical temperature and pressure, which require sophisticated and expensive equipment. This technique also has some restrictions for fresh plants and floral fragrances. Notwithstanding, the EO quality, having a composition similar to that of the raw material and a fresh flavour impression, offset most of these attributed disadvantages, besides being a fast and gentle process [9].. Supercritical carbon dioxide (CO_2) is the fluid most used for the supercritical extraction of EOs. It exhibits favourable properties as low critical pressure and temperature, high purity, chemically inertness, low cost and readily available, environmentally safe, bacteriostatic, and not flammable [7, 9].

Ultrasound-assisted extraction is commonly performed in association with other techniques (e.g., hydrodistillation and solvent extraction) to enhance the EOs extraction velocity/efficiency. It consists of immersing the herbal material in a liquid solvent and submitting the mixture to ultrasound, inducing acoustic cavitation bubbles that can rupture the plant cells, facilitating the EOs release, being an efficient, simple, and inexpensive technique suitable for thermosensitive compounds [7]. Another technique that results in increased yield and a shorter extraction time is microwave-assisted extraction. The process is based on the water within the plant matrix, whose cells are disrupted by absorbing microwave energy, releasing the chemical compounds into the extraction medium. The plant material's moisture content enables rapid and uniform heat distribution, and the extraction solvent (which has a distinct dielectric constant) remains cold, allowing the reduction of the sample temperature [10].

Nowadays, consumers have questioned synthetic compounds' safety, and the balance between their benefits and side effects, which contributes to the increase of research works aiming at the identification, chemical characterization, and evaluation of the biological activities of EOs. The EOs' biological properties have applications in various knowledge fields, for example:

1. Pharmaceutical, studies on combating bacterial resistance and biofilm formation, wound healing and enhancing transdermal drug delivery;
2. Food, as natural antioxidants and food preservatives;
3. Agroindustry, for crop protection [11, 12];

4. Veterinary, as a therapeutic alternative for inflammatory conditions, and so on [[6, 13–15].

3.2 BIOLOGICAL ACTIVITIES OF ESSENTIAL OILS

The term "biological activities" comprises all the beneficial or adverse effects a bioactive substance (e.g., EOs constituent) may exert on living beings. In plants, the volatile constituents of EOs, although not directly involved with the essential plant metabolism, exhibit multiple ecological functions, such as plant defence against pathogens, herbivores attacks, excess solar radiation, and chemical signalling for attraction of pollinators. The use of EOs in traditional medicine has been recognized and practised by humankind since ancient times [3]. Currently, the importance and potential applications of EOs are well documented. There is a continuous increase of research groups worldwide that develop basic and applied research with these compounds. The occurrence of synergism between major and minor EOs constituents is a point that deserves attention. In most situations, the biological effects of the whole EOs are significantly higher compared with the isolated constituents. Following is presented a description of important EOs' biological activities.

Antimicrobial activity: This is probably the most well-known biological activity of EOs. The scientific and medical literature shows numerous reports on the *in vitro* antimicrobial activity of EOs. Nowadays, the increased incidence of drug-resistant microorganisms, the main cause of serious bacterial and fungal infections, requires exploring new molecules and alternative approaches against resistant microorganisms. The antimicrobial activities of EOs depend on their composition, functional groups of the main bioactive molecules, and the synergistic interactions between their constituents. Some EOs have proven activity against gram-positive and gram-negative bacteria, yeast, filamentous fungi. The lipophilic nature of EOs allows them to interact easily with the lipids of the dense microbial cell membrane, and the susceptibility varies with the EOs composition and microorganism strains. Gram-positive bacteria are more susceptible than Gram-negative bacteria since the former does not have the rigid lipopolysaccharide outer membrane, restricting the diffusion of lipophilic substances [16]. EOs bioactive substances can interact, attach and accumulate in the cell membrane, disturbing its structural integrity, which affects the cell metabolism and can cause cell death [17].

Antiviral activity: Due to their lipophilic nature, EOs can also penetrate viral membranes, easily causing their rupture, leading to a reduction in the host's inflammatory responses [18].The EOs' constituents can act in synergy at different viral replication stages showing activity against

various viruses (e.g. influenza, human herpesvirus, human immunodeficiency, yellow fever, avian influenza). Activity against enveloped viruses, as the SARS-CoV-2, has also been demonstrated [19].

Antioxidant activity: The biochemical reactions involved in the normal human metabolism can continuously generate reactive oxygen species (ROS), including the free radicals, such as the hydroxyl radical (OH•), and the superoxide anion radical (O2•-), and non-radical species such as the hydrogen peroxide (H2O2) and the hypochlorous acid (HOCl). ROS are highly reactive molecules and are normally eliminated from our body by using endogenous and exogenous antioxidants [20]. Changes in the body's ability to eliminate them result in oxidative stress and, as a consequence, damage to cellular and extracellular constituents. An antioxidant bioactive is a compound capable of maintaining cellular structure and function by effectively inhibiting lipid peroxidation reactions, eliminating free radicals, and preventing oxidative damage. Essential oils, especially those with volatile phenolic compounds, are recognized as potent antioxidant ingredients due to their high reactivity with peroxyl radicals [21, 22].. However, some terpenoids and other volatile compounds, such as sulfur-containing components, also exhibit significant antioxidant activity [23].

Anti-inflammatory and antinociceptive activities: The inflammatory process involves mechanisms linked to cellular and vascular responses, aiming to eradicate the source of the damage and prevent its spread to other parts of the body. This process's clinical manifestations are heat, redness, swelling, pain, and, in more extreme cases, loss of tissue or organ function [24]. Many aromatic species are used in traditional medicine for anti-inflammatory and antinociceptive purposes to relieve pain and inflammatory diseases. Various experimental studies have demonstrated the anti-inflammatory and antinociceptive activity of the various EOs' active constituents [25, 26].

Antimutagenic activity: The antimutagenic activity of essential oils is related to several mechanisms, which includes the ability to inhibit the metabolic conversion of pro-mutagens into mutagens; to inhibit the penetration of the mutagenic agent into the cells; to make the antioxidant capture of radicals produced by mutagens; to activate the enzymatic detoxification of mutagens. The antimutagenic activity depends on both the mutagen and antimutagen doses used [27].

Anticancer activity: Interestingly, more than half of the anticancer agents developed and approved between 1940 and 2006 were natural products and their derivatives [28]. Various EOs

have shown anticarcinogenic/antimutagenic/antiproliferative effects and can be used for cancer prevention [27].

Immunomodulatory activity: Some EOs can impact the inflammatory process and the expression of interleukins. Active inflammatory responses are associated with increased circulating cytokines and substantial lymphopenia. EOs can act by reducing the release of pro-inflammatory cytokines from monocytes and macrophages [19].

Antiprotozoal activity: Protozoa are single-celled eukaryotic microorganisms. Protozoan diseases are serious public health problems, and, in recent decades, studies evaluating the antiprotozoal effects of EOs have become increasingly available, both for human and animal health [4].

Antiplatelet activity: Platelets are responsible for preventing blood loss from damage to blood vessels by the clotting process. However, the clotting process can be especially dangerous when it occurs in healthy blood vessels. Because it is irreversible, platelet aggregation can block blood flow, compromise oxygen transport to cells, and lead to stroke, pulmonary embolism, and heart attack [22]. The high prevalence of these diseases has led to the continuous search for new antithrombotic agents; with low adverse effects. Aspirin® is one of the most widely used synthetic drugs for "blood-thinning", although it is associated with an increased risk of developing gastrointestinal side effects, even in low-dose therapy [29]. Some essential oils have shown influence on the blood clotting process, and their effectiveness and safety have been investigated [30].

3.2.1 Mechanisms of Action of Essential Oils

The modes of action of the EOs are closely related to their composition. Due to the multicomponent and complex nature of EOs, they can influence biological systems through multiple mechanisms of action. The synergistic effect between two or more compounds can also play an important role in the biological activity of EOs [23]. The biological activities of some essential oils with great potential developing technological products are highlighted in Table 3.2, together with an overview of their mechanism of action.

TABLE 3.2. Biological activities of essential oils.

Biological activity	Essential oil	Mechanism of action	Reference
Antibacterial	*Syzygium aromaticum* *Melaleuca alternifolia* *Cymbopogon citratus* *Thymus vulgaris* *Cinnamomum verum* *Origanum ulgare* *Eucalyptus globulus*	Due to the complexity of the composition of EOs, there are several mechanisms of action involved in its antibacterial activity, among which the following stand out the disturbances in cell membrane integrity and the inhibition of cell wall synthesis, leading to cell lysis by the leakage of protein and lipid contents.	[16, 31, 32]
Antifungal	*Lippia sidoides* *Thymus vulgaris* *Cymbopogon citratus* *Rosmarinus officinalis* *Ocimum sanctum*	The antifungal activity of EOs can be promoted by different mechanisms: EOs can interact with fungal cell membrane, causing disruption, alteration or even inhibition of the wall formation; they can also inhibit mitochondrial enzymes, affecting the mitochondrial effectiveness and cellular methabolism; inhibit efflux pumps, modifying the fungal cell physiology; and they can also influence the ROS production in fungi.	[16, 33]
Antiviral	*Laurus nobilis* *Eucalyptus globulus*	*Laurus nobilis* - Inhibition of viral replication from SARS-CoV-2. *Eucalyptus globulus* - eucalyptus EO and its active constituent (eucalyptol) inactivate free influenza A (H1N1) virus and disrupt the envelope structures of the virus.	[19, 34]
Anti-inflammatory	*Rosmarinus officinalis* L.	The anti-inflammatory activity of rosemary EO can be mainly attributed to its major monoterpenes (1,8-cineole and α-pinene), by decreasing the activity of the transcription factor NK-κB, which impedes the synthesis of pro-inflammatory mediators. The antioxidant activity of rosemary EO also contribute to attenuate inflammation-induced injury by neutralizing the reactive species produced in inflammation.	[35]
Antinociceptive	*Thymus capitatus*	The carvacrol-rich essential oil from *T. capitatus* induces antinociception in orally treated mice by exerts its antinociceptive activity through peripheral nervous excitability blockade.	[25]
Antimutagenic	*Citrus sinensis* and *Citrus latifolia*	Both EOs can act by several antimutagenic mechanisms: reduce alkylated DNA damages through a reduction in the expression of base-substitution mutations; reduce the activation of pre-mutagens; and as possible ROS-scavenging mixtures.	[36]
Anticancer	*Melissa officinalis* L. *Melaleuca alternifolia*	*Melissa officinalis* L. – Induces apoptosis in human glioblastoma multiforme cell lines by DNA fragmentation and activation of caspase-9 and caspase-3. *Melaleuca alternifolia* - inhibit the growth and induce caspase-dependent apoptotic cell death in melanoma cells, due to the interaction between the	[37, 38]

		lipophilic components of essential oil and the phospholipid bilayer of melanoma cell membranes	
Antioxidant	*Syzygium aromaticum* L.	Some EOs are rich in phenolic compounds (or some terpenoids and sulphur containing components). These compounds are able to stop or delay the aerobic oxidation	[31]
Immunomodulatory	*Melaleuca alternifolia*	Tea tree essential oil derivative (terpinen-4-ol) reduces the expression of IL-8 (one of the major inflammatory mediators produced by oropharyngeal candidiasis) through mechanisms related to the protein synthesis inhibition. This EO also inhibits other inflammatory processes, as contact hypersensitivity and histamine-induced edema/weal/flare-reaction in human skin.	[39]
Antiprotozoal	*Lippia sidoides* *Cymbopogon citratus* *Thymus vulgaris*	The mechanism of antiprotozoal action of EOs is believed to be twofold: by direct effect on protozoa (disruption of flagellar membranes, mitochondrial swelling, and alterations in the organization of the chromatins); and by interfering with the isoprenoid pathway present in protozoa.	[4, 40]
Antiplatelet	*Foeniculum vulgare* L. *Ocotea quixos* *Artemisia dracunculus L.* *Wasabia japonica*	EOs containing a higher concentration of phenylpropanoids are related to an inhibition of arachidonate cascade and thrombin activity.	[30, 41]

3.3 ENCAPSULATION OF ESSENTIAL OILS

Essential oil constituents may show instability, high volatility, and sensitivity to external factors (for example, light, oxygen, and heat). During the production chain of EOs (extraction, transport and storage), some labile compounds can undergo oxidation and other degradation reactions, losing their function or even forming toxic derivatives [7].

Fortunately, these limitations can be overcome with the correct use of encapsulation technologies, which can provide new properties to EOs. The use of micro_/nanoencapsulation technology can solve several problems related to natural compounds' stability and bioavailability. *Microparticles* can be defined as spherical particles whose size is at the micrometre level (1-250 μm). Nanoparticles are solid colloidal systems whose particle size ranges from 10 nm to 1 μm, although for some pharmaceuticals applications, the suggested maximum size lies below 100 nm. The encapsulation of EOs consists of their incorporation in a carrier system (wall material), which can be composed of carbohydrates, gums, proteins, lipids, natural or synthetic polymeric materials, or mixtures between them. The product's

characteristics - aqueous solubility, release rate, bioaccessibility, bioavailability) - depend on product size (macro, micro or nanoparticles), wall material (e.g. polymeric and lipid nanoparticles), and lipid system used [42]. Reducing EOs volatility and protecting it against environmental factors allows its use in many innovative applications (for example, incorporation in textiles, surface coating, compositions for agricultural spraying, active packing, among others) [3]. The application potential and the biological activity of the finished product depend on its physical and chemical properties. Detoni et al. [43] encapsulated the essential oil of *Zanthoxylum tingoassuiba*, rich in α-bisabolol, in liposomes to assess both the increase in oxidative stability and the ability to reduce the cell viability of glioblastoma. It was concluded that liposome encapsulation protected the EO from temperature-induced oxidation. The encapsulated system performed well against glioma cells, with potential for the treatment of glioblastoma. Similarly, Hădărugă et al. [44] demonstrated the protective capacity of β-cyclodextrin against the oxidation of labile compounds of the EO of *Ocimum basilicum*.

Besides the increase of EOs stability, the encapsulation has also shown to be efficient for modulating their release and increasing the bioactivity, therefore, improving the EOs effectiveness. Donsì et al. [45] reported that the nano-encapsulated terpenes extracted from *Melaleuca alternifolia* showed enhanced antimicrobial activity against food-borne microorganisms (*Lactobacillus delbrueckii* and *Escherichia coli*), with minimal changes in the organoleptic properties of the treated juice. Baldim et al. [33] encapsulated the EO of *Lippia sidoides* in lipid nanosystems and evaluated its antifungal efficacy against *Candida albicans*. The encapsulated EO nanosystem successfully retained its antifungal activity. From a technological perspective, this result is very interesting since the lipid nanosystems are more stable, biocompatible and biodegradable. Saporito et al. [32] encapsulated EOs of *Eucalyptus globulus* or rosemary in naturally-based lipids nanosystems to enhance skin wounds' healing properties. They reported that nanoparticles loaded with the *Eucalyptus* EO showed good bioadhesion, increased cell proliferation *in vitro*, cytocompatibility, antimicrobial activity, and better healing process of skin wounds in the model of burns in rats.

3.3.1 Encapsulation of Essential Oils in Lipid Nanoystems

Recently, encapsulation in lipid systems has increased, mainly in the pharmaceutical and food sectors. Hydrophilic and lipophilic compounds can be encapsulated in these systems, which also present ease and low production cost, low toxicity, increased product aqueous solubility, bioavailability, and stability, and allow sustained-release/drug targeting. These systems are also preferred due to their higher drug loading capacity, economic viability,

suitability for large scale production and versatility, being useful in topical, dermal, transdermal, oral and parenteral formulations [46]. These systems are attractive and efficient methods for the EOs encapsulation, showing advantages over conventional systems such as improved permeation through biological barriers, better bioavailability, and controlled delivery of the bioactive compounds. Most lipid systems are derived from natural sources, being biocompatible and biodegradable, and have the GRAS status [42]. Nanoemulsions, microemulsions, solid-lipid nanoparticles and liposomes are typical lipid systems that have been used to encapsulate EOs [42, 47]. Figure 3.1 shows a schematic representation of different lipid nanosystems, which can be explored for EOs encapsulation. A brief description of lipid systems useful for EOs encapsulation is presented following.

Emulsions

Emulsions are tiny colloidal dispersions formed by mechanical mixing of two immiscible liquid phases plus a surfactant system. They can be classified as water-in-oil (W/O), oil-in-water (O/W), water-in-oil-in-water (W/O/W), or oil-in-water-in-oil (O/W/O) emulsions, being the O/W and W/O/W adequate for EOs encapsulation. Emulsions are classified as coarse emulsions (conventional emulsions - 200 nm – 100 mm), nanoemulsions (< 200 nm) and microemulsions (10 – 100 nm). Emulsions and nanoemulsions are thermodynamically unstable systems, although kinetically stable. They are susceptible to instability processes like coalescence, creaming, phase separation, flocculation, and Ostwald ripening. Their stability and functional performance are strictly related to the choice and concentration of appropriate emulsifiers systems [48, 49].

Emulsions in the nano-size range (e.g., droplet diameters below 200 nm), also known as nanoemulsions, have functional properties that differ considerably from emulsions. The encapsulation of bioactive in nanoemulsions may increase its bioactivity [50]. Nanoemulsions' production requires high energy mixing equipment, as high-pressure homogenizers and microfluidizers. However, the concentration of surfactants is relatively less than that required for the formation of microemulsions. They are kinetically stable systems whose stability (even over gravity) can last for several years, being especially attractive to the industry, especially the food and pharmaceutical. The surfactant choice is also a critical step since it is desirable that the emulsifier rapidly cover the countless new surfaces formed. However, despite the high kinetic and gravitational stability, the reduced droplet size of nanoemulsions favours the growth of larger droplets inside the emulsion, resulting in the phenomenon of instability known as Ostwald ripening [50]. With the decreasing droplet size, the oil solubility in water also

increases, favouring this phenomenon. Essential oils are unsuitable as the oily phase of nanoemulsions since they are partially miscible in water. EOs should be mixed with a highly lipophilic oil to be load in a nanoemulsion to prevent Ostwald ripening [51].

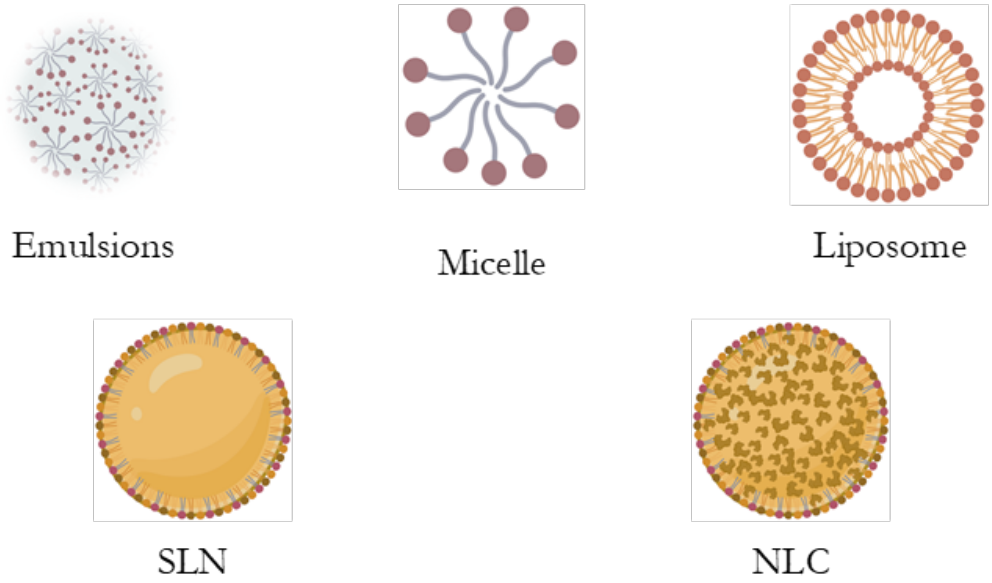


FIGURE 3.1. Schematic representation of distinct lipid nanosystems.

Microemulsions are optically transparent systems, having high thermodynamic stability and low viscosity. These systems are emulsified by a surfactant, normally associated with a co-surfactant [52]. The formation of microemulsions is a very simple and inexpensive process. They are formed spontaneously due to the surfactants' hydrophobic groups' tendency to associate, reducing their contact with the polar solvent. [50].. Microemulsions, however, may be unsuitable for some pharmaceutical applications due to the significant larger amounts of surfactants needed to stabilize them, which can be potentially toxic [51]. Moreover, its loading capacity is limited, and an excess can cause phase separation [50].

Micelles

Micelles are dispersed nanosystems structured in the form of a hydrophobic core enclosed by a hydrophilic shell. Some of their main advantages are the high loading capacity, stability, improved solubilization, prolonged circulation, very small size (1- 50 nm) and targeting potential. The shell stabilizes the system and interacts with different molecules, such as proteins and cells. Versatility is another strength of micelles, as it allows them to assume several shapes and incorporate various solutes with different structures. Surfactant-based micelles are self-assembled when the surfactant concentration exceeds the so-called "critical micelle

concentration" (CMC), reducing the unfavourable interactions between the lipophilic portion of the surfactant and water molecules.

Based on micelles' ability to incorporate lipophilic molecules within their structures, they serve as a suitable delivery system for several functional compounds, among them EOs. The lipophilic bioactive may either be located within the micelle's interior or become part of the surfactant layer. The bioactive is released by diffusion, controlled by pH, chemical, and concentration variations [53].

Liposomes

Liposomes are among the most widely studied colloidal delivery systems and formed the first group of lipid-based nanocarriers administered in *vivo* models. They were invented in the mid-1960s but developed for drug delivery purposes only in the 1970s [51].. Liposomes are self-assembled spherical structures formed from amphiphilic lipids (e.g., phospholipids). Cholesterol or ergosterol are also added to give stability, fluidity and to influence membrane permeability) [50]. The phospholipids are organized in a bilayer, which surrounds the aqueous core, with a diameter ranging from 50 to 5000 nm [54]. Depending on both the mean diameter and the number of bilayers, liposomes can be classified into three groups: (i) one bilayer forming unilamellar vesicles, with size usually up to 100 nm and low aqueous-lipid ratio; (ii) one bilayer forming large unilamellar vesicles, with size between 100 nm and 1 μm; and (iii) multilamellar vesicles, with multiple lipid bilayer membrane and size higher than 100 nm [55]. Liposomes may be used to encapsulate hydrophilic (into aqueous core), lipophilic (into lipid bilayer) and amphiphilic drugs (partitioned at the surface of the bilayers). The amphiphilic character of phospholipids molecules is also an attractive approach to encapsulate EOs [56].

The reduced antigenicity and toxicity and high encapsulation efficiency for different biomolecules are some of the advantages of liposomes. In contrast, difficulties in scale-up and reduced long term stability (by problems as oxidation, hydrolysis and aggregation of the vesicles) are the most limiting disadvantages for their administration. Although liposomes production is a relatively simple process, the final product costs and formulation instability are some considerable disadvantages that limit their full industrial scale-up [57].

SLNs

SLNs were developed in 1990 in parallel by M. R. Gasco, R. H. Müller, and J. S. Lucks, as an alternative transport system to the traditional ones, as emulsions, liposomes, and polymeric nanoparticles [58]. Solid lipid nanoparticles (SLN) are colloidal drug delivery

systems composed of a biocompatible and biodegradable lipid matrix, in which active molecules can be incorporated. The lipid matrix structure is stabilized by a suitable surfactant, very similar to oil-in-water nanoemulsions, except for the solid lipid (at physiological temperatures), instead of the liquid. Usually, the SLN sizes ranged between 150 and 300 nm, though larger and smaller sizes can also be obtained.

The SLNs can encapsulate lipophilic and hydrophilic molecules. These systems exhibit excellent biocompatibility and toxicity, promote target release, and have a fabrication process easy and scalable and do not use organic solvents. Hence, it is an excellent nanosystem for EOs encapsulation. On the other hand, its highly crystalline structure and the unexpected dynamics of polymorphic transitions reduce the load capacity of the SLN and favour the undesirable drug leakage during storage [42, 58].

NLCs

The second generation of lipid nanoparticles, the so-called nanostructured lipid carriers (NLC), was proposed in 1999. NLCs brought solutions to some of the limitations of SLN, such as high crystallinity, low drug loading, and drug leakage during storage. In these systems, the lipid matrix is composed of a mixture of solid and liquid lipids, which increases its ability to incorporate actives, and reduces the degree of crystallinity and consequently the occurrence of polymorphic transitions [59]. The strong immobilization and reduced expulsion of encapsulated molecules during storage are other key advantages of NLCs. These properties make these carriers a convenient system for encapsulating lipophilic compounds, such as the EOs. The lipophilic nature of EOs favours greater retention efficiency, and the release can be modulated by changing the composition of the lipid matrix [58].

3.3.2 Production and Characterization of the Lipid Based Encapsulation Nanosystems

Several lipid nanosystems may be used for EOs encapsulation, such as emulsions (coarse emulsions, microemulsions and nanoemulsions), auto-emulsifying systems SLNs, NLCs, liquid crystals, micelles, and liposomes, among others [60]. Excipients used to produce these systems include fatty acids (e.g., oleic acid), vegetable oils (e.g., soybean oil), semi-synthetic glycerides (e.g., Miglyol®, Capmul® MCM), polyoxyethylene glycols derivatives or macrogolglycerides (e.g., Labrasol®, Labrafil®, Gelucire®), ethoxylated glycerides (e.g., Cremophor®), polyalcohol fatty acid esters (e.g., Solutol®, Tween®), cholesterol and phospholipids (e.g., egg and soybean lecithin) [42]. The excipients' selection should consider the desired product

properties (e.g., sustained release, biodegradability, protection) and the intended administration route.

The methods usually used to encapsulate EOs in lipid systems can be classified into high energy and low energy emulsification methods [21, 46, 47].

High energy emulsification methods

These homogenisation methods require high mechanical energy and are carried out in equipment such as high-speed rotor-stator mixers (ultra-turrax), high-pressure homogenisers, microfluidisers and ultrasonic probes. A positive displacement pump feeds the lipid composition at high pressure through a homogenising valve promoting its emulsification in the high-pressure homogenisers. In contrast, the microfluidisers have an interaction chamber where the fluid is injected and homogenised by cutting, impact and cavitation. Factors such as temperature, viscosity, and concentration of the emulsified system's internal phase and the equipment used have significant effects on the physical-chemical characteristics of the final product. High-pressure homogenisers and microfluidisers apply very high energy to the system. Then, the coalescence of newly formed droplets becomes inevitable. Thus, the optimisation of the process must be carried out together with the appropriate selection of the emulsifying agent's type and concentration to generate a stable submicrometric emulsion with small and homogeneous emulsion droplet sizes and to minimise the need for over-processing [61].

The production of lipid nanosystems by using ultrasound probes has also been widely described in the literature. In homogenisation by ultrasound, reducing the droplet size of a coarse suspension or emulsion is caused using cavitation forces generated by ultrasonic waves generated by an ultrasound probe. Depending on the cavitation energy and homogenisation time, nanosuspensions or nanoemulsions with predefined properties can be produced. However, most of the ultrasonic processing systems still in use cannot be well suited for the aseptic production of pharmaceutical products since ions or particles may be emitted into the product by the cavitation abrasion of the ultrasonic probes, although it is possible to minimise this problem in more sophisticate ultrasonic homogenisers [62].

High-pressure homogenisation and microfluidization techniques are easy to scale up. They are widely used in laboratory research and industrial production, while ultrasound homogenisation is still more used on a laboratory scale. The main disadvantage attributed to high energy emulsification methods is their low energy efficiency. Only a small parcel of the

energy furnished is effectively used for emulsification [63], thus requiring a high energy level to obtain nanometer-scaled droplets.

Low energy emulsification methods

Low energy emulsification methods are frequently more energy-efficient and can produce smaller droplets sizes than the high energy methods (require only gentle stirring) but usually allow lower oil-to-surfactant ratios. These methods generally depend on the modulation of interfacial phenomena/phase transition and intrinsic physicochemical properties of surfactants, co-emulsifiers/co-surfactants and oil to obtain nanometer-sized droplet emulsions. They are usually called phase inversion and self-emulsification methods, depending on the variations or not of the surfactant system's spontaneous curvature during the emulsification process, respectively [63].

Examples of low energy emulsification protocols commonly used are the phase inversion temperature (PIT), the phase inversion composition (PIC), the solvent diffusion (also named solvent displacement), and solvent evaporation methods. In the PIT method, the spontaneous emulsification is carried out by changes in temperature at a fixed composition, while in the PIC, the composition (and interfacial properties) is changed at a constant temperature. PIT method uses the temperature-dependent solubility of nonionic surfactants, such as the polyethoxylated surfactants, to modify their affinity for water and oil as a function of temperature. The oily phase, water and the nonionic surfactant are mixed at room temperature. In the solvent evaporation method, the oil phase is dissolved in a water-miscible organic solvent such as acetone, ethanol and ethyl methyl ketone. The organic phase is then placed in contact with the aqueous phase containing the surfactant for spontaneous emulsification by rapid diffusion of the organic solvent. The organic solvent is then removed from the emulsified system by evaporation under reduced pressure; thus, requiring an additional process step. This technique is simple to be performed on the laboratory scale, but its scale-up for industrial production is tricky.

The main challenges to develop stable emulsified systems reside in the selection and evaluation of their main constituents, namely: emulsifiers (tween, span, phospholipids, amphiphilic proteins and polysaccharides), texture modifiers (sugars, polyols, polysaccharides and proteins), fillers agents (lipophilic materials) and coalescing retardants or Ostwald ripening agents (e.g., lipophilic materials with low water solubility such as long-chain triglycerides) [53].

Physicochemical characterisation of lipid nanosystems

The characterisation of the lipid system is mandatory for product quality control. Particle size, surface area, and structural characteristics of the lipid systems play important roles in controlling the release rate of EOs from the capsules core. The most used techniques for routine particle size measurements are photon correlation spectroscopy (PCS) and laser diffraction (LD). PCS, also known as dynamic light scattering, covers from few nanometers to about three μm. The measurements are based on changes in the intensity of the light scattered by the particle caused by Brownian motion. For larger particles, the LD method provides better results. This technique uses static light scattering and is based on the diffraction angle's dependence on the particle radius. LD measurement range varies from nanometers to millimetres [64].

The zeta potential allows the measurement of the electrical characteristics of a colloidal dispersion. This determination is very important because it allows predicting the nanosystems stability during storage. In general, increasing the zeta potential (modulus) minimises particle aggregation [64].

Although particle size and zeta potential are useful properties for characterising lipid nanosystems, they are not sufficient. The degree of crystallinity and polymorphic transitions also requires special attention since they are linked to the drug incorporation and release rate. The greater the packing density and the thermodynamic stability of the lipids, the lower is the rate of drug incorporation. Differential scanning calorimetry (DSC) and X-ray diffraction are widely used techniques for these characterisations. DSC assesses lipid modifications, determining their melting points and enthalpy of fusion. With X-ray diffraction, it is possible to obtain information on the length of the lipid network's long and short spacings. The drug incorporation decreases in the following order: supercooled fusion <modification α <modification β′ <modification β [64]. Table 3.3 shows techniques widely used to homogenise and reduce the particle size of several lipid nanosystems, as well as recommended methods to characterise them.

TABLE 3.3. Methods commonly used for homogenization and characterization of lipid nanosystems.

Lipid system	Homogenization methods	Characterization methods	Reference
Microemulsion	Requires low energy emulsification methods to be formed, since they are spontaneous self-assembled (dilution of an oil-surfactant mixture with water)	- Emulsion microstructure: optical microscopy - Particle size distribution: static light scattering; dynamic light scattering (d < 2 μm droplets) - Zeta potential - DSC and X-ray diffraction	[65, 66]
Nanoemulsion	- High energy techniques: high-shear stirring, high pressure homogenization and ultra-sonication - Low energy techniques: phase inversion temperature, emulsion phase inversion and spontaneous nanoemulsification	- Emulsion microstructure: electron microscopy (d < 500 nm droplets) - Particle size distribution: static light scattering; dynamic light scattering (d < 2 μm droplets) - Zeta potential - X-ray diffraction and DSC	[47, 66]
Micelle	Similar to microemulsion, micelles are spontaneously formed self-assembled colloidal structures, requiring only the solubilization of surfactant in water	- Particle size and zeta potential	[50, 67]
Liposome	- High energy techniques: ultra-sonication, high-pressure extrusion (through polycarbonate membranes, after lipid hydration) - Low energy techniques: detergent dialysis and solvent injection	- Particle size and zeta potential - Degree of crystallinity and lipid modification (differential scanning calorimetry and X-ray diffraction)	[47, 67]
SLN	- High-pressure homogenization (hot and cold) - Emulsification-solvent diffusion - Emulsification-solvent evaporation - Microemulsion technique - Membrane contactor technique - Solvent injection - Phase inversion - Ultra-sonication	- Particle size and zeta potential - Degree of crystallinity and lipid modification (differential scanning calorimetry and X-ray diffraction)	[42, 64]
NLC	- High-pressure homogenization (hot and cold) - Emulsification-solvent diffusion - Emulsification-solvent evaporation - Microemulsion technique - Membrane contactor technique - Solvent injection - Phase inversion - Ultra-sonication	- Particle size and zeta potential - Degree of crystallinity and lipid modification (DSC and X-ray diffraction)	[42, 64]

3.4 DRYING OF LIPID-BASED COMPOSITIONS LOADED WITH ESSENTIAL OILS

The development of dry, free-flowing lipid-based encapsulation nanosystems has attracted interest from several research groups. These systems may exhibit significant advantages over the liquid and semisolid forms, such as greater stability and shelf-life, easy handling, lower storage volume, and high drug load. Moreover, the dry lipid systems can be directly used to produce conventional solid dosage forms such as pills, tablets and capsules, enhancing patient compliance [68]. Redispersion of the dry product can recover totally or partially the initial lipid nanosystem characteristics when needed. The properties of the encapsulating composition, drying adjuvants (or cryoprotectants), drying method, and processing conditions affect the product redispersion capability. Several methods, including spray drying, spray chilling/cooling/congealing, fluidized bed coating, freeze-drying (lyophilization), centrifugal extrusion, and the physical adsorption on solid carriers (suitable for liquid lipids systems at room temperature), may transform the lipid-based encapsulation nanosystems into free-flow powders or granules [68].

However, the production of dry lipid based nanosystems loaded with EOs and other active phytopharmaceutical ingredients is challenging since it involves several steps, namely: the definition of the original lipid-based encapsulation system, selection of the composition constituents, the choice of the drying method and the drying adjuvants or cryoprotectants, the definition of the processing conditions. Depending on the choice of the original lipid system composition, drying excipients, and drying processing, it is possible to obtain various particulate systems, including solid dispersions, proliposomes, dry emulsions, dry SLN and NLC, among others [68–70].

However, most of the lipids' excipients are liquid at room temperature or have low melting points, and the EOs have high volatility. Thus, the most efficient dryers for these systems are those working at low temperatures, such as freeze-drying and spray freeze drying or those that work at high temperatures but with a short residence time of the product in the dryer. A brief description of drying systems widely used for the produce dry lipid-based encapsulation nanosystems is presented following.

3.4.1 Spray drying

Spray drying is a versatile drying technology, which has been widely used in industry to produce dry, free-flowing powders directly from liquid feeds. This technology has been successfully used to manufacture a wide range of products including, pharmaceuticals,

cosmetics, clays, pigments, soaps and detergents, among other products. A great advantage generally attributed to spray drying is its capability to control the product's physicochemical properties by the correct specification of feed composition properties and dying conditions [71].

Spray drying involves atomization of a liquid feed into a heated flowing gas (normally air), resulting in extremely rapid solvent evaporation (e.g., water). The process is divided into different stages; namely, atomization of the feed liquid, mixing the liquid with the hot gas, solvent evaporation and powder formation, separation and collection of the dry product. Typical values of the inlet gas temperature during spray drying are around 150 – 220 °C (sometimes higher). However, solvent evaporation occurs very quickly, and the residence time of the product inside the dryer is short. Figure 3.2 presented the main steps of the spray dryer process [72].

The versatility, low operating costs, the short residence time of the product inside the dryers, capability to process thermosensitive materials and feasibility for continuous and large-scale production have made spray drying a lead drying and encapsulation technology. This technology's successful use to encapsulate volatile substances, including EOs and flavours, is widely described in the literature [73]. The operation relies on applying a protective coating layer surrounding the EOs constituents to protect from the environment, improve product stability, taste masking, easy handling, among others. The encapsulated product must show a high retention efficiency of the EOs constituents, which should be maintained during the product shelf-life. The encapsulating composition constituents and spray drying conditions affect product properties, such as product granulometry, microstructure, redispersion capability, and bioactive compound retention. These characteristics affect the product functionality, stability and other technological and biopharmaceutical properties [73, 74]. Wall materials successfully used for EOs' spray drying encapsulation include mesquite gum [75]. Acacia gum, blends of maltodextrin (DE 16-20) and whey protein/or modified starch/or small molecule surfactant [76], skimmed milk powder and whey protein concentrate [77], molecular inclusion in cyclodextrins [78], and more recently lipid nanosystems [69].

The main factors affecting the volatiles' retention during spray drying encapsulation are the feed composition's physicochemical properties (e.g., solids content, density, viscosity, surface tension, type and concentration of wall materials and EOs) and the spray drying operating conditions (e.g., feed flow rate and pressure of atomization gas, feed flowrate of the encapsulating composition, inlet and outlet temperature of the drying gas, drying gas flow rate, gas humidity, and residence time inside the drying chamber) [75, 79]. The solids content is one

of the most important factors linked to the retention of volatile compounds in the encapsulated product. The higher the formulation's solids content, the faster is the formation of the superficial crust, which acts as a sem-permeable membrane promoting the high retention of volatiles [79]. On the other hand, the volatile constituents' retention tends to decrease conversely with the EO amount added to the encapsulating composition. Usually, the ratio of EO: wall material are 1:4, or lower [75, 79].

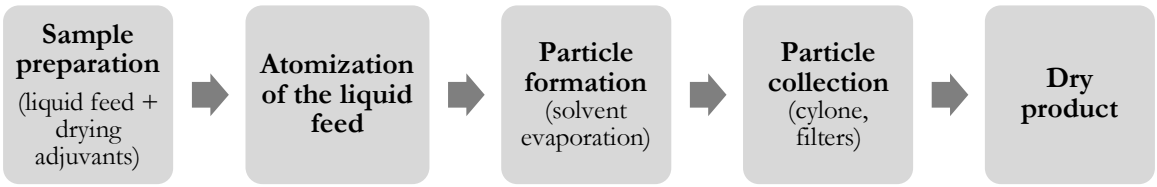


FIGURE 3.2. Main steps involved in spray drying.

3.4.2 Freeze-drying

The freeze-drying or lyophilization involves the rapid cooling of the material at temperatures and vacuum pressure below the triple point of water (0.01 ° C and 6.1 mbar). In these conditions, the sublimation of ice occurs. The speed of the freeze-drying is generally increased by reducing the pressure with a minimum value. The vapour condenser temperature must be kept below that of the frozen product [80], maintaining a temperature gradient with the frozen sample. Freeze drying is a drying method widely used in the pharmaceutical industry, and several freeze-dried products are regularly marketed.

The typical freeze-drying operation involves three stages (see Figure 3.3): freezing, primary drying, and secondary drying. The freezing phase is a cooling step where most of the solvent (e.g., water) is separated from the formulation components, resulting in ice formation. The primary drying is initiated when the chamber pressure is reduced to a few millibars. The shelf temperature is increased to supply a sufficient amount of heat to the frozen sample for water sublimation. The water is desorbed from the frozen formulation at a slightly higher temperature and low pressure during the secondary drying stage [81].

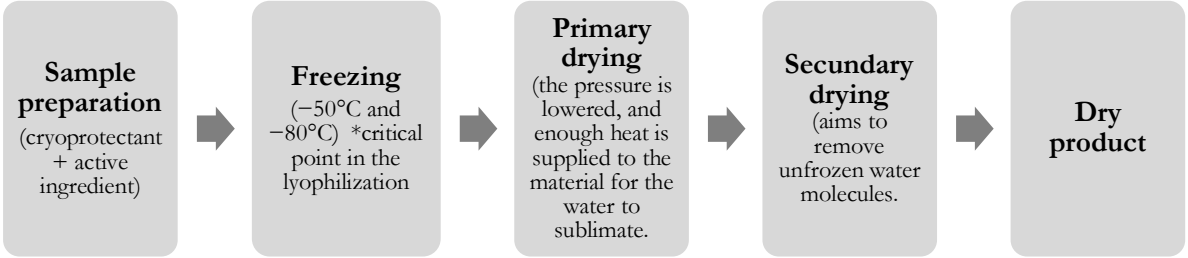


FIGURE 3.3. Main steps involved in the freeze-drying (lyophilization) procedure.

During the freeze-drying, the product temperature remains, and oxidisable substances are protected under vacuum conditions. The product' reconstitution is easier and quicker (microscopic pores are present in the dry sample and the constituents of dried material remain homogeneously distributed). The flavours and smells generally remain unchanged, with little product contamination owing to an aseptic process. It can be mentioned as disadvantages:

1. The loss of volatile compounds by the system's high vacuum and the expensive processing costs.
2. Amorphous (glassy) materials must be maintained at conditions below their critical point to prevent sample thaw or collapse during primary and secondary drying.
3. If too much heat is added, the material's structure could be altered.
4. Freezing damage can occur with labile products such as liposomes, emulsions, proteins, and viruses.

The residual moisture influence product stability during long-term storage [82].

The formulation of a freeze-dried product may require the addition of various excipients, like buffers (phosphate buffer), bulking agents (mannitol, sucrose or one of the other disaccharides), tonicity adjusters (mannitol, sucrose, glycine, glycerol, and sodium chloride), stabilisers (sucrose, trehalose, glucose, lactose, maltose) aiming to protect and to maintain product structure; generating a product of high quality and elegant appearance. The final product quality depends on the freeze-drying conditions (temperature and cooling rate, vacuum operation, heat supply rate), type and amount of excipients, among other factors [80, 82, 83].

3.4.3 Spray Freeze-Drying

Spray freeze drying (SFD) is a relatively novel technique used in the pharmaceutical field. This process combines spray drying and freeze-drying. Basically, SFD involves the atomisation of a feed solution into a cryogenic medium (e.g., liquid nitrogen) followed by the frozen dispersion's lyophilisation. As heat stress is avoided, this technology may be suitable for processing thermosensitive substances, allowing the formation of powdered particles with optimised particles characteristics [81]. It has proven benefits over other drying methods in producing products with improved structural integrity (the shrinkage is minimised), superior quality, and better storage stability [86]. Compared to spray drying, this process produces light and porous particles with enhanced aerosol performance, and the production yield is almost 100% [84].

The process steps involved in the spray freeze drying include the droplets generation (atomisation), almost instantaneous droplets freezing, primary drying, and secondary drying (Figure 3.4). Similar to spray drying, the SFD involves the spraying of the liquid composition; however, instead of atomising into a heated gaseous stream, the liquid feed is atomised directly into a cryogenic medium, and fast freezing of droplets takes place. The frozen droplets are collected by sieves or following the evaporation of the cryogen. The frozen particles are then transferred to pre-chilled shelves of a freeze-dryer, following the conventional freeze-drying. One advantage of SFD is that sublimation and secondary drying of the frozen particles are faster than those achieved in conventional freeze-drying due to the increased surface area of the frozen sample [85, 86]. The SFD is a good option to dehydrate lipid-based encapsulation nanosystems such as emulsions, liposomes, SLNs and NLCs, forming a product with high aqueous dispersibility.

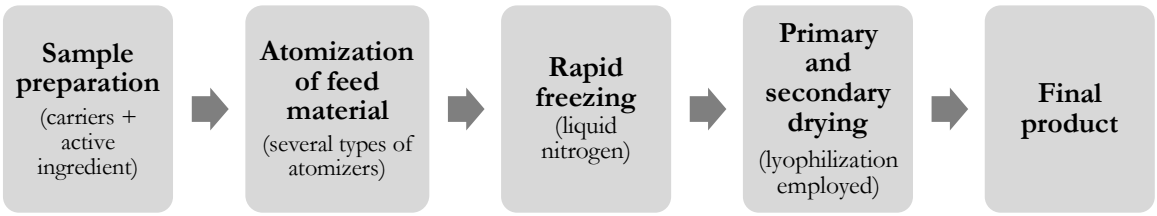


FIGURE 3.4. Steps involved in spray freeze drying.

The atomisation step is a key factor of the SFD, which affects the particle size distribution of the sprayed droplets directly. Several atomisers can be used for this step, such as centrifugal, ultrasonic, and two-fluid, three fluid or four-fluid nozzles. Factors influencing atomisation include feed properties (e.g., surface tension, rheology, solids content) and the atomising conditions (e.g., feed flow rate of the composition, feed flow rate, and pressure atomising fluid). Hence, to achieve smaller droplet sizes, high atomisation pressures (or energy) are required. The atomised composition's fast freezing is carried out below the sub-zero temperatures in a cryogenic fluid such as liquid nitrogen. Surfactants and cryoprotectant can be added to the feed composition to minimise the stresses caused by atomisation, composition freezing and drying steps. The solid content of the feed composition has significant effects on particle porosity. In general, solid content and particle porosity follow an inverse relation. However, a persistent reduction in the solid content would result in spherical structures' breakage due to individual particles' mechanical instability [86].

The physical-chemical properties of SFD particles (sizes, densities, and high specific surface area) show superior stability in the lungs, nasal mucosa, intestine and skin than those obtained by other drying technologies [86]. SFD processing of poorly water-soluble drugs

formulated with polyvinyl alcohol, polyvinylpyrrolidone, poloxamer and other polymers improved their wetting and dissolution capability [85, 86]. The ability to encapsulate poorly water-soluble drugs and the unique aerodynamic qualities of the porous particles produced have made this process particularly attractive for producing particles for pulmonary delivery [89].

In general, SFD offers some advantages over conventional freeze-drying, including faster drying times, less energy consumption during the dehydration stage and flexibility during scale-up. However, some of these advantages seem to be offset by energy consumption during freezing and difficulties inherent in spray-based processes. Significant amounts of cryogenic fluids may also be required to freeze the atomised composition, and processing under aseptic conditions can be challenging [87].

3.4.4 Spray Congealing

Spray congealing, also known as spray chilling, spray cooling or prilling, when a hot molten mixture is atomised in a cooling chamber, which solidifies the resulting droplets to form almost spherical powder particles. The active compound, matrix material and spray-frozen particles are also referred to as drug/core, carrier and microparticles, respectively. The matrix material must exist as a solid at room temperature and have suitable melting point ranges, typically from 50 to 100 ° C [88]. The lower melting points can cause product agglomeration, and the high melting point can lead to process malfunctioning, such as blocking the feed lines and atomisation systems. It is a low cost, simple, versatile and ‘green’ method to prepare microparticles without solvent use, having applications in the food, nutraceutical and pharmaceutical fields [89].

Spray congealing represents an interesting and successful approach to obtaining solid dispersions/solutions for various purposes, such as mask unpleasant taste and odour, improve the bioavailability of poorly water-soluble substances, and control the drug release from drug delivery systems. The technique has aroused great interest in the pharmaceutical sector due to the possibility of using lipids as carriers in drugs and biopharmaceuticals delivery systems [90].

The spray congealing principle is similar to spray drying and includes atomisation, particle formation and product collection. The key difference between both processes resides in that spray congealing use a cooling chamber instead of a drying chamber. The cooling chamber’s temperature is kept well below the matrix material’s melting point, which can overcome some limitations of spray drying, particularly concerning the volatiles’ thermal loss. Another difference between spray drying and freeze-drying is that spray chilling allows lipid-based

carrier materials (e.g., fats, waxes, polyethene glycols, fatty acids, and fatty alcohols) alone [91]. The production process is often faster because solvents are not used (100 % of solids content), and high encapsulation efficiency values (90-100%) can be reached [89]. The ability to control powder characteristics (particle size, morphology, density) without the need for other downstream processing methods (e.g., secondary drying, granulation, milling, pelletisation) offers a remarkable advantage over other particle formation methods. A major disadvantage of spray congealing is that the drug must be stable at the temperature required to melt the matrix material [88]. Figure 3.5 present the main steps involved in the spray congealing procedure.

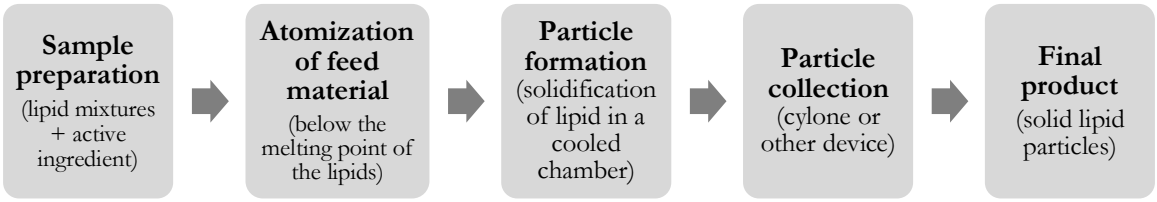


FIGURE 3.5. Main steps involved in the spray congealing procedure.

For successful operation of the spray congealing, the processing conditions must be accurately specified. For example, the change in the feed system's temperature may alter its viscosity and affect the product properties. The heat losses in the feed lines can cause product solidification inside, causing their blockage. The nozzles or atomizers used in spray congealing must handle viscous mixtures at high temperatures. The cooling chamber dimensions depend on the nozzle, but different chamber designs can be used) [92].

Spray dryers can be changed to spray congealing units since both processes exhibit high similarity. In spray drying, a hot gas flow is used to evaporate the water or solvent from the atomized droplets; in spray congealing, a cold gas flow is used to freeze the atomized molten droplets [91]. The main factors affecting the spray-congealed microparticles can be classified into two groups: equipment configuration and process variables (chamber design, atomizer types, operation temperatures) and formulation variables (matrix constituents, additives, and actives). Microparticles with desired physicochemical properties can be produced by selecting spray congealing processing conditions. The technique can be used to encapsulate proteins and peptides, probiotic microorganisms, vitamins, minerals, and flavours [90].

3.5 APPLICATIONS OF ENCAPSULATED ESSENTIAL OILS IN FOOD AND PHARMACEUTICAL PRODUCTS

The encapsulation of EOs in lipid-based nanosystems is a relatively new and promising strategy, permitting the development of innovative products, expanding the application

potential in a wide range of products. There are many lipid materials with unique distinct physicochemical properties and diversified production methods (see section 3.3.1), permitting the design of lipid-based encapsulation systems with preset characteristics. Several lipid systems can be used, such as nanoemulsions, microemulsions, solid lipid nanoparticles, nanostructured lipid carriers, and liposomes, among others. These systems can be produced by low- and high-energy emulsification methods, as already described in section 3.3.2. A well-designed system can enhance the antimicrobial and antioxidant activity of EOs, improve their stability, and facilitate their incorporation and distribution in food systems without causing changes in the organoleptic properties [93].

Encapsulated EOs have been applied in various commercial products for several purposes. Typical examples include:

- Food additives (natural antimicrobials and antioxidants, food flavours);
- Pharmaceuticals (antimicrobials, permeation promoters, herbal medicines);
- Cosmetics (perfumery, cosmeceuticals, mouthwashes);
- agroindustry (crop protection);
- veterinary medicine (natural anti-inflammatories) and as growth promoters for farm animals.

Encapsulated EOs can also be used in several other applications, such as antimicrobial and antioxidant active packings, edible packing and coating, insect repellent, medical textiles and membranes (smart textiles), among others [94, 95].

In this section, we present a brief overview of some food and pharmaceutical applications of EOs, in which the use of the encapsulated products may provide better results.

3.5.1 Food Antimicrobials

Food spoilage microorganisms and pathogens are responsible for significant losses and wastes in the agricultural and food sectors and can cause serious illnesses and even death of the consumers, respectively. EOs of aromatic and medicinal plants have demonstrated efficacy against several organisms which cause harmful effects over food products, such as insects, parasites, fungi and bacteria. Several *in-vitro* and *in-situ* studies have shown the efficacy of EOs against a broad spectrum of food pathogens, which usually may contaminate processed, and unprocessed food products. Common food pathogens include [6, 96]: a) Fungi (*Fusarium* spp., *Alternaria* spp., *Penicillium* spp., *Aspergillus* spp.); b) Bacteria (*Salmonella* spp., *Rhizoctonia*

solani, *Clostridium perfringens*, *Campylobacter spp.*, *Staphylococcus aureus*, *Escherichia coli O157:H7*, *Listeria monocytogenes*); c) Virus (norovirus, hepatitis A virus); and d) Parasites (*Toxoplasma gondii*, *Cryptosporidium* spp., *Trypanosoma cruzi*). EOs can also overcome the problems of microorganisms' resistance to conventional synthetic preservatives; and can be used alone or combined with other products (e.g., artificial preservatives).

Successful studies concerning the encapsulation of phytochemicals, EOs and extracts of aromatic in lipid nanosystems have been reported recently and include: rosmarinic acid (SLN)[97], Rosemary extract (NLC and proliposomes[70, 98], Sage and Savoury extracts (SLN)[97], Clove buds extract (NLC)[99], Clove EO (SLN and NLC)[100], Pepper-rosemary EO (*Lippia sidoides*, NLC)[33], Frankincense and Myrrh EO (SLN)[101], and others.

The synergistic effect of EOs constituents when loaded in different lipid-based nanosystems (nanoemulsion; SLNs, liposomes) for the preservation of grains and related food products (e.g., cooked rice, rice flour, sliced bread) has been discussed by Kaliamurthi and coworkers [93]. The authors emphasize the importance of exact quantification of the EOs constituents in the final lipid nanosystems, which is not well addressed in several studies reported in the scientific literature.

The increase of the EOs' antimicrobial activity, when incorporated into the lipid nanosystems, can be caused by distinct mechanisms of action, namely [102]):

- Disturbance and disruption of the integrity of the membrane followed by leakage of intracellular content and permeation of extracellular content into the cell interior;
- Diffusion of EOs constituents into the cell interior causing the dissociation/disruption of the proton transfer dynamics of the cell, which act simultaneously due to the larger number of substances present.

3.5.2 Natural Antioxidants for Food and Pharmaceutical Products

The oxidation of unsaturated oils and fats in food products is one of the most common food degradation processes, marked by the characteristic rancid odour (rancidity). Rancidity is caused by transforming the unsaturated constituents of oils and fats into hydroperoxides (caused by oxygen). Hydroperoxides can decompose into various volatile compounds (for example, alcohols, aldehydes, ketones and hydrocarbons), some of which are responsible for the unpleasant odours exhaled by rancid foods. Oxidation can also degrade proteins, pigments and other substances present in processed and unprocessed foods. These changes in the product's

characteristics significantly reduce its quality (and shelf life), making them unsuitable. To delay the occurrence of these harmful changes during the whole product shelf life, synthetic and natural antioxidant substances are normally added in small concentrations compared to the oxidizing substance [103].

Antioxidants are also beneficial to human health. Many diseases occur due to an overload of oxidative reactions arising from excessive meat, sugar, and fat consumption. There is increasing evidence that links degenerative diseases, such as diabetes, dementia, cancer, heart disease, and others, with the cell damage caused by reactive oxygen species (ROS), comprising both the free and non-free oxygen intermediates. Common ROS includes superoxide and hydroxyl radicals, hydrogen peroxide, singlet oxygen, and nitrogen dioxide. Antioxidants are synthetic or natural substances able to protect organisms from oxidative stress prevent free radical-induced tissue damage by preventing the formation of radicals, scavenging them, or by promoting their decomposition. Several scientific studies have reported that the presence of natural antioxidants in the diet could be an important tool for preventing degenerative diseases. These effects depend on the concentration of the natural antioxidant compounds. However, studies are showing that high concentrations of certain polyphenols can induce cellular DNA damage. Antioxidants, such as retinol and tocopherol, also shown to be toxic in high doses, whereas their use in small amounts showed effective antioxidative and antimutagenic activity [104].

Some toxic and even potential carcinogenic effects have been associated with the synthetic antioxidants usually used in food and pharmaceutical products, such as the butylhydroxy-anisol (BHA), butylhydroxy-toluene (BHT), tert-butylhydroxyquinone (t-BHQ), trihydroxy-butylphenone (THBP) and propyl gallate (PG) In recent years, as a result of these studies, the Joint Expert Committee on Food Additives (JECFA) of the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) has restricted the values of acceptable daily intake (IDA) of the synthetic antioxidants [105]. Thus, the search for effective, nontoxic natural antioxidant compounds for use in food or pharmaceutical products has been intensified [106].

The use of natural antioxidants in the food, cosmetic, and therapeutic industry is a promising alternative for synthetic antioxidants in respect of low cost, highly compatible with dietary intake and does not cause harmful effects inside the human body. Several phytochemicals, mainly the phenolic compounds, have been identified as free radicals' scavengers. Several studies show the antioxidant potential of fruits, berries, cherries, citrus, prunes, olives, and aromatic and medicinal plants [107]. Rosemary extracts, for example, are

commercially available for use as a natural antioxidant for foods in Europe and the USA and has received the GRAS status, and is considered safe and effective [108].

Various studies have been developed to evaluate the antioxidant activity of the EOs, most of which have demonstrated the strong radical scavenging capacity and ability to inhibit lipid peroxidation of fats and oils [109]. However, the antioxidant activity of some EOs constituents can also be produced by the transformation of hydroperoxides into stable substances (secondary antioxidant). Besides antioxidant activity, EOs pure and their isolated substances also exhibit other interesting biological activities, which can be beneficial to human health, most linked with their capacity to scavenge the ROS. Most of the antioxidant activities of EOs and plant extracts are due to the presence of phenolic constituents; such as the phenolic acids (e.g., caffeic and rosmarinic acids), phenolic monoterpenes (e.g., thymol, carvacrol, eugenol), phenolic diterpenes (e.g., carnosol and carnosic acid). Most of these compounds are present in high concentrations in various EOs and linked with their antioxidant activity [110].

The encapsulation of the EOs in different lipid-based nanosystems is an effective way to modulate their physicochemical properties, making it possible to use them as natural antioxidants in various food and pharmaceutical products having diversified characteristics.

3.5.3 Cosmetics

Today, consumers around the world are increasingly focused on health and beauty. The interest in natural cosmetics opened a window of opportunity for novel products or reformulation of others by adding phytochemicals and functional ingredients. The EU Cosmetics Regulation (Regulation (EC) 1223/2009) defines *cosmetics* as a product to be used on external parts of the body (for example, epidermis, hair, lips, nails, genitals) or on teeth and mouth mucosa for cleaning, perfuming, protecting or correcting bad smells [111]. EOs have played a key role in the perfume and cosmetics industry as fragrance ingredients due to their sweet and mild smell [112]. They can enhance the product properties and act as a preservative agent. The EOs characteristics allow the formulation of innovative products for health, beauty, and well-being, adding value to the products.

Cosmeceuticals are cosmetic products with medicinal properties. In essence, they are cosmetics products containing active ingredients (e.g., antioxidants, phytoextracts, phytochemicals, and UV filters). Sometimes, cosmeceuticals are named phytocosmetics when phytochemicals or phytoextracts are the main active ingredient. Phytocosmetics are currently used for various beauty treatments for skin, face, lips, hair, and nails with beneficial actions

against photoaging, inflammation, hair loss, lip care, psoriasis, ultraviolet toxicity (UV), and so on. Compared to synthetic cosmetic ingredients, phytochemicals/phytoextracts are generally milder, biodegradable, and have less toxicity. The use of preservatives can be minimised or even eliminated in most phytocosmetics containing EOs (for example, rosemary oil, eucalyptus oil) due to their antimicrobial and antioxidant activities [111]. However, the low solubility, low penetration, and physicochemical instability when applied to the skin are some of the problems to be overcome in the formulation of phytocosmetics containing EOs. Different nanotechnology-based systems, including lipid nanosystems, have been used successfully to solve these problems, and new products are already on the market [113]. The encapsulation can protect and prevent the loss of the EOs volatile aromatic ingredients and enhance their solubility, stability, bioavailability, and functionality of the EOs containing cosmetics. The use of lipid-based nanosystems offers interesting possibilities, with a vast quantity of biocompatible and biodegradable lipids and surfactants [42]. Lipid nanosystems loaded with EOs, phytochemicals, or phytoextracts are already in the market. They contributed to increasing consumers' interest in products containing them [112]. The effective production and implementation of phytocosmeceuticals is a multidisciplinary task since, and involves selecting and characterising the active ingredients, activity and toxicity assays, product formulation, safety assessment, and product performance and efficacy testing in humans [114].

3.5.4 Mouthwashes

Oral hygiene is an important aspect of periodontal health. There is a balance in a person’s oral microbial population, which helps prevent opportunistic microorganism proliferation. An optimum mouthwash has some advantages, such as having antiseptic effects on the mouth, washing the food residue on the gingival (gum) medium and teeth, reducing the mouth bacteria, masking and neutralizing halitosis, and introducing a good taste and sense of freshness in the mouth [115].

There is growing interest in oral health care professionals searching for therapeutic agents that complement and enhance the mechanical removal of biofilms in the oral cavity. Oral biofilms are mainly constituted by gram-positive and gram-negative bacteria, which produce various metabolites that induce gingival inflammation. The mechanical plaque removal is not performed sufficiently by the majority of the population. Hence, antimicrobial mouthwashes that increase daily home care can provide an effective means of removing or controlling bacterial plaque to limit gingivitis and periodontitis.

The most prevalent oral infectious diseases, such as dental caries, periodontal inflammations, and gingivitis, are produced by dental biofilm formation in the oral cavity. *Dental plaque* is a multifaceted biofilm that builds upon the surface of teeth, including more than 500 bacterial species. Moreover, it has recently been shown that oral microorganisms can cause other severe or chronic infectious diseases. The value of mouth and teeth hygiene has been known from ancient times until now [115].

A mouthwash may be recommended to treat infection, reduce inflammation, relieve pain, reduce halitosis or deliver fluoride locally for caries prevention. Among the active compounds most used in obtaining mouthwashes, it can be mentioned [116]:

1. chlorhexidine gluconate (cationic bis-guanide with broad-spectrum antimicrobial activity);
2. benzydamine hydrochloride (analgesic, anti-inflammatory, antimicrobial and anaesthetic properties), cetylpyridinium chloride (quaternary ammonium compound with antiseptic and antimicrobial properties);
3. sodium benzoate (act by dispersing fatty, proteinaceous and carbohydrate substances);
4. triclosan ([2,4,4'-trichloro-2'-hydroxydiphenyl ether], increase the ability of mouthwashes to bind to the oral mucosa and thus be available for longer periods).

Normally, oral hygiene products based on synthetic substances predominate over those containing natural compounds, perhaps due to their easy availability and faster results. Many of these products have been criticized for their adverse effects on teeth, gums and mucous membranes. DNA damage in cheek cells is one of the most common side effects of mouthwashes [117]. Thus, phytochemicals are being investigated and added to oral hygiene products as protective agents (antimutagenics. Most EOs have a strong antimicrobial and antioxidant activity, having a high potential for application in products for oral health. Examples of EOs used in oral care products include cumin, clove, rosemary, thyme, black sesame, cinnamon, and black pepper. Mouthwashes containing four phenol-related essential oils (thymol, eucalyptol, menthol and methyl salicylate (in up to 26% alcohol) claim to penetrate the plaque biofilm and kill microorganisms that cause gingivitis. These mouthwashes display broad-spectrum antimicrobial activity, prevent bacterial aggregation, slow bacterial multiplication, retard plaque maturation and decrease plaque mass and pathogenicity. Their mechanism of action is thought to involve bacterial cell destruction, bacterial enzyme inhibition, and endotoxin extraction from Gram-negative bacteria. They also have anti-

inflammatory and prostaglandin synthetase inhibitory activity and act as antioxidants by scavenging free radicals.

The EOs reduced plaque, gingivitis and halitosis, as proven by clinical studies. This activity was linked to their bactericidal and plaque-permeating capabilities. Mouthwashes containing essential oils have been recommended as an adjunct to mechanical oral hygiene, particularly in patients with poor oral hygiene and those who suffer from gingival inflammation, despite regular brushing and flossing. Mouthwashes containing EOs offer additional benefits such as control of malodor by killing the odour-causing bacteria. EOs mouthwashes inhibited plaque and gingivitis and killed some subgingival plaque bacteria. In patients with dental implants, rinsing with EOs mouthwashes reduces plaque and gingivitis indices, oral malodour and also reach areas that are difficult to access or highly vulnerable to plaque accumulation [118].

3.5.5 Phytotherapy

The knowledge of the composition and several biological activities of the EOs have been useful for developing herbal derived products, including pharmaceuticals, medicinal supplements, and nutraceuticals, which can be used for preventive and curative purposes. Products containing EOs have been used as the main or complementary medicine agent in several nations' traditional and complementary medicine systems, and these products' interest has constantly been growing [119]. EOs have been used as antimicrobial, antiviral, antioxidant, antihypertensive, gastroprotective agent, anti-atopic dermatitis, antitussive, anti-inflammatory, immunomodulator, antimutagenic, anticancer, antiprotozoal, among other uses [120].

EOs were effective for the treatment of several pulmonary related disorders, such as cough and nasal congestion (ex. *Eucalyptus* EO, Mint EO, Thyme EO, menthol, 1,8-cineole), improvement of the lung health (Ginseng EO), chronic obstructive pulmonary disease (Lavender EO), and others. Common examples of medicinal products containing EOs are the "Vicks Vapourub" family and several muscle strains creams and gels. Recently in Brazil, the pharmaceutical company Aché launched the topical anti-inflammatory Acheflan®, which contains in its composition 2.3-2.9% of α-humulene from the EO of *Cordia verbenaceae* (Erva-baleeira), a native Brazilian plant [121].

In the Aromatherapy practice, EOs are applied in inhalation, vaporization, and massages, aiming to relieve various ailments, as aches, pains, muscle strains, depression, stress, anxiety, and energy and short-term memory enhancement [122].

At present, the emergence of several pathogens resistant to conventional treatments is one of the most serious public health problems in the world. Resistant microorganisms are generally associated with hospital infections that are difficult to treat. Hence, there is a need for the pharmaceutical market to develop new drugs to fight these drug-resistant microorganisms. The EOs, antimicrobial action occurs by several mechanisms since they are constituted by several active molecules acting synergistically. Thus, they are a promising and innovative alternative source of antimicrobial agents to develop new therapies to combat antimicrobial resistance, either in isolated form or in association with traditional antibiotics [33, 123].

3.6 FINAL REMARKS AND FUTURE PERSPECTIVES

The encapsulation of EOs is a research subject that has attracted the interest of Academia, Industry, and consumers due to several biological properties and the high potential of technological applications. The EOs encapsulation protects them from environmental factors (e.g., oxygen, light), among others. The EOs' encapsulation may improve their physicochemical properties and can enhance their biological activity, water-solubility, bioavailability, reduce volatilization, taste and odour, and skin irritation. Encapsulation in lipid nanosystems is a promising strategy since different systems can be produced according to the desired objectives. Most lipid materials are also biocompatible, biodegradable, and non-toxic, making them ideal for developing pharmaceutical and food products, even for oral and parenteral use.

In the perfumery and cosmetics sector, encapsulated EOs are used in skin creams, body lotions, balms, shampoos, soaps and perfumes, and mouthwashes and breath fresheners. This sector invests significant resources in developing new products to satisfy the consumer's demand, which prefers products formulated with natural ingredients. The use of lipid nanosystems loaded with EOs has enormous potential for developing a future generation of novel "smart products", expanding applications [114].

In the food & beverages industry, the EOs encapsulation expands the range of applications. For example, encapsulated EOS can be easily incorporated into hydrophilic food systems. Several conventional and new applications in the food sectors are already being explored, including natural preservatives, flavouring agents, insect repellent to protect stored foods, antimicrobial and antioxidant edible films and active packings to increase the shelf life of perishable foods. The market of encapsulated flavours for use in most diverse food systems is also very relevant and is growing significantly.

Another sector with a high potential to use encapsulated EOs is aromatherapy. In Europe, for example, there is a high demand for aromatherapy products, as consumers look for alternative methods to improve their health conditions. Essential oils have a wide range of stress-relieving, anti-bacterial, soothing, refreshing, and invigorating properties. They are also used to treat or alleviate symptoms of indigestion, stress, insomnia, anxiety, depression and eczema. The encapsulation can improve the EOs properties, which can increase the adherence of the consumer in this practice, and ever furnish better results.

The production of dry, free-flowing and dispersible lipid nanosystems loaded with EOs is also an important subject and increases the possibilities of applying EOs in the most diversified products. These innovative nanoparticulate systems can be produced by several technologies and involve two stages, the preparation of the emulsified systems (high or low energy methods) and the selection of the drying technology (spray drying, spray freeze-drying, freeze-drying, spray congealing, and so on). Spray freeze-drying and the spray congealing methods are promising encapsulation technologies since they are performed at low temperatures, minimizing the degradation or losses of thermosensitive or volatile constituents of EOs, compared to spray drying.

This chapter presented a comprehensive overview of the general aspects of EOs, characterization methods, biological activity, the potential of application mainly in food and pharmaceutical sectors, and their encapsulation in lipid nanosystems, describing several systems and production methods. The production of particulate dispersible lipid nanosystem loaded with EOs, describing several particle formation methods, is also addressed. The information presented is not exhaustive, and readers interested in more information can consult the references cited.

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CHAPTER 4. ROTAS TECNOLÓGICAS PARA ENCAPSULAÇÃO DE ÓLEO ESSENCIAL EM LIPOSSOMAS: PREPARAÇÃO E CARACTERIZAÇÃO

Esse artigo foi apresentado na forma oral no Simpósio comemorativo dos quarenta anos da área de sistemas particulados do DEQ/UFSCar, em 16 e 17 de maio de 2019, e publicado na forma de artigo completo nos Anais.

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4.1 INTRODUÇÃO

Atualmente existe uma tendência na utilização de ativos naturais devido às evidências científicas comprovando os efeitos benéficos à saúde. A atividade biológica de produtos de origem vegetal está associada a metabólitos secundários sintetizados pelas plantas e incluem diversas classes de compostos, como por exemplo, os carotenoides, alcaloides, polifenóis, terpenos e saponinas. Muitos destes compostos exibem significativas propriedades biológicas, como por exemplo, antioxidante, antimicrobiana, anti-inflamatória, e antitumoral, com grande potencial de exploração nas indústrias farmacêutica, alimentícia, química e cosmética.

Estudos mostram que o óleo essencial de *Lippia Sidoides* (Alecrim-pimenta) apresenta atividade antimicrobiana contra diversos fungos, como *Microsporum canis*, *Candida spp* e *Cryptococcus neoformans* (1,2) e bactérias, como *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* e *Bacillus subtilis* (3,4). Seu componente majoritário é o Timol (68,5%), seguido pelo p-cimeno (10,7%), e os demais componentes correspondem a 7,5% do óleo essencial.

Compostos fitoquímicos, em geral, possuem sabor e odor desagradável o que pode prejudicar seu uso como medicamento ou suplemento/aditivo alimentar. Esses compostos podem ainda ser voláteis, termossensíveis e susceptíveis à oxidação, encontrando-se muitas vezes na forma líquida nas condições ambientais, podendo sofrer alterações irreversíveis de suas propriedades físico-químicas quando expostos sem proteção ao contato com outros materiais ou agentes externos.

Portanto, o desenvolvimento de mecanismos de proteção que possam manter a estabilidade dos ativos até o momento do uso e proporcionar sua liberação em um alvo fisiológico específico, é de fundamental importância. Estes sistemas podem conferir inúmeras vantagens a produtos naturais incluindo o aumento da solubilidade, melhoria da atividade biológica, aumento da estabilidade, liberação sustentada, redução da degradação devido a ação de fatores físicos e químicos, dentre outras vantagens (5,6). A encapsulação de ativos vegetais consiste na inclusão do agente ativo em um invólucro protetor o qual pode ser um filme composto de derivados de carboidratos, gomas, proteínas, lipídios ou polímeros.

Lipossomas são vesículas que possuem um núcleo aquoso cercado por uma ou mais bicamadas de fosfolipídios. São estruturas capazes de encapsular moléculas hidrofílicas no núcleo aquoso, hidrofóbicas na bicamada de lipídeos e anfifílicas na interface entre lipídeo e água (7). São biocompatíveis, biodegradáveis e possuem baixa toxicidade, sendo assim eles têm potencial aplicação farmacêutica (8). Porém possuem baixa capacidade de encapsulação de moléculas hidrofóbicas, que podem ser facilmente liberadas da bicamada lipídica (9).

Por outro lado, complexos de inclusão de ciclodextrinas são alternativas viáveis para aumentar a solubilidade em água e a estabilidade de ativos. McCormack e Gregoriadis (1994) foram os primeiros a idealizar a utilização conjunta de lipossomas e complexos de ciclodextrina, de maneira a melhorar a encapsulação de moléculas hidrofóbicas, por meio da complexação destas com ciclodextrina, e a encapsulação desse complexo no núcleo aquoso de lipossomas, e Sebaaly e colaboradores (2016a) realizaram esse processo com óleo essencial de Cravo, obtendo bons resultados de eficiência de encapsulação.

Esse trabalho empregou o óleo essencial de *Lippia sidoides*, visando investigar técnicas para a obtenção de sistemas lipossomais para sua estabilização, enfatizando-se processos de preparação, tipo de excipientes e procedimentos para a caracterização físico-química e estabilidade.

4.2 MATERIAL

Óleo essencial de *Lippia sidoides* (Pronat - Produtos Naturais LTDA, Brasil). Reagentes, solventes e padrões analíticos: água purificada por osmose reversa; água ultrapura Mili-Q; álcool (n) butílico e álcool etílico absoluto (Labsynth, Brasil), acetonitrila e metanol, ambos adquiridos da Sigma-Aldrich Chemical Co (São Luis, EUA). Agentes encapsulantes e carreadores de secagem: Lecitina de soja (Phospholipon 90H - Lipoid, Alemanha), colesterol (Sigma-Aldrich, Brasil), goma xantana, manitol (Natural Pharma Produtos Farmacêuticos Ltda,

Brasil) e hidroxipopil-beta-ciclodextrina (Sigma-Aldrich, Brasil), goma arábica (Fibregum® B, Colloides Naturels, Brasil), Lacprodan (concentrado proteico de soro de leite - Arla Foods Ingredients S/A, Argentina), lactose e Aerosil® 200 (Evonik Degussa, Alemanha) e Capsul (National Starch Food Innovation, Brasil).

4.3 OBTENÇÃO DE LIPOSSOMAS PELO MÉTODO DE INJEÇÃO DE ETANOL

Esse método pode ser utilizado para produzir lipossomas unilamelares pequenos e grandes (SUV e LUV). Nesse método, os lipídeos são dissolvidos no solvente (etanol ou éter) e então injetados em solução aquosa aquecida, seguido de evaporação do solvente (12).

4.3.1 Adaptação do método injeção de etanol

Método baseado em Sebaaly e colaboradores (2016) Sebaaly e colaboradores (2016b) com algumas alterações para incorporação de óleo essencial. Esse método consiste em aquecer tanto a fase aquosa (água destilada) quanto a lipídica (etanol absoluto, Phospholipon 90H, Colesterol e óleo essencial de *Lippia sidoides*), sob agitação magnética e verter lentamente a fase lipídica sobre a aquosa na temperatura de 55ºC, simulando o método de injeção de etanol. Homogeneizar a suspensão de lipossomas em Ultra-turrax (3 min a 10.000 rpm) e remover etanol e parte da água por rotaevaporação. Em seguida a formulação foi dividida e cada parte incorporada separadamente a uma solução aquosa de carreadores de secagem: Lacprodan ou goma arábica, estando os carreadores na proporção de 1:1 em relação a quantidade de Phospholipon 90H, Colesterol e óleo essencial. As amostras foram secas por *spray drying*, e os pós obtidos foram analisados quanto à densidade, potencial zeta e tamanho da partícula.

4.3.2 Método injeção de etanol padrão

A padronização teve por objetivo realizar alguns ajustes do método anterior, visando melhorar as propriedades dos sistemas obtidos. Utilizou-se os mesmos procedimentos descritos anteriormente, no entanto, a formulação foi mantida sob agitação magnética (2 h) antes da rotaevaporação. Também foram alterados os carreadores de secagem a serem avaliados, porém a proporção foi mantida. Para esses sistemas foram usados Lacprodan e lactose, e as amostras foram secas por *spray drying* e liofilizador. As amostras líquidas e secas são analisadas quanto ao potencial zeta e tamanho de partícula. A quantificação da retenção de timol foi feita por HPLC, utilizando a curva padrão do timol, e as amostras são analisadas por cromatografia gasosa acoplada a espectrometria de massas (CG-MS) para avaliar qualitativamente a presença dos componentes do óleo essencial nas amostras. Além disso, também são analisadas alíquotas

retiradas no decorrer do processo de produção dos lipossomas para verificar as perdas de óleo essencial (através da quantificação de timol).

4.3.3 Método injeção de etanol padrão usando Capsul como carreador de secagem

Esse estudo teve por objetivo alterar o carreador de secagem (Capsul) e avaliar as propriedades do pó quando diferentes proporções de Capsul e diferentes temperaturas de secagem em *spray drying* são utilizadas. O método de obtenção dos lipossomas foi o mesmo descrito anteriormente, no entanto, após a rotaevaporação a formulação é dividida e incorporada na solução de carreador de secagem Capsul, nas proporções de 1:1 e 1:2 em relação à quantidade de Phospholipon 90H, Colesterol e óleo essencial. As formulações resultantes são liofilizadas e secas por *spray drying* (nas temperaturas de 70 e 100ºC). São determinadas as densidades dos pós obtidos, a percentagem de retenção de timol por HPLC, e o potencial zeta e tamanho da partícula das amostras secas.

4.4 OBTENÇÃO DE LIPOSSOMAS COM β-CD

Dentre os diferentes tipos de ciclodextrinas (CD), a mais utilizada é a β-CD por possuir alta solubilidade em água e toxicidade segura (14). Esses carboidratos são capazes de complexar com compostos bioativos hidrofóbicos, e os complexos formados podem ser encapsulados em lipossomas, se alojando em seu núcleo aquoso. Tendo em vista a reduzida eficiência de encapsulação de compostos hidrofóbicos em lipossomas, a complexação com ciclodextrina permite uma maior retenção desses compostos nesse tipo de estrutura (10).

4.4.1 Adaptação do método injeção de etanol com β-CD

Método baseado em Chen et al. (2014), com modificações: 4 mmol de β-CD são hidratados com álcool etílico absoluto (2,5% m/v) e a solução é mantida em repouso à temperatura ambiente por 30min. O OE é gotejado sobre a solução de β-CD sob agitação magnética, e a mistura permanece sob agitação (3h). Colesterol e Phospholipon 90H são adicionados ao complexo formado entre OE e β-CD, e essa mistura e a fase aquosa são aquecidas até 60ºC, quando a fase lipídica é vertida lentamente sobre a fase aquosa, sob agitação. A formulação então é rotaevaporada para eliminação do solvente, dividida e separadamente incorporada à solução de carreadores de secagem Lacprodan e Aerosil (na proporção de 1:1 em relação a quantidade de Phospholipon 90H, Colesterol e óleo essencial). É realizada a secagem (*spray drying*), e o timol retido é quantificado por HPLC.

4.5 CARACTERIZAÇÃO DOS SISTEMAS PARTICULADOS LÍQUIDOS E SÓLIDOS

4.5.1 Tamanho de partícula, índice de polidispersão e potencial zeta

Parâmetros medidos por espalhamento de luz dinâmico, utilizando o Zetasizer (modelo Nano ZS90, Malvern, Reino Unido), equipado com ângulo de espalhamento 90º. Medidas feitas em triplicata a 25 °C, com diluição de 1:400 (v/v) das amostras em água Mili-Q. Os valores de potencial zeta foram calculados a partir da média de valores de mobilidade eletroforética pela equação de Smoluchowsk.

4.5.2 Retenção de timol

O ativo foi quantificado por cromatografia líquida de alta eficiência (HPLC). As condições foram baseadas em Benelli e colaboradores (2013), com algumas modificações. As análises foram realizadas em cromatógrafo LC-20A Prominence Shimadzu com injetor automático, bomba LC-6A (Shimadzu Corporation, Kyoto, Japão) e detector de arranjo de diodos (SPD-M20A), coluna C18 (Shimadzu Shim-Pack CLC(M) 4,6 mm x 25 cm, 5 μm), tempo de corrida de 20 minutos, vazão de 1 mL/min, volume de injeção de 20 μL, temperatura do forno de 30 °C. A fase móvel foi composta por água (A) e acetonitrila (B) segundo o gradiente: 0–2 min, 10% B; 2–7 min, aumento linear de B para 78%; 7–17 min, 78% B; 17– 20 min, aumento linear de B para 100%; 20–23 min, 100% de B; 23–26 min, redução linear de B para 10%, 23–32 min, 10% de B. O cromatograma foi analisado a 276 nm.

O método de preparo das amostras consistiu na diluição de uma quantidade pré-determinada em metanol, sendo homogeneizadas em ultrassom e mantidas sobre agitação magnética por 30 minutos de forma a extrair o timol. Após esse período as amostras foram centrifugadas por 5 minutos a 5000 g, sendo o sobrenadante analisado por HPLC, após filtração em filtro Millipore 0.45 μm.

4.5.3 Análise qualitativa de eficiência de encapsulação

Baseia-se na presença dos componentes do óleo essencial nas formulações líquidas, utilizando CG-MS. A análise foi feita em cromatógrafo gasoso acoplado ao um espectrômetro de massa (GC-MS modelo GCMS QP-2010, Shimadzu, Japão), usando a coluna capilar EN5-MS (30 m x 0.25 mm x 0.25μm). H2 foi utilizado com gás de arraste, com temperatura programada de 60°C a 240 °C com rampa de 3 °C/min. As demais condições cromatográficas foram as seguintes: temperatura do injetor de 240 °C; vazão de H2 de 1,30 mL/min; temperatura do detector de 260 °C, a razão de split de 1:50. Os principais constituintes do OE foram

identificados pela comparação de seu espectro de massa com os espectros publicados na biblioteca eletrônica da Wiley (WILEY7.LIB), e dos índices de Kovats (IK) relativos a uma série de alcanos (C9-C20) com os valores reportados em Adams (2007). O método de preparo das amostras seguiu a mesma metodologia descrita anteriormente para retenção de timol em HPLC.

4.5.4 Umidade e atividade de água

O teor de umidade dos prolipossomas foi determinado pelo método de titulação por Karl Fischer, empregando-se um equipamento Karl Fischer 870 Titrino Plus (Methrom, Suiça). Análises de atividade de água foram realizadas no medidor de atividade de água Aqua Lab 4TEV (Decagon Devices, Pullman, EUA), utilizando o eletrodo capacitivo. Os resultados foram expressos como média e desvio (medidas realizadas em triplicata).

4.5.5 Propriedades de fluxo do pó

A razão de Hausner (HR) e o índice de Carr (IC) foram utilizadas como propriedades de fluidez. Esses parâmetros são determinados segundo as Equações 3.1 e 3.2, descritas a seguir:

$$H_R = \frac{\rho_{t,1250}}{\rho_b} \quad (3.1)$$

$$I_C = \frac{\rho_{t,1250} - \rho_b}{\rho_{t,1250}} \times 100 \quad (3.2)$$

onde ρb é a densidade aparente ($\rho_b = m_0/v_0$), $\rho_{t,1250}$ é a densidade compactada, determinada usando o volume ocupado por uma amostra de pó em uma proveta após 1250 quedas a partir de uma distância de 14,0 mm. O equipamento Caleva® (Tapped Density Tester, TDT, Frankfurt, Alemanha) foi empregada para a determinação das densidades aparente e compactada, de acordo com o método descrito na farmacopeia americana (18).

4.6 RESULTADOS

4.6.1 Método de Injeção de etanol

4.6.1.1 Adaptação do método injeção de etanol

A formulação preparada por esse método foi seca por spray drying para avaliar a influência de cada carreador de secagem sobre o rendimento do processo de secagem. As condições de secagem podem ser observadas na Tabela 4.1, e a porcentagem de recuperação do

produto e tempo de secagem referentes a cada carreador, seus respectivos valores do conteúdo de umidade e a atividade de água, constam na Tabela 4.2. A porcentagem de recuperação do produto foi calculada baseada na massa de sólidos alimentada.

Tabela 4.1 Condições de secagem em *spray dryer* das formulações contendo Lacprodan e Goma arábica

Parâmetro	**Valor**
T secagem (ºC)	100
Vazão ar de secagem (m^3/h)	60
Bico atomizador (mm)	1
Vazão alimentação (g/min)	4
Pressão entrada (Kgf/cm^2)	3
Pressão ar de secagem (Lpm)	17

Tabela 4.2 Parâmetros de desempenho da secagem por *spray drying*

Parâmetro	**Lacprodan**	**Goma arábica**
Recuperação (%)	65,8	46,5
Tempo de secagem (s)	681	720
X_p (%)	6,0 ± 0,5	6,8 ± 0,1
A_w (-)	0,39 ± 0,00	0,37 ± 0,00

*X_p: umidade, A_w: atividade de água.

Os resultados apresentados na Tabela 4.2 apresentam maior porcentagem de recuperação da formulação contendo Lacprodan, sendo mais eficiente do que a Goma arábica para o processo em questão. O conteúdo de umidade e a atividade de água de ambos os pós foi semelhante, assim como o tempo de secagem. Foi possível avaliar apenas as propriedades de fluxo do pó produzido na secagem da formulação com Lacprodan como carreador de secagem, pois a quantidade de pó produzida pela secagem da formulação com a Goma arábica, foi insuficiente para que essa análise pudesse ser feita. Os resultados obtidos para densidade aparente (ρ_a), densidade de compactação (ρ_c), Razão de Hausner (RH) e Índice de Carr (IC), são apresentados na Tabela 4.3.

Tabela 4.3 Densidade dos pós provenientes da secagem em *spray drying* das formulações contendo Lacprodan e Goma arábica como carreadores

Parâmetro	**Lacprodan**	**Goma arábica**
ρ_a (g/mL)	0,157	ND
ρ_c (g/mL)	0,203	ND
RH (-)	1,3	ND
IC (%)	29,3%	ND

*ND: não determinado.

Valores baixos de HR e IC indicam baixa coesividade e, portanto, boa fluidez (19). Materiais pulverulentos que apresentam pequena fricção entre partículas adjacentes apresentam HR por volta de 1,2. Valores de HR superiores a 1,6 em geral são observados para partículas coesivas e de dimensão reduzida, apresentando escoamento restrito (20). Materiais com IC < 10% são considerados de excelente fluidez, de 11 a 15% boa fluidez, de 16 a 20% razoável, 21 a 25% aceitável, 25 a 31% ruim, 32 a 37% muito ruim, e >38% fluidez extremamente ruim (18). Sendo assim, considerando os resultados da Tabela 3, o pó analisado apresenta fluxo pobre tanto pelo índice de Carr quanto pela Razão de Hausner.

O tamanho da partícula, índice de polidispersão (PDI) e o potencial zeta dos pós também foram analisados, e duas técnicas de ressuspensão dos mesmos em água foram comparadas. Foi realizada diluição de 1:400 da amostra com Goma arábica e Lacprodan em água Milli-Q. A ressuspensão do pó contendo Goma arábica foi realizada utilizando agitação magnética por 1h 30 min, o mesmo processo foi utilizado para ressuspensão do pó contendo Lacprodan, porém foi também avaliada uma maneira alternativa de ressuspensão desse pó, utilizando um Ultraturrax por 2 min a 15.000 rpm seguido de 30 min de agitação magnética. Posteriormente foi realizada medida do tamanho da partícula, PDI e potencial zeta, cujos dados obtidos são apresentados na Tabela 4.4.

Tabela 4.4 Comparação entre a ressuspensão dos pós com Ultra-turrax e com agitação magnética

Parâmetro		**Ultra-turrax**	**Agitação magnética**
Lacprodan	dp (nm)	1465,7 ± 235,4	2026,3 ± 327,3
	PDI	1 ± 0,0	1 ± 0,0
	Z (mV)	-26,4 ± 0,4	-34,5 ± 0,5
Goma arábica	dp (nm)	ND	2146,3 ± 185,3
	PDI	ND	1 ± 0,0
	Z (mV)	ND	-39,8 ± 1,6

*dp: diâmetro médio de partícula; PDI: índice de polidispersão; Z: potencial zeta; ND: não determinado.

A partir dos dados apresentados na Tabela 4.4, é possível observar que nenhuma das ressuspensões dos pós foi adequada, visto que todas obtiveram um alto índice de polidispersão, sendo assim, não foi possível comparar os métodos de ressuspensão propostos.

4.6.1.2 Método injeção de etanol padrão

O método foi padronizado e foi realizada uma análise detalhada das perdas de óleo essencial durante o processo, por meio de alíquotas da formulação que foram retiradas em várias etapas do processo, e acondicionadas em local refrigerado para posterior análise. Os carreadores

de secagem Lacprodan e lactose, foram utilizados para proteger os componentes da formulação durante os processos de secagem, que consistiram em *spray drying* e liofilização. Lacprodan foi escolhido novamente como carreador de secagem, devido ao bom rendimento de secagem em *spray drying* verificado nos resultados apresentados anteriormente. As condições de secagem foram as mesmas apresentadas na Tabela 4.1 e a Tabela 4.5 esquematiza os respectivos rendimentos de secagem das formulações contendo Lacprodan e lactose.

Tabela 4.5 Parâmetros de desempenho da secagem por *spray drying* das formulações contendo Lacprodan ou lactose como carreadores

Parâmetro	Lacprodan	Lactose
Recuperação (%)	69,4	41,7
Tempo de secagem (s)	1.939	1953
X_p (%)	5,5 ± 0,4	4,9 ± 0,1
A_w (-)	0,40 ± 0,01	0,43 ± 0,00

*X_p: umidade, A_w: atividade de água.

O conteúdo de umidade não difere muito entre os dois pós, todavia, durante a secagem e manuseio do pó contendo lactose foi percebido que este apresentava alta higroscopicidade pois formava grumos com facilidade. Diferentemente do pó contendo proteína, que assim como da última vez utilizado nesse projeto, obteve bom rendimento no *spray drying* e se mostrou um pó bastante "solto".

O pó contendo Lacprodan obtido do processo de secagem por liofilização, também apresentou aspecto bastante "solto", assim como o contendo lactose demonstrou possuir alta higroscopicidade. Os valores do conteúdo de umidade e atividade de água desses pós podem ser observados na Tabela 4.6, e diferente do observado nos pós obtidos por *spray drying*, os valores obtidos para os pós liofilizados demonstram que o pó contendo lactose é mais higroscópico do que o que contém Lacprodan.

Tabela 4.6 Conteúdo de umidade e atividade de água dos pós liofilizados das formulações contendo Lacprodan e lactose

Parâmetro	Lacprodan	Lactose
X_p (%)	2,7 ± 0,1	3,2 ± 0,1
A_w (-)	0,08 ± 0,01	0,26 ± 0,02

Foram retiradas alíquotas da formulação antes e depois da rotoevaporação, e após adição dos carreadores de secagem Lacprodan e lactose. O tamanho da partícula, PDI e potencial zeta das alíquotas e dos pós ressuspensos foram avaliados. Foi realizada diluição de 1:400 das

alíquotas para realizar a leitura desses parâmetros e os resultados estão esquematizados na Figura 4.1.

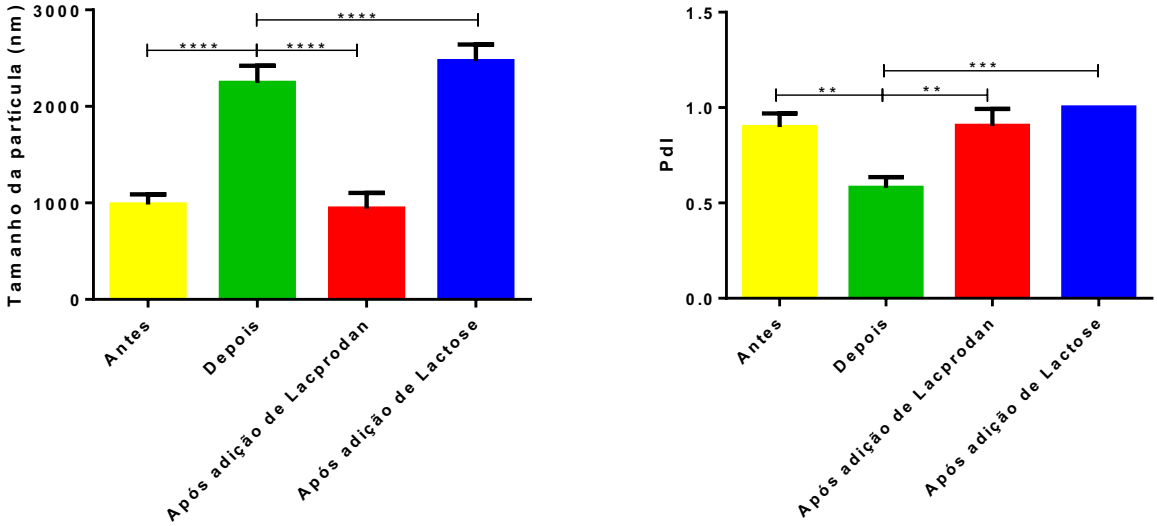

Figura 4.1 Tamanho da partícula e PDI da formulação antes e depois da rotaevaporação, e após a adição de Lacprodan e lactose. (Dados analisados pelo teste de Bonferroni, com significância *p < 0,05)

É possível perceber que após a rotaevaporação, o tamanho das partículas aumentou, todavia, o PDI diminuiu, o que significa uma distribuição de tamanho mais homogênea na formulação. A seguir, foi preparada foi apenas uma formulação, e esta foi dividida em duas partes, de maneira que cada parte fosse incorporada a um dos carreadores de secagem. A formulação antes de ser incorporada aos carreadores, possuía, como pode ser observado na Figura 4.1, um PDI estatisticamente menor do que o observado após a adição dos carreadores. Este PDI elevado, que se originou com a adição dos carreadores, se manteve mesmo após os processos de secagem. Isso indica que o alto PDI observado na Figura 4.2 provém da adição dos carreadores de secagem. Não houve diferença estatística entre o tamanho das partículas antes e depois da secagem, seja por spray drying ou por liofilização para ambas as formulações com Lacprodan e lactose, e o mesmo foi observado para o PDI. A Tabela 4.7 apresenta os resultados do potencial Zeta determinados para todas as amostras utilizadas nessa análise.

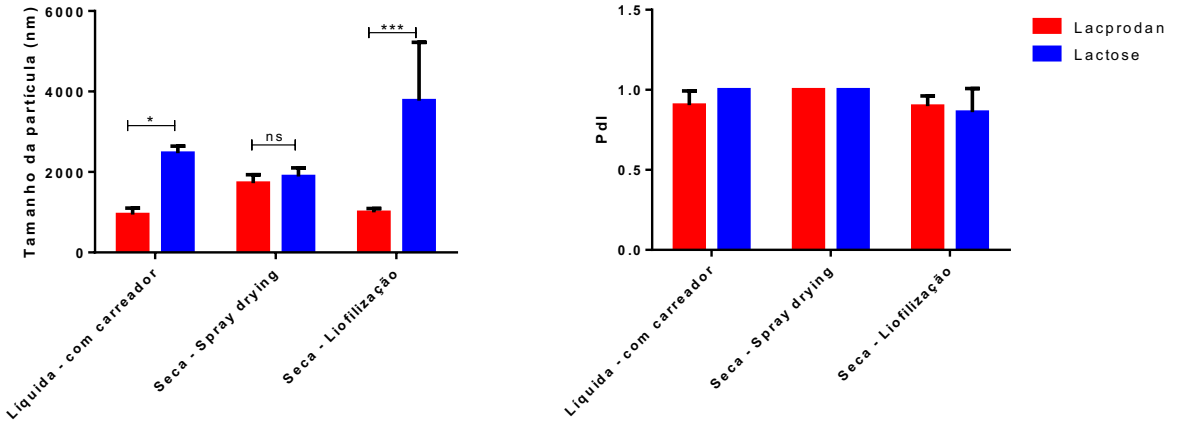

Figura 4.2 Tamanho da partícula e PDI da formulação após a adição de Lacprodan e lactose, e dos pós obtidos das secagens por spray drying e liofilização (dados foram analisados pelo teste de Bonferroni, com significância *p < 0,05)

Tabela 4.7 Potencial zeta das formulações antes e depois da rotaevaporação, após a adição de Lacprodan e lactose, e dos pós obtidos por *spray drying* e liofilização

Potencial zeta (mV)			
Rotaevaporação		Antes	- 27,4 ± 3,9
		Depois	-34,1 ± 2,6
Após adição de carreador de secagem		Lacprodan	-32,2 ± 1,3
		Lactose	-34,3 ± 1,3
Após secagem	*Spray drying*	Lacprodan	-27,1 ± 1,7
		Lactose	-22,9 ± 4,7
	Liofilização	Lacprodan	-29,8 ± 0,3
		Lactose	-29,8 ± 1,4

A eficiência de encapsulação do timol (composto majoritário do óleo essencial de *Lippia sidoides*) e as perdas ao longo do processo foram avaliadas por HPLC. A Figura 4.3 representa os resultados obtidos para a quantificação.

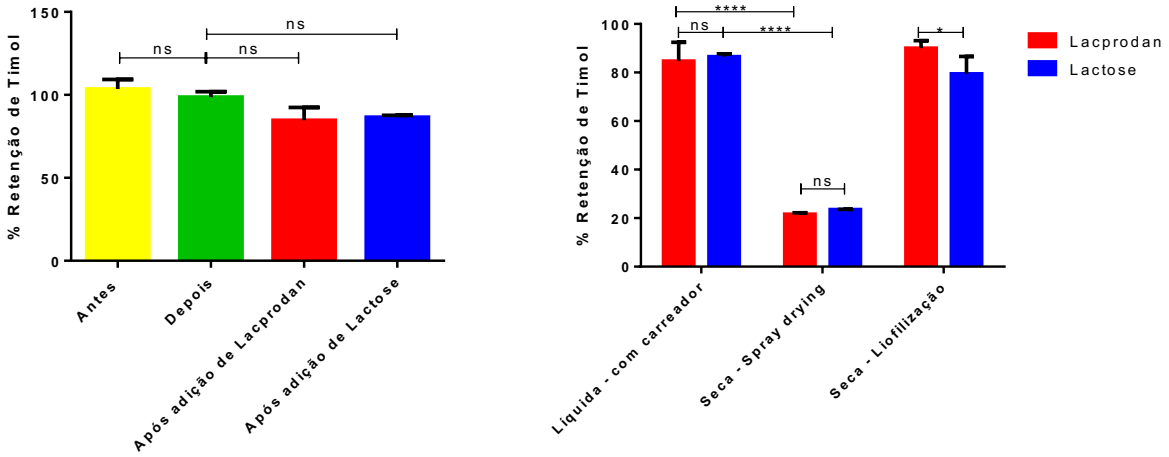

Figura 4.3 Retenção de timol das formulações antes e depois da rotaevaporação, após a adição de Lacprodan e lactose, e dos pós obtidos por *spray drying* e liofilização (Dados analisados pelo teste de Bonferroni, com significância * $p < 0,05$; ns: não significativo)

É possível concluir por meio da Figura 4.3, que durante o processo que precedeu a secagem da formulação, não houve diferença estatística na porcentagem de retenção do timol em nenhuma das etapas analisadas. É válido ainda dizer, que não houve perda significativa de timol durante a rotaevaporação, validando o processo de produção dos lipossomas. Também foi possível perceber que após o processo de secagem por *spray drying*, a retenção de timol diminuiu muito, tanto na formulação com Lacprodan quanto na com lactose. Enquanto não houve diferença estatística entre a retenção de timol antes e depois da secagem por liofilização, porém a retenção de timol na formulação com Lacprodan foi maior do que na formulação com lactose após esse processo de secagem.

Também foi realizada uma análise por cromatografia gasosa acoplada a espectrômetro de massas (CGMS), a fim de verificar, de maneira qualitativa, a presença de alguns compostos do óleo essencial de *Lippia sidoides*. Na amostra coletada após a rotaevaporação da formulação, e nos pós ressuspensos provenientes da secagem das formulações com Lacprodan e lactose em *spray drying* e liofilização, foram encontrados em comum os seguintes compostos presentes também no óleo essencial: timol, óxido de cariofileno, 4-carvomenthenol e cariofileno. Também foi possível verificar, pelo número de compostos apresentados, que nem todos os compostos do óleo essencial estavam presentes nas amostras, isso se deve aos processos aos quais o óleo presente nas amostras passou, resultando na perda de alguns de seus componentes.

4.6.1.3 Método injeção de etanol padrão usando Capsul como carreador de secagem

A formulação preparada foi pelo mesmo método discutido no item anterior, porém adicionando-se o óleo essencial após a rotaevaporação, foi incorporada no carreador de

secagem Capsul, que estava nas proporções de 1:1 e 1:2 em relação à quantidade de sólidos da formulação. A secagem foi realizada por liofilização e *spray drying*, nesse último ela foi feita nas temperaturas de 70 ºC e 100 ºC. As condições de secagem no *spray drying* são as mesmas já apresentadas na Tabela 1, exceto a temperatura de secagem (70 ºC e 100 ºC para ambas as proporções de carreadores). Foi feita duplicata da secagem da formulação com carreador na proporção de 1:1 a 70 ºC no *spray drying* para validação dos dados. A porcentagem de recuperação do produto dos pós produzidos em *spray drying*, e a atividade de água e o conteúdo de umidade, dos pós produzidos por *spray drying* e liofilização, podem ser observados na Tabela 4.8.

Tabela 4.8 Recuperação do produto (R), umidade (X_p) e atividade de água (A_w), dos pós contendo Capsul nas proporções de 1:1 e 1:2, obtidos por *spray drying* e liofilização

Spray drying					
	1:1 Capsul			1:2 Capsul	
	70 ºC		100 ºC	70 ºC	100 ºC
R (%)	49	48	42	49	47
X_p (%)	8,4 ± 0,3	7,6 ± 0,6	6,4 ± 0,3	7,0 ± 0,0	6,2 ± 0,5
A_w (-)	0,52 ± 0,00	0,49 ± 0,00	0,37 ± 0,01	0,39 ± 0,01	0,373 ± 0,01
Liofilização					
	1:1 Capsul			1:2 Capsul	
X_p (%)	3,61 ± 0,750			4,46 ± 0,361	
A_w (-)	0,230 ± 0,010			0,193 ± 0,011	

Observando os valores de porcentagem de recuperação do produto, é possível perceber que quando a proporção de Capsul era 1:1 em relação a quantidade de sólidos, a secagem em spray drying na temperatura de 70 ºC resultou em uma porcentagem de recuperação um pouco maior do que a 100 ºC. Já com o Capsul na proporção de 1:2, a secagem a 70 ºC também apresentou maior porcentagem de recuperação do produto do que a 100 ºC, porém essa diferença foi menor do que a observada quando a proporção de Capsul era 1:1. Não houve diferença na porcentagem de recuperação do produto entre a formulação com Capsul na proporção de 1:1 e a com Capsul proporção de 1:2, quando sendo comparadas nas mesmas temperaturas de secagem no *spray drying*. Além disso, a porcentagem de recuperação do produto foi semelhante entre as amostras em duplicata, com proporção de carreador 1:1 e secagem à 70 ºC, validando o teste.

Os valores de umidade e atividade de água foram semelhantes entre os pós obtidos por spray drying, e entre os pós obtidos por liofilização, todavia esses últimos se mostraram menores. Os resultados obtidos para as propriedades de fluxo dos pós estão apresentados na Tabela 4.9.

Tendo em vista os valores de referência, podemos concluir que os valores de IC se encontram classificados como fluxo pobre (pós coesivos), ou fluxo muito pobre, assim como os valores de RH indicam fluxo fraco e fluxo pobre. Portanto, de maneira indireta é possível supor que os pós não apresentam boa escoabilidade. Os resultados apresentados para porcentagem de retenção de timol por HPLC de cada tratamento podem ser observados na Figura 4.4.

Tabela 4.9 Densidade aparente (ρ_a), da densidade de compactação (ρ_c), da Razão de Hausner (RH) e do Índice de Carr (IC), dos pós obtidos pela secagem das formulações com Capsul nas proporções de 1:1 e 1:2, por *spray drying* e liofilização

Spray drying					
	1:1 Capsul			1:2 Capsul	
	70ºC		100ºC	70ºC	100ºC
ρ_a (g/mL)	0,2	0,2	0,2	0,21	0,2
ρ_c (g/mL)	0,2	0,3	0,25	0,28	0,27
RH (-)	1,3	1,3	1,3	1,3	1,4
IC (%)	26	25	25	33	35
Liofilização					
	1:1 Capsul			1:2 Capsul	
ρ_a (g/mL)	0,34			0,35	
ρ_c (g/mL)	0,42			0,46	
RH (-)	1,23			1,31	
IC (%)	23,5			31,43	

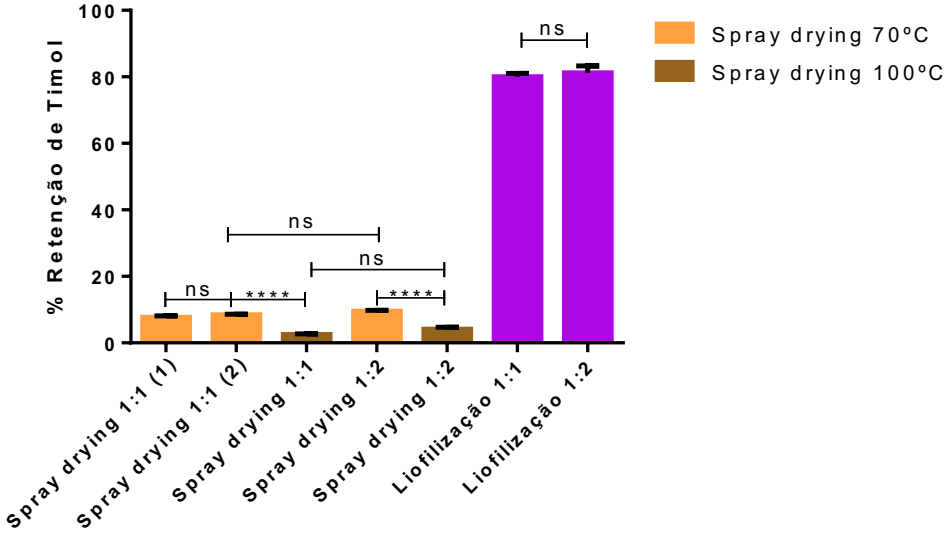

Figura 4.4 Retenção de timol dos pós obtidos por *spray drying* e liofilização, das formulações contendo Capsul nas proporções de 1:1 e 1:2. (Dados foram analisados pelo teste de Bonferroni, com significância *p < 0,05; ns: não significativo).

Foi possível perceber que não houve diferença entre a retenção de timol obtida entre os pós oriundos das formulações com proporção de carreador 1:1 e secas à 70 ºC, validando o método. A Figura 4.4 também revela que o processo de secagem em *spray drying* ocasiona grande perda da quantidade de óleo essencial encapsulada nos lipossomas, enquanto o processo de secagem por liofilização permite maior porcentagem de retenção do ativo. Isso se deve, tendo como base os dados encontrados, à temperatura a qual a formulação é exposta durante o processo de spray drying, visto que as formulações que foram secas a 70 ºC obtiveram uma maior porcentagem de retenção do ativo do que as secas à 100 ºC, independentemente da proporção de carreador de secagem utilizado. Quanto a variação na proporção do carreador de secagem Capsul (1:1 e 1:2), não houve diferença entre elas no que se refere à porcentagem de retenção de timol, tanto nas secagens realizadas em spray drying quanto nas por meio de liofilização. Talvez a variação na quantidade de carreador não tenha sido de uma magnitude capaz de produzir diferenças perceptíveis. O tamanho da partícula e o PDI podem ser observados na Figura 4.5, e o potencial zeta é apresentado na Tabela 4.10.

Tabela 4.10 Potencial zeta dos pós contendo Capsul nas proporções de 1:1 e 1:2, obtidos por *spray drying* e liofilização

Potencial zeta (mV)		
Spray drying 70 ºC	1:1 (1)	-18,9 ± 0,4
	1:1 (2)	-19,3 ± 0,8
	1:2	-15,4 ± 0,4
Spray drying 100 ºC	1:1	-19,07 ± 1,5
	1:2	-21,7 ± 0,8
Liofilização	1:1	-28,6 ± 1,5
	1:2	-23,5 ± 0,7

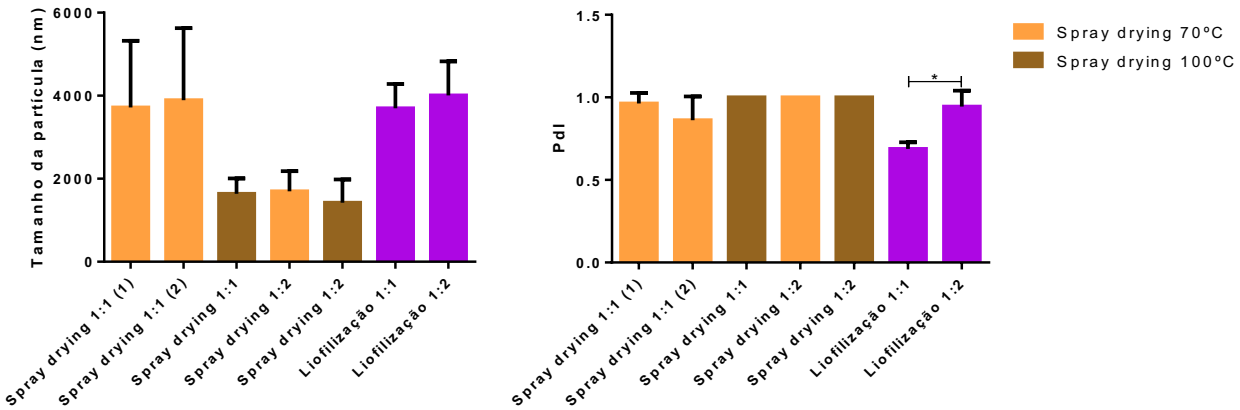

Figura 4.5 Tamanho da partícula e PDI dos pós contendo Capsul nas proporções de 1:1 e 1:2, obtidos por *spray drying* e liofilização (Dados analisados pelo teste de Bonferroni, com significância *p < 0,05).

Os dados apresentados na Figura 4.5 mostram que o pó obtido da formulação com proporção de carreador de secagem 1:1 liofilizada, foi a que obteve melhor índice de polidispersão, enquanto nas demais, o PDI foi próximo de 1 indicando uma distribuição bastante heterogênea de tamanho da partícula, o que explica os desvios padrões altos observados no tamanho das partículas. Sendo assim, ambas as formulações com proporção de carreador 1:1 e 1:2, secas por liofilização, obtiveram altas porcentagens de retenção de timol, porém apenas o pó obtido da formulação com proporção de carreador 1:1, após ressuspensão, obteve um índice de polidisperso baixo e tamanho da partícula acima da escala nanométrica.

4.6.1.4 Métodos de produção de lipossomas que se utilizam de β-CD para aumentar a retenção de compostos bioativos hidrofóbicos

As formulações contendo β-CD foram acrescidas de Lacprodan e Aerosil e secas em *spray dryer* utilizando as mesmas condições de secagem já descritas na Tabela 4.1. Os resultados para as secagens estão esquematizados na Tabela 4.11.

Tabela 4.11 Recuperação do produto (R), umidade (X_p) e atividade de água (A_w) dos pós contendo Lacprodan e Aerosil

Parâmetro	Lacprodan	Aerosil
R (%)	55,7	30,02
X_p (%)	6,1 ± 0,2	3,5 ± 0,2
A_w (-)	0,33 ± 0,01	0,29 ± 0,01

Novamente, o Lacprodan demonstrou obter melhor porcentagem de recuperação do produto após secagem em *spray drying*, assim como aconteceu quando comparado à Goma arábica e à Lactose. A recuperação do produto contendo Aerosil foi bem inferior ao produto com Lactose, o que indica que o Aerosil não é um bom carreador de secagem para o produto. A porcentagem de retenção do timol após o processo de secagem é apresentada na Figura 4.6.

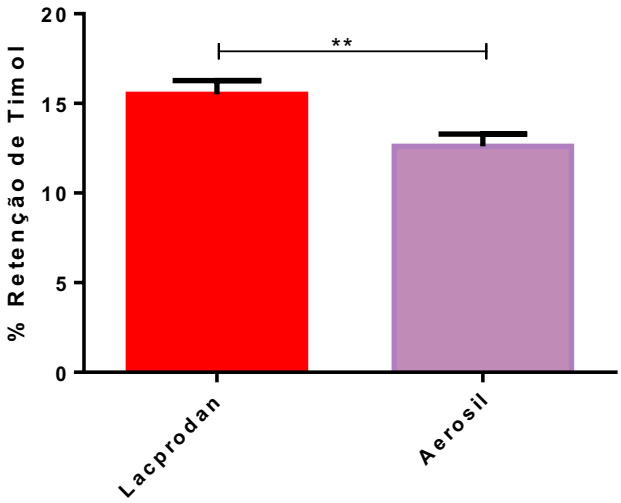

Figura 4.6 Retenção de timol dos pós contendo Lacprodan e Aerosil, obtidos por *spray drying* (Dados analisados pelo teste t pareado com correção de Welch, com significância *p < 0,05).

A porcentagem de retenção do timol foi consideravelmente maior na formulação com Aerosil como carreador de secagem na presença de β-CD, do que em formulação teste também com Aerosil, porém sem β-CD, que foi em média de 3% (dados não apresentados); essa mesma formulação teste possuía por sua vez um método um pouco diferente de preparo dos lipossomas e não contava com a presença da β-CD. Isso revela que a β-CD foi capaz de aumentar a retenção de timol quando utilizamos Aerosil como carreador de secagem. Todavia, a porcentagem de retenção de timol da formulação com Lacprodan após a secagem, foi um pouco menor do que a observada com o uso desse mesmo carreador na Figura 4.3. O que indica que esse novo método com adição de β-CD talvez não seja capaz de aumentar a retenção de timol quando utilizamos Lacprodan como carreador de secagem, durante a secagem por *spray drying*.

4.7 DISCUSSÃO

No decorrer do trabalho foram realizados planejamentos experimentais distintos que visaram avaliar, sob condições variadas, os processos de preparação de lipossomas contendo óleo essencial de *Lippia sidoides* e as características físico-químicas das formulações liquidas e dos pós produzidos por esses processos. Tendo isso em mente, pudemos perceber que, em se tratando do método de preparo de lipossomas contendo óleo essencial, o que mais foi explorado e que apresentou melhores resultados foi o método adaptado de injeção de etanol, tendo se mostrado um método que permite a formação de lipossomas que podem ser facilmente incorporados a carreadores de secagem. Além disso, também foi possível observar que a adição

de óleo essencial antes da rotaevaporação não acarretou perdas significativas durante o processo, informação bastante importante quando se trabalha com esse tipo de processo.

De maneira geral a secagem por liofilização, independentemente dos carreadores utilizados, demonstrou produzir menor interferência nas partículas lipossomais, resultando em maior porcentagem de retenção do ativo e melhor estabilidade, perceptível por meio dos resultados de tamanho da partícula, PDI e potencial zeta. Enquanto a alta temperatura envolvida na secagem em *spray drying* pode ter promovido algum tipo de modificação nas partículas lipossomais, o que resultou na perda considerável do óleo essencial durante o processo, visível por meio da baixa porcentagem de retenção do ativo obtida dos pós oriundos desse processo de secagem.

Os resultados obtidos também permitiram observar que a formação de complexos de ciclodextrina com o óleo essencial de *Lippia sidoides* de fato aumentaram a porcentagem de retenção do ativo após a secagem em *spray drying*, em nosso estudo esse resultado foi obtido com o uso do carreador de secagem Aerosil.

4.8 CONCLUSÃO

Esse trabalho teve como objetivo principal investigar rotas tecnológicas para a obtenção de lipossomas contendo óleo essencial de *Lippia sidoides*, enfatizando-se processos de preparação, tipo de excipientes e procedimentos para a caracterização físico-química e aumento da retenção de ativo. Tal objetivo foi atingido e os resultados obtidos foram bastante válidos para a compreensão do comportamento do óleo essencial de *Lippia sidoides*, no que diz respeito à sua encapsulação em sistemas lipossomais por métodos distintos, capacidade de complexação com β-CD, e secagem em *spray drying* e via liofilização. Além disso, o trabalho permitiu avaliar a atuação de vários carreadores frentes aos processos de secagem, e às formulações lipossomais obtidas por métodos diversificados. Etapas futuras consistiriam na avaliação da atividade biológica dos produtos produzidos, a fim de averiguar as propriedades inerentes do óleo essencial de *Lippia sidoides*.

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CHAPTER 5. CYCLODEXTRINS-IN-LIPOSOMES: A PROMISING DELIVERY SYSTEM FOR *LIPPIA SIDOIDES* AND *SYZYGIUM AROMATICUM* ESSENTIAL OILS

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5.1 INTRODUCTION

Essential oils (EOs) are known for showing a multitude of biological effects due to their variable terpene, terpenoid, and phenolic contents (1). Numerous studies have highlighted the properties of EOs as antimicrobial (2–4), antiviral (5), pesticide (6), larvicidal (7), anti-inflammatory (8), and antioxidant (9) agents, among others. These compounds are widely accepted by consumers because of their natural status and safety profile (1). In particular, the essential oils of *Lippia sidoides* (LEO), popularly known as pepper rosemary, and clove (*Syzygium aromaticum*) (CEO) have been extensively studied for their antimicrobial effects (2,10). These EOs are rich in thymol and eugenol, respectively, compounds that are linked to their antimicrobial activity. Despite the high potential use as active ingredients, EOs are volatile liquids, very sensitive to environmental variations; and susceptible to degradation, especially when exposed to light, oxygen, heat, and humidity. To widen their applicability, encapsulation technologies have been proposed [2,11,12].

Liposomes have been widely used as drug delivery systems for sustained-release purposes. They are non-toxic lipid-based carriers consisting of concentric vesicles formed by one or more phospholipid bilayers (11,12). The amphiphilic character of phospholipid molecules offers the liposomes the ability to encapsulate both hydrophilic and lipophilic compounds, being an attractive approach for the loading of EOs (13). In contrast, problems such as oxidation and hydrolysis of the phospholipids lead to leakage of the encapsulated active ingredient and aggregation of the vesicles (14). Moreover, the entrapment of lipophilic compounds is limited to the inner hydrocarbon chains of the lipid bilayers, and can thus be rapidly released from the liposomes (15). Cyclodextrins (CDs), on the other hand, are suitable to form inclusion complexes with a large variety of molecules due to their ability to establish weak intermolecular interactions and increase the solubility of lipophilic compounds (16), both

in the solid and aqueous state (17,18). This process has, as its main advantages, high encapsulation efficiency and long retention time. The possibility of transforming liquid compounds into crystalline forms, masking possible odors and unpleasant tastes, and increasing the physicochemical stability of volatile compounds are other notable advantages (18). It is also noteworthy that CDs are generally recognized as safe by the United States Food and Drug Administration (US FDA) agency for use as protectants and additives in food products, and also as flavor carriers (18).

Beta-cyclodextrin (β-CD) is a cyclic polysaccharide, which presents a hydrophilic external surface and a hydrophobic internal cavity (6). Its derivative 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) has attracted interest due to its improved ability to form inclusion complexes, greater water solubility, and lower toxicity toward biological membranes (19). Furthermore, the presence of CDs in the aqueous core of liposomes preserves the integrity of the membrane without affecting the characteristics of the liposomes. Hence, drug-in-cyclodextrin-in-liposomes (DCL) can be an interesting approach, since it combines the advantages of both carriers, cyclodextrins, and liposomes, in providing controlled drug release (19). DCLs are based on the entrapment of CD/drug inclusion complex into the inner cavity of liposomes. This technique allows an increase in the solubility of the bioactives and in the stability of the vesicles; being particularly suitable for the delivery of volatile compounds (18), like essential oils. Additionally, compared to conventional liposome production methods, the double loaded technique provides rapid release of the actives from the outer phospholipid bilayer, as well as prolonged release due to the presence of the inclusion complex in the inner aqueous core (20).

To ensure the stability and increase the shelf-life of liposomes containing volatile compounds, freeze-drying can be used very conveniently. Such drying processes occur at low temperatures and without the presence of water in the liquid form, favoring both greater stability and the retention of bioactives. The particulate-based forms, known as proliposomes, generate liposomal suspension upon hydration under appropriate stirring conditions (21,22).

Although many recent studies have used DCL as carriers for encapsulating lipophilic compounds (18,21,25,26), few attempts have been made on the encapsulation of more than one essential oil in the same structure. This is a challenging strategy as EOs have a complex and varied composition, including terpenes, phenylpropenes, and oxygenated compounds. There can be over a hundred different compounds in a single EO (1). Despite presenting challenges in the production stage, the inclusion of more than one bioactive (or more than one EO) has the

possibility of a producing a synergistic effect and can potentially reduce the dose required for single drug usage with increased drug-efficacy, and subsequently lower drug toxicity.

Therefore, the purpose and the novelty of the present study is the development of proliposomes of DCLs combining two EOs (or the combination of their major isolated bioactives). The proliposomes of DCLs were prepared by the double loading technique, where the bioactives were added both in the organic phase and in their inclusion complex in the aqueous phase. We used different bioactives combinations (both *Lippia sidoides* and clove essential oils in combination and isolated, and their respective major components, thymol, and eugenol). Based on its remarkable antimicrobial activity, the pepper rosemary and clove EOs, and their major compounds, thymol, and eugenol, were chosen as models for this study. Proliposomes were obtained by freeze-drying, and their reconstituted liposomes were characterized concerning the mean hydrodynamic diameter, polydispersity index, zeta potential, differential scanning calorimetry, X-ray diffraction, and retention of the bioactive major compounds.

5.2 MATERIALS AND METHODS

5.2.1 Materials

2-Hydroxypropyl-β-cyclodextrin (HP-β-CD) was supplied by Roquette (Lestrem, France), and hydrogenated soybean Phospholipon 90H was purchased from Lipoid GmbH (Ludwigshafen am Rhein, Germany). Eugenol (Eug), eugenyl acetate (Eug-Ac), thymol, absolute ethanol, cholesterol, and methanol-HPLC grade were purchased from Sigma–Aldrich (Darmstadt, Germany). CEO (essential oil from *Syzygium aromaticum*), having as the main compounds Eug (86.89%), Eu-Ac (2.91%), trans-caryophyllene (9.04%), α-caryophyllene (0.97%), and the butyl acetate (0.09 %), was bought from a clove essential oil producer located in Valença (BA, Brazil). LEO (essential oil from *Lippia sidoides*), having thymol (68.5%), p-cimeno (9.43%), trans-caryophyllene (7.72%), β-myrcene (2.84%), γ-terpinene (2.71%), α-terpinene (1.16%), and thymol methyl ether (0.97%) as the main compounds, was obtained from PRONAT (Produtos Naturais Ltd.a, Recife, PE, Brazil).

5.2.2 Preparation of Drug-in-CD-in-Liposomes

Liposomal formulations were prepared by the ethanol injection method adapted from Sebaaly et al. (15). Briefly, the aqueous phase, consisting of the inclusion complexes of HP-β-CD with bioactives was prepared by dissolving HP-β-CD in ultra-pure water and adding the

bioactive (CEO and/or LEO, eugenol, and thymol). This solution was stirred for 24 h at 25 ± 2 °C. Subsequently, the organic phase was prepared by dissolving Phospholipon 90H and cholesterol in absolute ethanol under magnetic stirring and heated at 55 °C (above the transition temperature of the phospholipids), when the previously heated bioactive (CEO, LEO, thymol, and Eug) was added. A peristaltic pump was used to inject the organic phase into the aqueous phase at a flow rate of 1 mL/min. The obtained dispersion was kept under magnetic stirring for 15 min, at room temperature. Finally, ethanol and some of the water were removed by rotary evaporation at 45 °C (Rotavapor Fisatom model 802, Perdizes, SP, Brazil). The storage of all dispersions prepared took place in a dark room at 4 °C. Table 5.1 shows the composition of the produced EOs-in-cyclodextrin-in-liposomes.

Table 5.1. Composition of the EOs-in-cyclodextrin-in-liposome systems.

Phase	Component (g)	Formulation			
		CDC	**CDL**	**CDCL**	**CDET**
Organic phase	Phospholipon 90H	0.4	0.4	0.4	0.4
	Cholesterol	0.2	0.2	0.2	0.2
	L. sidoides EO	-	0.1	0.05	-
	Clove EO	0.1	-	0.05	-
	Thymol	-	-	-	0.05
	Eugenol	-	-	-	0.05
	Ethanol	31.6	31.6	31.6	31.6
Aqueous phase	HP-β-Cyclodextrin	2	2	2	2
	L. sidoides EO	-	0.33	0.165	-
	Clove EO	0.27	-	0.135	-
	Thymol	-	-	-	0.11
	Eugenol	-	-	-	0.12
	Water	80.0	80.0	80.0	80.0

5.2.3 Preparation of Proliposomes

Freshly prepared liposomal suspensions were frozen at −20 °C for about 12h in plastic tubes (50 mL) and for a further 4h at −80 °C before freeze-drying (MicroModulyo, Thermo Fisher Scientific, Waltham, USA). Lyophilization time lasted for 72 h. The lyophilized liposomes were stored at −20 °C for further analysis and characterization. Figure 5.1 shows the production scheme of EO loaded carriers.

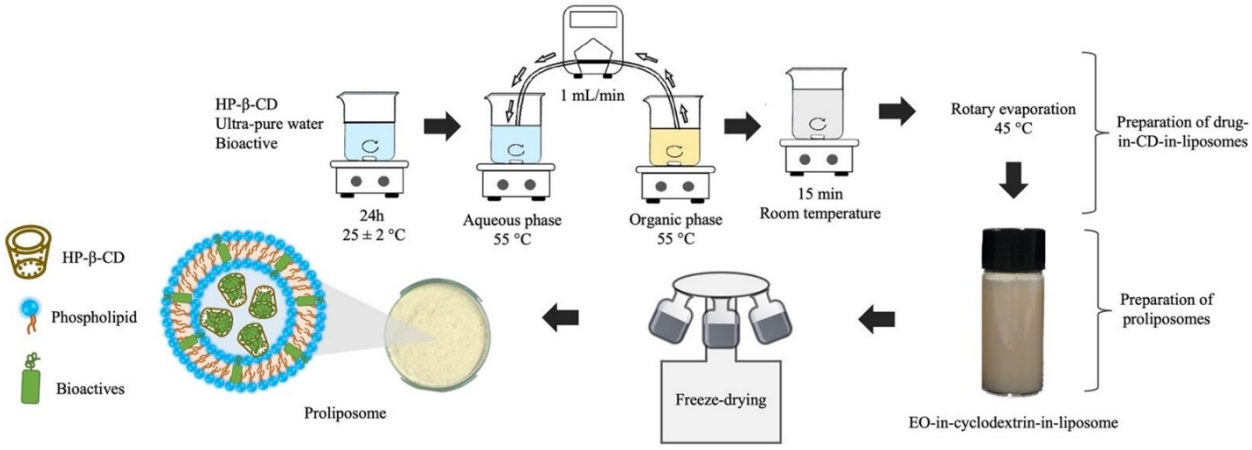

Figure 5.1 Diagram of EOs-in-cyclodextrin-in-liposome and proliposomes production.

5.2.4 Mean Hydrodynamic Diameter, Polydispersity Index, and Zeta Potential

The mean hydrodynamic diameter and polydispersity index of the EOs-in-cyclodextrin-in-liposomes were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS90 (Malvern, UK). Zeta potential was measured by micro electrophoresis, with the same equipment. All the liquid samples were diluted in MilliQ® water (Millipore, Billerica, MA, USA) at a ratio of 1:10 (v/v) and measured in triplicate at 25°C. The measurements were repeated on the 1st, 15th, and 30th days after production. For the analysis of proliposomes, these were firstly redispersed in MilliQ® water at the original concentration, stirred for 30 min, and diluted to 1:200 (v/v) before measurements.

5.2.5 Differential Scanning Calorimetry

A differential scanning calorimeter (Shimadzu DSC-50, Shimadzu Corporation, Kyoto, Japan) was used to study the thermal transformations of the components of the formulations and their relationship with particle structure, according to the method described by Zhang et al., with some modifications (23). Measurements of each component of the formulations, of the physical mixture, and the proliposomes were performed. The samples were exactly weighted in aluminum pans and heated from 20 °C to 250 °C following cooling to 20 °C at a heating and cooling rate of 10 °C/min.

5.2.6 X-ray Diffraction Study

The X-ray powder diffraction patterns were obtained using a Rigaku Rotaflex RU200B X-ray diffractometer (Tokyo, Japan) with Cu-Kα radiation (λ = 1.5418 Å). Samples were

scanned at a current of 100 mA and a voltage of 50 kV. Patterns were obtained using a step width of 1.2°/min from 0° to 50° at room temperature on a 2θ scale.

5.2.7 Retention of Bioactives

The amount of the major bioactive compounds (eugenol and thymol) in the liquid and dried products was monitored by high-performance liquid chromatography with diode array detection (HPLC-DAD), following a method previously developed by Leal et al. (24) and validated by our group. Analyses were performed in a Shimadzu HPLC (Shimadzu Corporation, Kyoto, Japan) using a C-18 column (Shimadzu Shim-Pack CLC(M) 4.6 mm × 25 cm, 5 μm, 100 Å) at an oven temperature of 30 °C, with a volume injection of 20 μL. The mobile phase used was water (A) and acetonitrile (B) using gradient elution: 0–2 min 10% B in A; 2–7 min 10–78% B in A; 7–17 min 78% B in A; 17–20 min 78–100% B in A; 20–23 min 100% B in A; 23–26 min 100–10% B in A; and 23–32 min 10% B in A. The chromatograms were recorded at 276 nm. The samples were diluted with methanol, homogenized in an ultrasound bath, and kept under magnetic stirring for 30 min. After the extraction, the samples were centrifuged for 5 min at 5000× *g* and the supernatants were filtered in a 0.4 μm Millipore membrane and analyzed by HPLC.

5.2.8 Statistical Analysis

Results were expressed as mean ± SD. A two-way analysis of variance (ANOVA) using a Bonferroni post-hoc test was used to compare the levels of significance between the samples and $p < 0.05$ was considered statistically significant.

5.3 RESULTS AND DISCUSSION

5.3.1 Stability Analysis of Liposomes

For most commercial applications the long-term stability is an important parameter to be considered for a delivery system. We, therefore, carried out a series of tests to evaluate the physicochemical stability of liposomes over one month. The mean hydrodynamic diameter, PDI, and ZP of the liposomal formulations were measured over a storage period of 30 days at 4 °C. The freshly prepared liposomal solutions exhibited a homogeneous whitish appearance. Figure 5.2 demonstrates the distribution of nanometric and micrometric particles, varying according to the encapsulated bioactive. In general, the systems showed an appreciable increase in the hydrodynamic diameter throughout 30 days of storage at 4 °C, which suggests vesicle coalescence. On the other hand, the liposome formulation containing clove essential oil (CDC)

had a mean particle size perceptibly smaller than the other samples. We hypothesized that the differences in particle size are at least partly due to the type of the encapsulated essential oil, because we obtained larger liposomes containing *Lippia sidoides* essential oil when compared to the liposomes containing clove essential oil, produced by the same method in our pre-formulation studies (data not shown).

The polydispersity index (PDI) was determined to indicate the width of particle size distribution, whose values ranges from 0 (monodisperse system) to 1 (very polydisperse distribution), reflecting the tendency of the particles to aggregate. The PDI values were similar for all the formulations, slightly increasing upon storage. Similar findings were obtained by other authors (25,26), possibly because HP-β-CD might replace the drug molecules from the hydrophobic core with cholesterol (or another lipid component), favoring the destabilization of the liposomal structure (27). This problem is based on a question of affinity of the asset with the HP-β-CD, the greater the affinity of the bioactive for the HP-β-CD, the more stable the inclusion complex will remain. Therefore, to overcome this problem, the types and concentrations of CD, and the affinity with the drug molecules should be intensively investigated during the pre-formulation studies. The selection of lipids with lower affinity for cyclodextrin than cholesterol or the drug itself should be preferable (27). As an indicator of vesicle stability, ZP values were reduced over the storage time, corroborating the evidenced changes in particle size and PDI.

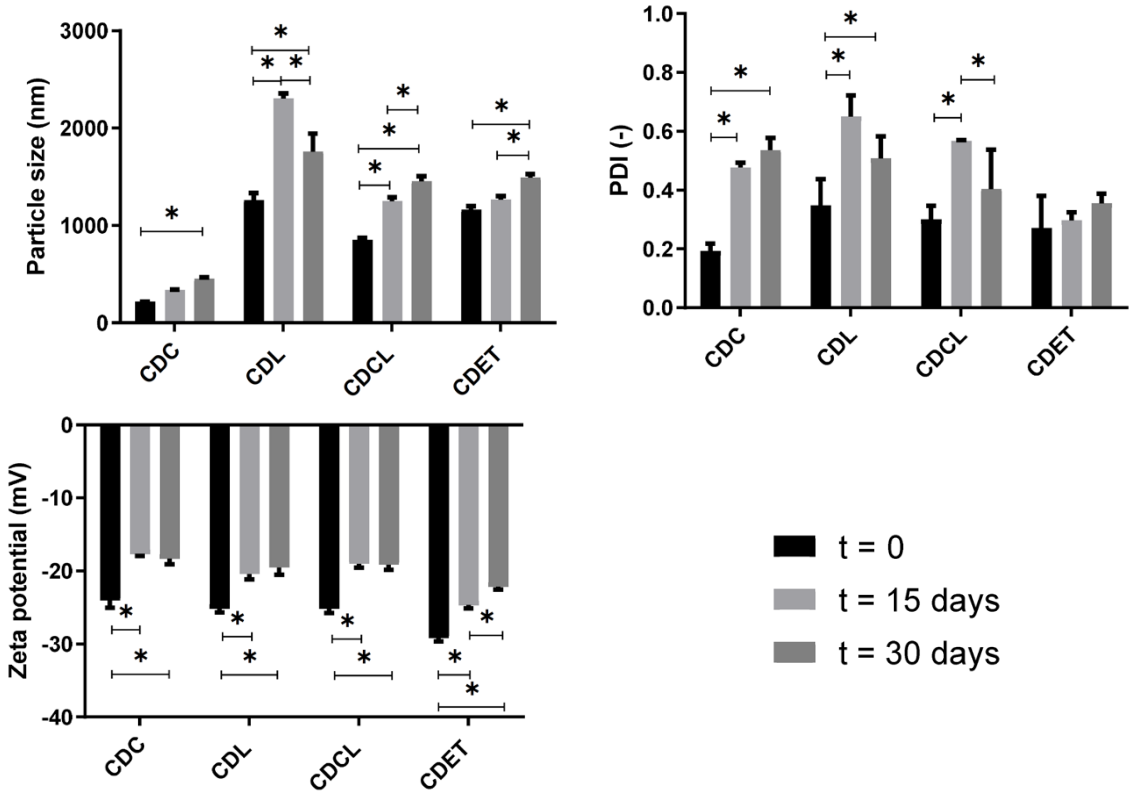

Figure 5.2. Stability parameters of EOs-in-cyclodextrin-in-liposome systems during storage at 4 °C, at t = 0 days, t = 15 days, and t = 30 days. The symbol (*) means significant difference at $p < 0.05$ (Bonferroni post-hoc test).

5.3.2 Proliposomes Properties

Reconstituted proliposomes were characterized and compared in terms of mean diameter, PDI, and ZP (Table 5.2). Significant differences were found between the sizes of the liposomal systems. The presence of LEO affected the reconstituted liposomes mean size significantly; CDL and CDET showed similar sizes. PDI was also similar among the developed formulations, slowly increasing from 0.3 to ≈ 0.4, indicating the relative homogeneity of proliposomes. The ZP is a parameter linked to the electrostatic repulsion between suspended or emulsified systems and generally is used as indicative of system stability (28). All formulations showed negative values of zeta potential, ranging from -22.3 to -26.5 mV. Particularly, the larger proliposomes (CDCL) showed significantly higher zeta potential than the others, in agreement with literature results (28).

Table 5.2. Particle size, PDI, and zeta potential of proliposomes obtained by freeze-drying.

Formulation	Particle Size (nm)	PDI (-)	Zeta Potential (mV)
CDC	354.3 ± 27.1 [a]	0.41 ± 0.05 [†]	−22.3 ± 0.7 *
CDL	1648.0 ± 106.4 [b]	0.41 ± 0.05 [†]	−23.3 ± 0.6 *
CDCL	3300.3 ± 476.8 [c]	0.31 ± 0.21 [†]	−26.5 ± 1.3 **
CDET	1493.0 ± 70.6 [b]	0.29 ± 0.02 [†]	−23.6 ± 0.3 *

Same letter (a, b, c) or symbol (*, **, [†]) means no significant difference according to Bonferroni's multiple comparison test ($p < 0.05$).

Determination of an inclusion complex between host and guest molecule depends on a variety of parameters and requires multivariate analysis for this description. Therefore, complementary characterization methods, including differential scanning calorimetry (DSC) and X-ray diffraction (XRD), were employed to verify the occurrence of complexation. DSC is the most commonly used technique for determining the thermal effects of the material. It quickly provides accurate information on both the physical and energetic properties of the material (29) and indirect evidence about the formation of the cyclodextrin inclusion complex (6). In this study, DSC was used to study the influence of bioactive compounds on lipid membrane organization.

It is well known that some constituents of the essential oils, among them thymol and eugenol, can increase the fluidity of the liposomal membrane by reducing the phase transition temperature of the phospholipid (30,31). The DSC analysis of the proliposomes (Figure 5.3 and Table 5.3) showed no melting peak of the actives (represented by red or green lines in Figure 5.3), indicating the absence of any significant level of crystallinity in the analyzed product, confirming its amorphous state. Moreover, the bioactive compounds were able to interact with the phospholipidic membrane, causing variations in the thermodynamic parameters (peak temperature—T_m and enthalpy difference—ΔH) (Table 5.3). Such variations were more pronounced in proliposomes loading thymol, eugenol, and clove EO. According to Cristani et al. (32), these terpenes act as substitutional impurities, causing a decrease in T_m and ΔH values. The disappearance or even gradual decrease of the melting point of a crystalline guest compound provides indirect evidence of the inclusion complex formation with cyclodextrin. However, DSC alone is not a suitable technique to confirm the formation of the cyclodextrin inclusion complex when using volatile substances as guest compounds (33), as the essential oils, thymol, and eugenol. For such guest molecules, X-ray powder diffraction is the most useful technique to detect the inclusion complex formation (18).

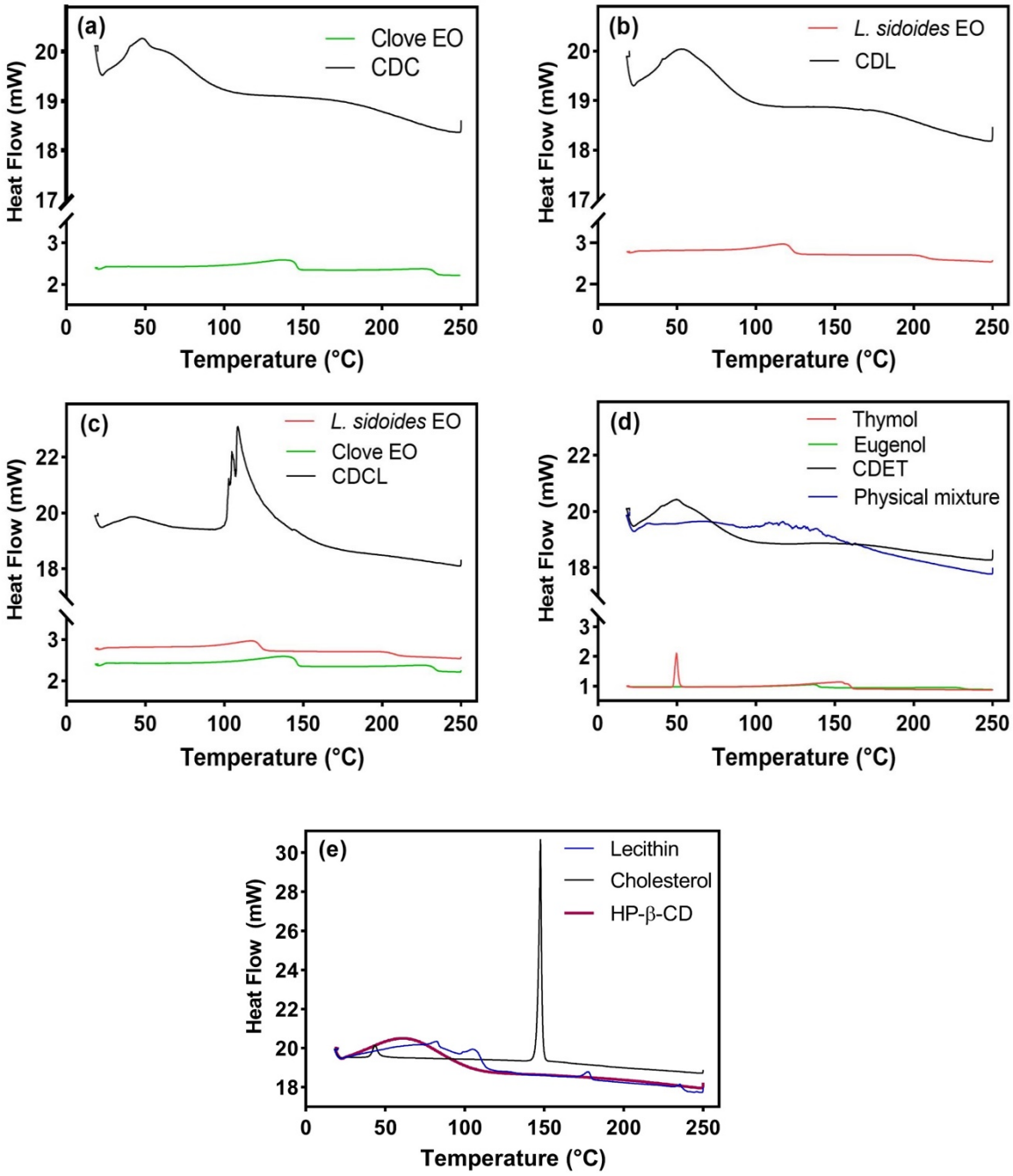

Figure 5.3. DSC thermograms of proliposomes, essential oils, isolated bioactives, and raw materials: (**a**) CDC proliposome loaded by clove essential oil and pure clove essential oil; (**b**) CDL proliposome loaded by *L. sidoides* essential oil and pure *L. sidoides* essential oil; (**c**) CDCL proliposome loaded by both essential oils (*L. sidoides* and clove), and the pure essential oils; (**d**) CDET proliposome loaded by both isolated bioactives (thymol and eugenol), the pure isolated bioactives, and physical mixture; (**e**) bulk raw materials (lecithin, cholesterol, and HP-β-CD). Up: endothermic heat flow.

Table 5.3. DSC parameters of proliposomes, bulk materials, bioactive compounds, and essential oils.

Sample	Onset (°C)	Peak (°C)	Δ*H* (J/g)	Area (mJ)
CDC	32.1	48.7	80.8	177.8
CDL	31.7	54.3	93.9	197.3
CDCL	107.1	108.6	101.5	314.8
CDET	28.0	49.9	113.2	237.7
Lecithin	97.8	106.5	12.1	31.4
Cholesterol	145.8	147.7	67.7	148.9
HP-β-CD	23.2	63.3	160.3	368.7
Eugenol	110.9	136.6	111.7	234.6
Thymol	47.8	49.8	125.6	314.0
L. sidoides EO	91.5	117.7	97.8	195.5
Clove EO	101.5	138.4	156.3	343.9

X-ray diffraction is also able to characterize and identify the structure of lipid and drug molecules (34). The X-ray powder diffraction patterns of pure compounds and loaded proliposomes are presented in Figure 5.4. The peaks patterns from HP-β-CD are also present in the samples (CDC, CDL, CDCL, and CDET) and the physical mixture, but are broader and with lower intensity. The addition of guest compounds to the HP-β-CD increased the lattice disturbance of the latter, as can be seen by comparing the patterns of the HP-β-CD with the proliposomes. This interaction resulted in the appearance of new peaks in the proliposome samples, producing a diffractogram that differs from the one of non-complexed HP-β-CD, evidencing the formation of the inclusion complexes. Concerning lecithin, its main peak was presented in CDC, CDL, and CDET proliposomes, also with a decrease in intensity. The decrease in the intensity of this peak may indicate a change in the crystalline structure of lecithin and a less ordered structure of proliposomes. The broad diffraction peaks also reflect a reduction in crystallinity (6,35). Such an amorphous state might contribute to the higher retention of bioactives (35). However, as the diffraction patterns presented by CDC, CDL, and CDET proliposomes were similar, retention of bioactives in these samples probably also remained similar.

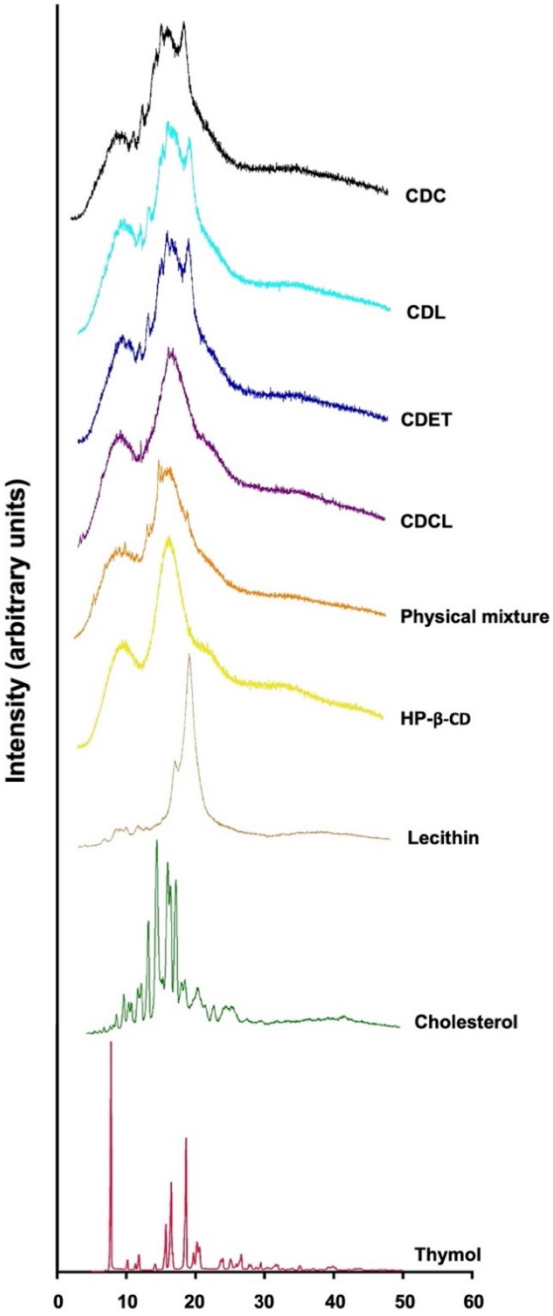

Figure 5.4. X-ray diffraction (XRD) profiles of proliposomes (CDL, CDCL, CDET), physical mixture (lecithin, cholesterol, HP-β-CD, thymol, and eugenol), and bulk raw components.

5.3.3 Retention of Marker Compounds in Proliposomes

The content of bioactives in the proliposomes is strongly associated to the previously investigated variables. The results of eugenol and thymol retention in the liposomal structures are shown in Figure 5.5. The eugenol retention reached near 90% for liquid samples and 72% for proliposomes, while for thymol the values were around 80% for both systems, with no statistical difference between the samples ($p < 0.05$). Indeed, the freeze-drying had a significant

influence on eugenol retention both for formulations containing essential oil (CDC and CDL) and the isolated bioactives (CDET). Furthermore, the liquid formulations achieved higher eugenol retention in comparison to thymol, but the effect was significantly lower for the dried proliposomes. Similar results for eugenol loss during the freeze-drying of β-CD inclusion complexes have also been reported (6). The relatively higher lipophilicity of thymol suggests favored partitioning both into the lipidic wall of proliposome and the hydrophobic internal cavity of HP-β-CD, rather than the external hydrophilic surface of the cyclodextrin. Hence, thymol is apparently better incorporated into the lipophilic parts of the delivery system, remaining retained, despite the water removal during the freeze-drying process. Molecular modelling studies could explain the differences between eugenol retention in liquid and proliposomes samples through the arrangement of bioactive molecules inside the CD cavity. On one hand, the eugenol molecule enters into the CD cavity from the wider rim, leaving OH and MeO groups outside and the allyl group keeps folded towards the phenyl ring (36). On the other hand, the thymol molecule is located inside the hydrophobic cavity of CD in a way that allows its OH group to come close to the hydroxyl groups of CD to form hydrogen bonds that further stabilize the inclusion complex (37). Thus, thymol tends to be more stabilized in the inclusion complex when compared to eugenol, despite the negative pressure and water removal from the freeze-drying drying process.

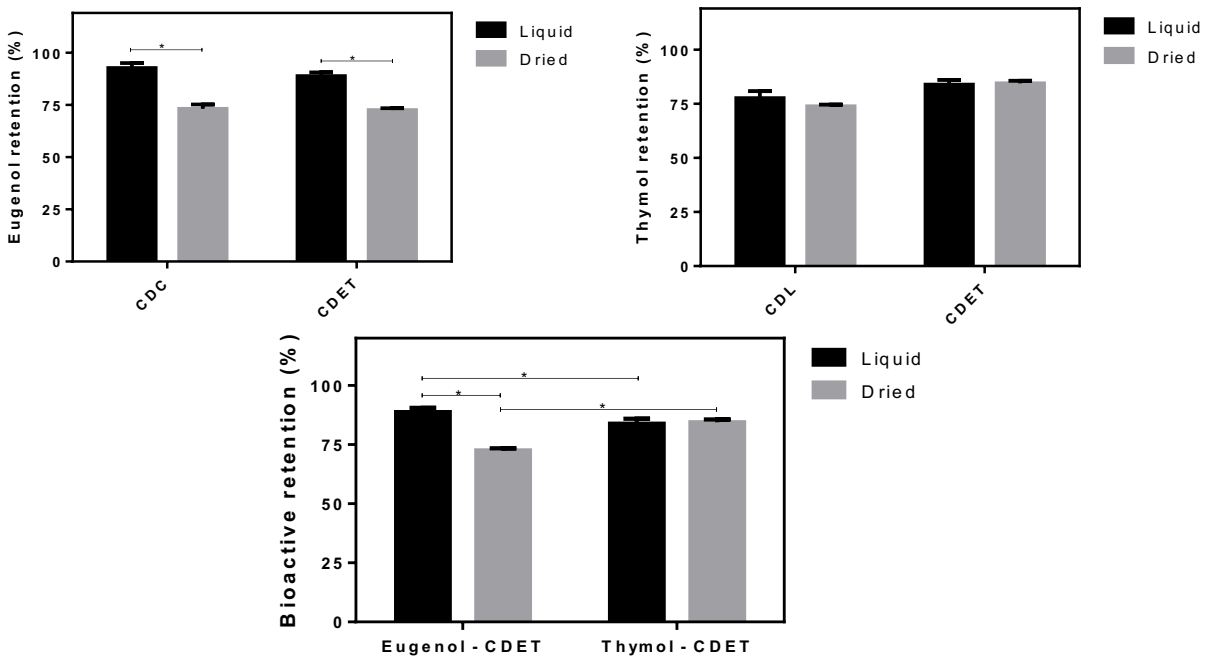

Figure 5.5. Eugenol and/or thymol retention of liquid and freeze-dried forms of EOs-in-cyclodextrin-in-liposome systems. The symbol (*) means significant difference at $p < 0.05$ (Bonferroni post-hoc test).

5.4 CONCLUSIONS

Taken together, the results described in the present study allow us to potentially propose drug-in-cyclodextrins-in-liposome as a carrier system to be loaded with *Lippia sidoides* and/or *Syzygium aromaticum* EOs, as well as the isolated EOs main compounds eugenol and thymol. With the selected preparation method relatively stable particles and proliposomes have been obtained. The liquid systems and reconstituted composition showed sizes between 350 nm and 3300 nm with a low polydispersity index and zeta potential around −23 mV. The samples also presented a low degree of crystallinity and high retention of the major marker compounds (eugenol and thymol). While not compromising the objectives of this study, which have been successfully accomplished, namely the demonstration that our proposed methodology has been successful for the encapsulation of EOs and other natural phytopharmaceutical and volatile compounds in cyclodextrin-in-liposomes, we share the opinion that some additional studies, namely, the assessment of the release and toxicological profiles, including scanning/transmission electron microscopy and Fourier-transformed infrared spectroscopy, as well as the evaluation of the promising biological activity, will certainly add value to the potential use of these systems. Overall, EOs-in-cyclodextrin-in-liposome has remarkable potential for use in a wide variety of products of food, cosmetic, and pharmaceutical industries.

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CHAPTER 6. SLN AND NLC FOR TOPICAL, DERMAL, AND TRANSDERMAL DRUG DELIVERY

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6.1 INTRODUCTION

Solid lipid nanoparticles (SLN) were developed in the early 1990s as alternative delivery systems to liposomes, emulsions and polymeric nanoparticles [1-3]. Owing to the lipid biocompatibility and versatility, SLN show many advantages over polymeric and inorganic nanoparticles for the delivery of a set of drugs [4-7]. Advantages, such as reduced toxicity [3,4], higher loading capacity [8], chemical versatility [9], biodegradability of lipids [9], possibility of large-scale production [8,10,11], and a wide range of applications in various fields [4,10], reason the interest of academia and industry in seeking new applications of lipid nanoparticles as delivery systems. SLN are composed of a solid lipid matrix with a melting point above 40ºC, in which the drug is incorporated and/or attached. As nanoparticles remain solid after administration, they offer controlled release of the loaded drug [10].

The second generation of lipid nanoparticles is known as nanostructured lipid carriers (NLC). Unlike SLN, the lipid matrix of NLC consists of a blend of solid and liquid lipids (oils), which provides a reduction in the melting point of the solid lipid, the matrix yet still remaining solid at room and body temperatures [3]. NLC are also stabilized in aqueous dispersion using a surfactant or a mixture of surfactants. The presence of oil in the composition avoids the recrystallization of the solid lipid over storage, contributing to increase the loading capacity, in particular, for lipophilic compounds [10]. Moreover, as the addition of oil prevents the recrystallization of solid lipids, a more thermodynamically stable system is obtained, less likely to expel the payload from the particle, also improving its release properties [4,10,12]. The mean particle size of SLN and NLC is in the submicron range, ranging from about 40 to 1000 nm, depending on the composition of the lipid matrix (i.e., lipid and surfactant combination) and on the production method [5]. Figure 6.1 illustrates the differences between SLN and NLC lipid matrix structure.

Literature describes different combinations of lipids and production methods to obtain SLN and NLC. The possibility of using generally recognized as safe (GRAS) materials or even raw materials of natural origin [13], increases the application range and the interest of different market segments in the use of these nanoparticles. In addition, the proper selection of components may allow the control of the morphology, structural and occlusive properties, as well as drug loading. This review focuses on the cutaneous application of SLN and NLC, giving an overview on commonly used materials (and selection criteria), production methods and drugs that have been successfully loaded in these nanoparticles.

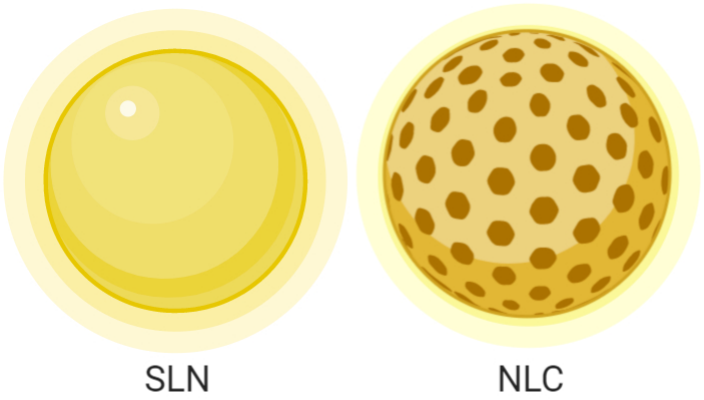

Figure 6.1 SLN and NLC matrix structure

6.2 SLN AND NLC INGREDIENTS FOR CUTANEOUS DELIVERY

SLN are composed of solid lipids (at room temperature), usually at a concentration between 0.1% (w/w) and 30% (w/w) [14,15]. In contrast, the matrix of NLC is derived from a combination of solid lipid and oil (liquid lipid), and the ratio of this blend may vary from 70:30 to 99.9:0.1 [16-18]. The total lipid content of the NLC may vary from 5-40% [16-19]. In both cases, the nanoparticles are stabilized in aqueous medium using at least one surfactant in a concentration ranging from 0.5-5% w/w [3,5,14]. Proper combination of ingredients can be achieved using factorial design experiments usually setting the mean particle size, polydispersity index (PI) and zeta potential (ZP) the dependent variables [13,20-26].

The selection of excipients is instrumental to ensure biocompatibility and safety [27-31]. When selecting components for SLN and NLC formulations, some parameters have to be considered, namely, drug solubility in the lipid, melting temperature of the lipid, compatibility and miscibility between the selected solid and liquid lipids, choice of surfactant and its Hydrophilic Lipophilic Balance (HLB) and also the method of production [5,32]. A summary of components typically used in formulation of SLN and NLC for topical drug delivery is presented in Table 6.1.

Table 6.1. Frequently used ingredients for SLN and NLC production.

Ingredient	Examples	Properties	References
Solid lipids	Beeswax	Natural wax with GRAS status, composed of a mixture of fatty acids and fatty alcohols esters, with melting point of 62-64˚C; it requires HLB of 9 to be emulsified.	[33-35]
	Carnauba wax	Natural wax with GRAS status high melting point of 82-85˚C, composed of monoesters; it requires HLB of 12 to be emulsified.	[34-36] [37]
	Cetyl palmitate	Synthetic wax produced by esterification of cetyl alcohol and palmitic acid, with melting point between 40.5-51˚C, depending of the composition; it requires HLB of 10 to be emulsified.	[1,33,36,38-40]
	Compritol® 888 ATO	Blend of different esters of behenic acid with glycerol; it holds acceptable safety profile, with a melting point of 69-74˚C, and is also stablished as emulsifier, with HLB ≈ 2.	[5,36,41-44]
	Dynasan®	Triglycerides series from Sasol; a group of natural and safe lipids, which includes Dynasan 112 (trilaurin; melting point 46˚C), Dynasan 114 (trimyristin; melting point 55-58˚C), Dynasan 116 (tripalmitin; melting point 61-65˚C), and Dynasan 118 (tristearin; melting point 70-73˚C).	[33,40,42,45,46]
	Gelucire®	Series of lipid from Gatefossé defined by their melting points between 33-70˚C and by the HLB between 1-18. The most frequently used for SLN/NLC is Gelucire 50/13 (stearoyl macrogol-32 glycerides) and is GRAS listed.	[5,47-51]
	Precirol® ATO 5	Glyceryl palmitostearate, is a mixture of mono, di and triglycerides of palmitic and stearic acid, of GRAS status, melting point of 58˚C, low HLB of 2.	[43,52,53] [33,54] [43]
	Softisan® 378	Blend of triglycerides with hydrocarbon chain length of C8-C18, low melting point of 35-42˚C and GRAS status.	[45,54,55]
	Stearic acid	Endogenous long-chain saturated fatty acid, GRAS listed, with melting point around 70˚C and HLB around 15.	[32,56-58]

Liquid lipids	Miglyol® 812	Triglycerides of capric and caprylic acid, are medium chain triglycerides with high stability against oxidation, holds GRAS status and high solubility for many drugs.	[32,36,45,48,55]
	Oleic acid	Pure substance used as emulsifying agent and penetration enhancer, with GRAS status.	[5,52,54,58]
	Squalene	Triterpene produced by human skin cells (as precursor for cholesterol).	[5,43,53]
	Vitamin E/α-tocopherol	Liquid lipid with the advantage of providing protection to oxidation sensitive substances.	[41,59]
Surfactants	Lecithin	Is an essential component of cell membranes, obtained from different sources (egg, vegetables) and used in a wide variety of pharmaceutical application as emollient, emulsifying and solubilizing agent, with HLB between 4-9.	[35,56,60] [19,54]
	Plantacare® 810	Caprylyl/capryl glucoside is a highly effective stabilizer for SLN and NLC, with HLB 15-16.	[1,39]
	Poloxamer® 188	Nonionic triblock copolymer with hydrophilic and lipophilic units, used as emulsifier and stabilizing agent in a wide variety of pharmaceutical formulations, it is nontoxic and nonirritant, with HLB > 24.	[5,45,54,57,59]
	Quillaja saponin	Natural saponin-based surfactant, isolated from the tree *Quillaja saponaria*, composed by a complex mixture of amphiphilic constituents. Exhibit antioxidant properties and HLB of 13.5.	[5,61,62]
	Sodium lauryl sulfate	Sodium dodecylsulphate is an anionic surfactant, widely used in cosmetics and pharmaceutical formulations, moderately toxic, but with GRAS status and HLB ≈ 40.	[54,63]
	Tween®80	Polyoxyethylene sorbitan monooleate, or polysorbate 80, is an O/W surfactant widely used in cosmetic, food and pharmaceutical formulations, including intravenous products. Holds GRAS status and HLB around 15.	[5,35,42,45,48,52-54]

6.2.1 Choice of lipids

The solid lipids employed in the formulation of SLN and NLC are biocompatible/physiological and biodegradable lipids, which may either be used independently (in SLN) or as mixture of two or more lipids in a specified ratio (NLC). The wide variety of lipids used in topical lipid nanoparticulate formulations may be classified as fatty acids, waxes,

steroids, partial glycerides and triglycerides. These lipids are melted during the fabrication of nanoparticles at high temperature *i.e.* above 80˚C. The liquid lipids (oils), typically found in the lipid matrix of NLC, are usually derived from natural sources and have been granted GRAS (Generally Recognised As Safe) status by regulatory bodies. Medium chain triglycerides, such as Miglyol® 812, together with oleic acid and linoleic acid, have been most commonly used as penetration enhancers [64]. Some authors have also explored oils from botanical sources, e.g. Mediterranean essential oils [16,65], alpha-pinene [66], citral [26], linalool [22], Siberian pure seed oil [46], sucupira oil [13], owing to their inherent dermatological benefits. Use of tocols has also been proposed in this regard [41,59,67].

The choice of lipids is dictated by the solubility properties of the active moiety (i.e. drug) to be loaded [68-70]. This can either be located between the lipid layers (possible only when the size of drug molecules is smaller by 20%, as compared to lipid molecules) or between the fatty acid chains and imperfections of the lipid matrix [3]. The type of lipids used, as well as the ratio of solid and liquid lipids forming the core of NLC, was found to influence the drug loading and structural properties of the particles, namely, type I (imperfect model), type II (amorphous model) or type III (multiple model) [68]. For SLN, the classical structures are the type I (homogeneous matrix model), type II (drug-enriched shell model) and type III (drug-enriched core model). Some general rules in this respect may be considered:

i. Highly ordered matrices, spatially similar lipids or monoacid glycerides of high purity (e.g. tristearin) lead to a decrease of the loading capacity, while accelerating the drug expulsion process [3,71].
ii. Admixture of dissimilar lipids enhances the loading capacity owing to the creation of imperfections in lipid matrix. Such mixtures can however result in the creation of supercooled melts. For instance, mixing triglycerides (possessing high melting points and high crystallization temperature) with lipids of small chain length, causes an increase in the supercooling effect. Likewise, addition of long chain length lipid molecules to lipid with low crystallization temperature, reduces supercooling. Differential Scanning Calorimetry (DSC) studies are commonly run for the identification of supercooling [20,41,72-74].
iii. Lipids, such as tricaprin, trilaurin, trimyristin and certain Witepsol bases are known for their tendency to yield supercooled melts. Thus, lipid nanoparticles meant for prolonged release and/or enhanced occlusion should not be formulated with these lipids [75,76].
iv. The structure of NLC varies with the lipid composition of the matrix. When a solid lipid is mixed with a small amount of a liquid one, an ‘imperfect crystal’ type of NLC arises.

The ‘amorphous type’ is obtained by mixing special lipids, such as hydroxyoctacosanyl hydroxy stearate, isopropyl myristate, which do not crystallize after cooling. The third type of NLC is derived from multiple water-in-oil-water (w/o/w) emulsions, comprising nanocompartments of liquid lipid within the lipid matrix (e.g. oil-fat-water type III NLC). These are obtained when the liquid lipid is present in a concentration that exceeds its solubility in solid lipid. In this case, the drug exhibits greater solubility in the liquid lipid than in the solid one, getting entrapped in the oily nanocompartments [77-81].

Yang et al. evaluated the effect of the liquid lipid in the crystallization and aggregation stability of tristearin NLC dispersions [82]. The amount of oil present in the NLC formulation significantly influenced the crystallization of NLC, their melting temperature and degree of polymorphism observed. An effective liquid lipid, such as triolein and tricaprylin, enhanced the ability of the surfactant to stabilize NLC dispersion. Additionally, oils (such as olive oil) offer highwe mobility at the interface, enabling the surfactant to completely cover hydrophobic surface during polymorphic transitions. The use of liquid lipids, which produce less ordered crystal lattice (e.g. pentadecane), results in more stable NLC formulations.

6.2.2 Role of Surfactant

Surfactants act by reducing the interfacial tension between the lipid and the aqueous phase, thereby contributing to the stability of the resulting formulation. Surfactants are amphiphilic in nature and their molecules get preferentially located at the interface. The surfactants of ionic nature (e.g. sodium deoxycholate) increase the nanoparticles surface charge , causing electrostatic repulsion and, thus, improving the physical stability [32,73]. Non-ionic surfactants (e.g. Poloxamer 188, Sorbitan monoesters and polysorbates) circumvent nanoparticle aggregation by virtue of steric stabilization effect. Addition of co-emulsifying surfactants possessing high mobility, also retards the gelation of colloidal nanoparticle dispersions [83].

Radomska-Soukharev carried out an in-depth investigation to study the stability of lipids in SLN formulations using different lipids and varying amounts of surfactants [84]. It was found that triglycerides yield more stable products as compared to mono and diglycerides. It was postulated that a binary mixture of surfactants imparts more stability than a single surfactant. It was further stated that the nature of surfactant and its concentration has an impact in its solubilising capacity for water in the lipid phase, and also brings about variations in the incorporation of the surfactant in the outer shell of SLN and its distribution in the molten lipid

phase. SLN dispersion can cause distortion in crystallization behaviour, thereby, lowering the melting enthalpy. Moreover, the effects of electrostatic and steric stabilization were found to be additive.

In practice, the selection of surfactant mix is realized in view of the HLB of the lipids constituting the nanoparticle matrix and their concentration in the lipid phase of the dispersion [32]. Surfactants belonging to the following categories have found application in formulation of SLN/NLC: Phospholipids, ethylene oxide/ propylene oxide, copolymers and sorbitan esters, polysorbates, alkylaryl polyether alcohol polymers and bile salts [71]. Table 6.2 lists the commonly used excipients in lipid nanoparticle formulations.

Table 6.2. Commonly used excipients in lipid nanoparticle formulations for cutaneous applications.

Solid lipids	Liquid lipids	Surfactants
Glyceryl behenate (Compritol® 888 ATO)	Oleic acid	Polysorbates
Glyceryl palmitostearate (Precirol® ATO5)	Castor oil	Sodium cholate
Cetyl palmitate	Caprylic/capric triglycerides (Miglyol® 812)	Soybean lecithin
Beeswax	Olive oil	Tyloxapol
Carnauba wax	α-Tocopherol	Poloxamer 188 (Pluronic® F68)
Glyceryl monostearate	Squalene	Sorbitan esters
Glyceryl tristearate	Labrafac	Phosphatidylcholine
Glyceryl tripalmitate	Isopropyl myristate	Egg lecithin
Glyceryl trimyristate	Transculol HP	Gelucire 50/13
Hydrogenated palm oil (Softisan® 154)	Linoleic acid	Sodium oleate
Stearic acid	Soybean oil	Solulol H515

6.2.3 Use of surface modifiers

Surface modification of SLN/NLC has been attempted by many formulators with the view to enhance their stability and/or increase their circulation systemically, avoiding their uptake by reticuloendothelial system (RES) [71]. This strategy involves the use of hydrophilic substances such as PEG [85-87], chitosan [88,89], lecithin [90-92] and dicetyl phosphate [93], which coat the hydrophobic surface of the nanoparticles. As a result, not only their stability and

dispersibility is improved but also their interaction with mucosal membranes can be customized for drug targeting. Additional advantages include reduced thrombogenicity of nanoparticles and the feasibility of providing a depot effect for the release of hydrophobic drugs from drug carriers bound by a hydrophilic coating [71]. Surface modification is particularly useful for drug transport through skin using lipid nanoparticles if the drug reaches systemic circulation via transdermal route [85]. Gao et al. reported an increased penetration and skin deposition of lornoxicam NLC, whose surface had been modified with polyarginine peptide [94]. On the other side, Silva et al. reported that surface coating of clobetasol loaded NLC increased the retention of the drug in the *stratum corneum* [95].

The perspectives presented above substantiate the influence of SLN/NLC components on their structure, stability and drug release. In the broader outlook of formulation development, certainly other aspects also draw our attention. These include the disease-specific parameters and route of the drug administration, propensity of skin irritation and delivery mode/vehicle. The following points must be taken into account:

i. The nature of the disease and the desired site of action dictate the route of drug administration. For transdermal drug delivery, lipid nanoparticles should have a mean size below 100 nm. Such small sized particles can be tailored by the appropriate choice of lipids, surfactant, and their ratio.

ii. Although lipid nanoparticles have been crowned as ‘nanosafe carriers’ [18], the surfactants employed in their formulation could be a cause of skin irritation [96], particularly when using new surfactants with less known toxicity profile [28,29,97].

iii. Depending upon the therapeutic requirements, the developed NLC/SLN could be embedded in a suitable semi-solid, such as hydrogel, cream, ointment [98]. This not only facilitates application at the desired site but the properties and ingredients of the semi-solid could act in synergistic fashion with the SLN/NLC to yield a therapeutic response or dermatological effect.

The interplay of various components forming a lipid nanoparticulate system determines their characteristics and performance specifications. Therefore, the selection of ingredients of lipid matrix and other excipients should be based on vigorous pre-formulation studies and sound literature review. These often-overlooked elements of formulation design and development hold much significance in determining the final outcome.

6.3 METHODS FOR SLN AND NLC PRODUCTION

6.3.1 High-pressure homogenization (HPH)

High-pressure homogenization is the main method stablished for SLN and NLC production [4,99]. The advantages of this technique go beyond the short production time. This method also allows laboratory scale production to be easily transposed to large-scale production [100]. Besides, the avoidance of organic solvents, yielding average particle size in the sub-micron region [9], and the variety of homogenizers brands and models at a reasonable price [101], make this a widely used technique in many industries. However, being a process that requires high energy intensity, it increases the temperature of the samples, which is not suitable for heat sensitive compounds [99]. This technique involves pushing the high-pressure sample into a very narrow gap (a few microns wide). High shear stress and cavitation forces reduce particles to submicron range [101]. High-pressure homogenization of SLN and NLC can be performed at both high and low temperatures (hot and cold homogenization, respectively). However, it is important to keep in mind that for both techniques it is necessary to dissolve or disperse the drug at a temperature about 5°C above its melting point [99,101].

6.3.1.1 Hot HPH

In hot homogenization, the entire process is carried out at temperatures above the melting point of the lipid. First, a pre-emulsion of the drug loaded lipid melt and the aqueous emulsifier phase (5-10°C above lipid melting point) is obtained by high speed stirring (e.g. Ultra-Turrax). The hot pre-emulsion is then homogenized at high pressure at controlled temperature. For SLN and NLC production, a single homogenization cycle is sufficient to produce a hot emulsion with particle size in the range of 250-300 nm [102], when the pre-emulsion lipid concentration is in the range of 5-10%. Finally, the nanoemulsion obtained is cooled to room temperature and recrystallizes, forming SLN and NLC [14]. It is also possible to homogenize emulsions whose concentration reaches 40% [101,103]. However, lipid concentrations above 30% cannot be used to form NLC, but rather highly concentrated SLN formulations [104]. Nonetheless, the number of cycles will depend on the emulsion lipid concentration, since the energy required to shear the lipid mass is directly proportional to its concentration in the formulation [104]. On the other hand, increasing the number of homogenization cycles often results in increased particle size, since particle kinetic energy increases, favoring coalescence [101,105]. The literature usually reports the use of three homogenization cycles at 500 bar [102]. Literature reports that, in general, hot homogenization

can be used even for temperature sensitive compounds, since the time of exposure to elevated temperature is relatively short [104]. The temperature employed in the process is nevertheless a limitation of this technique, especially for extremely temperature sensitive compounds and hydrophilic compounds, which, with the high temperature, can partition from the lipid phase to the aqueous phase [9,104].

6.3.1.2 Cold HPH

Cold homogenization has been developed to overcome the problems related to the hot homogenization [101]. This process is recommended for extremely temperature sensitive and hydrophilic compounds [104]. Although it minimizes thermal exposure, this technique does not fully prevent it, since the active substance must be dissolved in the melted lipid phase in the initial step [101]. Then, the melted mixture is rapidly cooled down to a solid state with dry ice or liquid nitrogen. This rapid cooling favors the homogeneous distribution of the active compound in the lipid phase. The formed solid is then ground to powder microparticles, and a pre-emulsion is formed by high speed stirring of the powder in a cold aqueous surfactant solution. The dispersion is subjected to the homogenizer at or below room temperature, usually for five cycles at 500 bar, to form the lipid nanoparticles [14,99]. The disadvantage of this technique lies on the need for high energy in the severe homogenization step. In other words, this is not an energy efficient process [99]. Moreover, compared to hot HPH, larger and more polydisperse particles are observed in cold HPH [101].

6.3.2 Microemulsion technique

This method consists of melting the lipid (or lipid blend) and heating the aqueous phase (containing surfactant) at the same temperature. The microemulsion is prepared by adding the aqueous solution to the lipid phase under mild stirring. Lipid nanoparticles are obtained by dispersing the microemulsion in cold water (2-10°C) under stirring. Finally, the system is washed with distilled water, filtered (to remove larger particles) and can be lyophilized to remove the excess of water [8,106]. This technique allows the formation of nanoparticles at mild temperature conditions. Nevertheless, the disadvantages include the need for relatively high concentrations of surfactants, the strong dilution of the particle suspension by pouring the microemulsion into water and obtaining a suspension with a very low particle concentration [8,102].

6.3.3 Emulsification-solvent diffusion

This method involves the formation of an oil-in-water emulsion with a partially water-miscible solvent with low toxicity. The process is based on the water miscibility in these solvents, which contains the drug. Once formed, this transient oil-in-water emulsion is transferred to water, under continuous stirring, which causes the solvent to diffuse into the outer phase, resulting in the solidification of dispersed phase and formation of nanoparticles. Depending on the boiling point, the solvent may be further removed by evaporation under reduced pressure. [107,108]. As advantages, this approach is versatile, reproducible and easy to implement; does not require high-energy sources; does not expose the drug to conditions of temperature stress and agitation; and results in narrow size distribution. On the other hand, it is necessary to clean up and concentrate the lipid nanoparticle dispersion [108].

6.3.4 Emulsification-solvent evaporation

In this technique, the lipid matrix is dissolved in a water-immiscible organic solvent and emulsified by the aqueous phase. The solvent is evaporated under reduced pressure, favoring the formation of nanoparticle dispersion by lipid precipitation in the aqueous medium. This is a totally heat-free technique that can result in very small nanoparticles up to 100 nm, depending on the components used. However, one drawback is the use of organic solvent, which can leave toxic residues in the sample [107].

6.3.5 Solvent injection (or solvent displacement)

This approach consists of dissolving the lipid matrix in a water-miscible solvent and rapidly injecting the mixture through an injection needle into a stirred surfactant-containing aqueous phase [109]. The technique is easy to implement, is versatile and efficient for obtaining lipid nanoparticles. Nonetheless, the use of organic solvent is a disadvantage. [109].

6.3.6 Phase inversion

This is a solvent-free technique that consists of mixing the formulation components (lipid matrix, drug, water and surfactant) under magnetic stirring and applying three temperature cycles (85-60-85-60-85°C) to reach the inversion process. Thereafter a thermal shock is applied by diluting the mixture in cold distilled water resulting in lipid nanoparticle formation [110]. This technique does not use organic solvents and the heating is only for a short period. However, it is a time-consuming process, requiring several steps.

6.3.7 Sonication or ultra-sonication

Like high shear homogenization, this is a dispersion technique. The method involves melting the lipid matrix (with the drug) 5–10°C above its melting point, followed by the dispersion in an aqueous phase containing surfactant at the same temperature, under high speed stirring, to form an emulsion. This is then sonicated to reduce droplet size and gradually cooled to form the nanoparticle dispersion [99]. The use of a very common equipment in laboratories is an advantage [14]. However, obtaining lipid nanoparticles requires long sonication times, which improves the risk of metal contamination from the probe. Moreover, as the energy distribution in the sample is not completely homogeneous, the resulting particles are highly polydisperse [111].

6.3.8 Membrane contactor technique

This method was developed for large-scale production of lipid nanoparticles. The molten lipid matrix containing the drug is pressurized through a porous membrane (usually with a pore diameter of 0.05 μm) to the aqueous phase containing a surfactant, maintained at lipid melting temperature. When passing through the pores, the lipid forms small droplets that precipitate as lipid nanoparticles, when the preparation is cooled to room temperature [12]. The method is scalable, simple and the particle size can be controlled by using membranes with a different pore size [8].

6.4 SKIN APPLICATIONS OF SLN AND NLC

6.4.1 Topical and dermal drug delivery by SLN/NLC

When considering the skin as a route of drug delivery, one has to consider the topical, dermal and transdermal administration. While all are applied onto the skin, only the transdermal formulations are aimed to penetrate and reach systemic circulation. Topical drug delivery stands for the drug action at the superficial layers (e.g. epidermis), whereas dermal drug delivery happens when the drug reaches the skin dermis. Hair follicles also provides a versatile penetration route, both for dermal and transdermal drug delivery, with special application for lipid nanoparticles, since SLN and NLC are formed by lipids, which also appear in the composition of sebum in hair follicles [112]. In order to provide an insight into the recent research undertaken in this domain, the relevant literature is summarized in Tables 6.3 and 6.4.

Navigating through the scientific reports, it is apparent that NLC have been widely appreciated for their merits as compared to SLN. However, it must be noted that SLN have the

same benefits as NLC, in context of cutaneous application. It is perhaps for this reason that SLN have also engaged the attention of formulation scientists till date. The few drawbacks associated with these latter can be meticulously dealt with, keeping in view the pharmaceutical considerations in light of their composition and design.

The available scientific data advocate lipid nanoparticles as the most promising drug delivery alternatives for cutaneous administration, owing to their inherent attributes. Moreover, incorporation of these particles in a suitable base (i.e. cream, gel, ointment, emulgel or lotion) enhances their benefits.

Topical delivery of drugs constitutes an important part of the therapeutic regimen for the management of skin disorders in which no systemic absorption is recommended. The preference for topical route can be ascribed to its advantages over parenteral and oral parenteral route. It not only circumvents the systemic side effects but also avoids fluctuations in plasma drug levels. Further, it enables a greater drug concentration to be delivered at the affected site and the first pass metabolism is by-passed. However, traversing the *stratum corneum* constitutes a major challenge in topical delivery of hydrophobic moieties (since most of the drugs used in the treatment of skin disorders are hydrophobic in nature). The tight packing of corneocytes in the stratum corneum imposes a barrier for xenobiotics [113]. In order to meet this challenge, two strategies are being currently employed: the use of penetration enhancers and design of nanoparticle-based formulations. Second approach has proved to be more promising, in view of the skin irritation potential of penetration enhancers. Nanoparticles, in general, act as drug reservoir, which maintains relatively higher drug concentration in the skin layers. In this respect, lipid based nanoparticles are a better alternative as compared to polymer-based nanoparticles, as the epidermis is chiefly composed of lipids [83]. Some examples are described ahead.

EI-Housiny et al. formulated a well-known antifungal drug, fluconazole (FLZ) in SLN topical gel in order to enhance its efficacy in Pityriasis Versicolor (PV). FLZ-loaded SLN were crafted employing ultrasonication technique and modified high shear homogenization, followed by their incorporation into Carbopol 934 gel. FLZ-loaded SLN exhibited reasonable colloidal size, no aggregation and were of spherical shape. The encapsulation efficiency ranged from 55.49% to 83.04%. Particles showed electrostatic stability (high ZP) and prolonged release profile *in vitro*. Further, clinical evaluation of FLZ-loaded SLN gel was carried out on PV patients comparing with commercial cream Candistan. FLZ-SLN gel showed remarkable enhancement ($p<0.05$) in therapeutic response, in comparison to commercial cream. Findings

of this study advocated superior therapeutic index of the prepared FLZ-loaded SLN gel over the marketed Candistan cream.

Montenegro et al. developed SLN for topical delivery of idebenone (IDE) to enhance its effectiveness [114]. For this purpose, IDE ester (IDEPCA) with pyroglutamic acid was synthesized. Then, IDEPCA was encapsulated in SLN. The prepared SLN were evaluated for *in vitro* antioxidant, antiglycation and *in vivo* hydrating effect, after topical application (in human volunteers) of IDEPCA-SLN gel and compared with IDE-SLN. All SLN displayed satisfactory technological characteristics (mean particle size, polydispersity index and stability). Results of antioxidant activity showed similar oxygen radical absorption capacity of IDEPCA and IDE-SLN, while for *in vitro* nitric oxide scavenging activity IDEPCA-SLN were found more effective. For antiglycation activity, both IDE and IDEPCA SLN depicted similar effectiveness in the inhibition of formation of advanced glycation products. *In vivo* findings established this as a better strategy to prepare topical nanoformulation with enhanced hydrating action.

Harde et al. developed a topical adapalene (Ada) SLN gel for ameliorating skin irritation behaviour of the drug commonly used in acne [115]. Ada-SLN were produced via hot homogenization method and optimized using a Box-Behnken design. The optimized formulation showed mean particle size of 102±5 nm, with encapsulation efficiency above 85%. Ada-SLN were embedded in a Carbopol gel. The obtained semi-solid exhibited an optimal viscosity of 24.57±0.27 Pa.S, with spreadability of 12.39±2.62 cm^2 appropriate for skin application. *In vitro* dermatokinetic results revealed enhanced dermal bioavailability for 0.1% w/w Ada-SLN gel (4.69-fold, ~0.48 µg/cm^2) and 0.1% w/w Ada-SLN gel (3.19 fold, ~ 0.37 µg/cm^2), in comparison to a gel containing 0.1% free Ada (non-loaded into SLN) (~0.12 µg/cm^2). Confocal microscopy illustrated significant follicular localization of lipid nanoparticles, followed by their diffusion into the dermis. Transepidermal water loss studies and skin irritation evaluation in Episkin (reconstituted human epidermis) supported higher skin tolerance of fabricated nanogel. Histological and visual findings further reinforced the enhanced anti-acne potential of the novel Ada-SLN gel, when compared to the gel containing non-loaded Ada.

Waghule *et al* proposed NLC embedded in a topical gel for the delivery of anti-fungal voriconazole (VCZ) to minimize the intensity and frequency of its adverse effects [116]. A Box-Behnken design was again used for the optimization of process and formulation parameters. The optimized formulation exhibited suitable mean particle size, and high encapsulation efficiency and drug loading. VCZ-loaded NLC depicted prolonged release of

drug up to 10 hours. The chosen formulation was embedded in a Carbopol gel and *ex vivo* permeation studies were performed. Results revealed enhanced permeation 66.45% and sustained release up to 11 hours in comparison to a gel containing free drug. The results reported the NLC embedded gel retained more drug in skin strata, preventing its systemic permeation and, as a result, minimizing the adverse effects associated with the free VCZ. *In vitro* evaluation of anti-fungal activity (*Aspergillus flavus*) showed significantly higher zone of inhibition (22.5±0.5 mm) of NLC formulation than the free drug counterpart (14.5±0.5 mm). This study aids an understanding regarding interaction between formulation and process variables. Further, it was reported that VCZ-loaded NLC gel, capable of targeting skin, could be a promising alternative for the management of topical fungal infections.

Moghddam et al. developed a nimesulide NLC for topical delivery [117], optimized through Box-Behnken design. Selecting the ratio of stearic to oleic acids and the concentrations of Poloxamer 188 and lecithin as independent variables, the particle size and encapsulation efficiency (the dependent variables) were optimized. Additionally, skin permeation assay, *in vitro* release, confocal laser scanning microscopy (CLSM) and stability evaluation were performed. The optimized nimesulide NLC demonstrated reasonable encapsulation efficiency, particle size and skin permeation. The results of preliminary studies displayed delayed drug release for the optimized batch, following a Higuchi release kinetics. CLSM revealed an improved penetration of Rhodamine loaded NLC to deeper skin layers. The findings of this study revealed NLC as a potential carrier for topical application of nimesulide.

Jain et al. produced and characterized a topical NLC gel, co-loading adapalene (Ada) and vitamin C (AP-Ascorbyl-6 palmitate) [118]. NLC were produced by HPH and then dispersed into a gel. Drug-loaded NLC gels were tested for skin permeation and biodistribution, and anti-acne therapeutic efficacy against testosterone-induced acne (male Wistar rat). NLC gel enhanced epidermal targeting and retarded systemic absorption. The findings of this research suggested not only the potentiality of NLC for dermal application of Ada, but also synergistic effect of vitamin C in topical acne therapeutics.

The therapeutic outcome in cutaneous disorders can be increased by means of drug targeting approach. The targeting benefits of lipid nanoparticles have been realized in the last decade. By virtue of the small particle size and controlled release property, a low concentration gradient is attained in the epidermal layer, which results in drug accumulation, preventing its further penetration into deeper layers [119]. Occlusive property of the SLN/NLC is a further advantage in this regard, which is complimented by the lipids and surfactants composing the nanoparticles [89,120].

Shrotriya et al. prepared resveratrol (RES) loaded SLN, as an alternative to topical corticosteroids in irritant contact dermatitis [121]. The challenges encountered in formulating RES, like poor solubility and bioavailability, were overcome. RES-loaded SLN were produced by ultrasonication technique, employing Precirol ATO 5 (lipid) and Tween 20 (surfactant) and further embedded into a Carbopol gel. RES-loaded SLN gels were studied for their *ex vivo* permeation, skin deposition (using human cadaver skin) and skin irritation (using New Zealand white rabbits). In addition, effect of the prepared nanogel was checked in BALB/c mice. RES-loaded NLC showed a mean particle size below 100 nm and encapsulation efficiency of 68-89%. Particles exhibited a controlled release of RES up to 24 hours. Further, skin deposition and irritation studies validated skin targeting potential, with no irritation. Finally, RES nanogel exhibited a decrease in skin water content and competent suppression of ear swelling in BALB/c mouse model, in contact dermatitis, in comparison to commercial gel. The findings of this work confirm the added value of lipid nanoparticles in the management of skin contact dermatitis, as suggested by expert opinion [122].

Raj et al. developed aceclofenac (ACF) SLN containing hydrogel for improved topical delivery of the non-steroidal anti-inflammatory drug (NSAID) [123]. SLN were prepared using ultrasonic emulsification and optimized for lipid content and stirring speed. Besides routine characterization for particle size, zeta potential, polydispersity index, encapsulation efficiency and surface morphology, *in vivo* anti-inflammatory studies were also performed. The *in vivo* data illustrated a prolonged inhibition of edema from ACF-loaded SLN hydrogel, in comparison to plain ACF gel (after 24 hours). Results of skin retention from CLSM validated skin targeting by ACF-loaded SLN gel, which can serve as a potential carrier for ACF in topical application.

Akbari et al. loaded naproxen (NAP) in SLN by ultrasonication to enhance skin permeation [124]. The performance of NAP-loaded SLN was evaluated in terms of *ex vivo* skin permeation and retention of the NSAID in skin layers. SLN contributed to increase the amount of NAP in skin strata, with low systemic absorption, and reduced side effects.

Bikkad et al produced halobetasol propionate (HP) SLN to minimize the side effects associated with the corticosteroid, and to provide controlled release [125]. HP-loaded SLN were fabricated employing solvent injection technique and optimized using 3^2 factorial design experiment. The optimized HP-loaded SLN was dispersed in Carbopol gel for skin application. The nanogels were compared with a commercial formulation, in terms of *in vitro* skin permeation, drug disposition (using human cadaver skin) and skin irritation. HP-loaded SLN displayed average size of 200 nm and encapsulation efficiency of 84-94%. A prolonged drug release up to 12 hours were obtained, while drug disposition and skin irritation studies

confirmed that HP-loaded SLN gel was capable of avoiding systemic uptake, with better accumulation of drug in upper skin layers with limited skin irritation, as compared to commercial formulation.

Silva et al. studied the epidermal targeting of clobetasol propionate (CP) NLC and chitosan coated clobetasol propionate NLC [95]. After physicochemical characterization, epidermal targeting was validated with extensive *in vitro* skin permeation experiments and drug quantification, in various skin layers. Results showed increased drug concentration in the epidermal layer, higher than 80-fold, with chitosan coated and uncoated NLC, in comparison to marketed formulation. Further, the uncoated NLC did not display dermal retention.

Zhao et al. loaded podophyllotoxin (POD) into NLC, in order to enhance its skin distribution [126]. For this, two types of POD-loaded NLC were produced. Their targeting efficacy in skin was compared via *in vitro* and *in vivo* experiments. Remarkably higher deposits of POD were detected in skin layers from *in vitro* and *in vivo* activity in rat skin. Additionally, to analyse the skin distribution of POD, Nile red loaded NLC formulations were prepared and checked via CLSM, suggesting higher skin targeting through NLC. Skin irritation of POD-loaded NLC was also investigated in damaged and intact rabbit skin. No irritation was observed, which suggested its safety for topical use.

Chen et al. prepared Coenzyme Q10 (Q10) NLC for epidermal targeting [127]. Formulation and process parameters were optimized using Box-Behnken design. The prepared Q10-loaded NLC were tested in rat skin. Results of skin permeation assay showed 10.11 times more accumulation of Q10 in the epidermal layer from Q10-loaded NLC, when compared with Q10-loaded emulsion. After 24 hours exposure to day light, the amount of Q10 available in Q10-loaded loaded NLC was diminished only by 5.59%, whereas a decrease of 24.61% was seen in the emulsion. These results suggest the protective effect of the lipid matrix against light degradation also contributing for a significant epidermal targeting potential of Q10.

Rocha et al. demonstrate the potential of NLC to enhance topical nail drug delivery by producing NLC as a delivery system for the antifungal drug voriconazole (VOR) [128]. VOR-containing NLCs were produced by the microemulsion technique and the in vitro drug penetration was evaluated in porcine hooves for NLC, NLC containing urea as a penetration enhancer and unloaded VOR. Results showed similar penetration for NLC and NLC added by urea, with a significantly higher amount of drug in deeper regions of hooves, when compared with the unloaded VOR, which indicates a very promising strategy for the onychomycosis management.

6.4.2 Transdermal drug delivery by SLN/NLC: A road less travelled

Other cutaneous application of lipid nanoparticles is the transdermal delivery of drugs, in which the skin is used as a route of administration of drugs for systemic distribution. In this case, lipid nanoparticles are used as carriers of drugs meant to treat disorders other than those affecting the skin, owing to the advantages of transdermal over oral and parenteral routes. Digging into the literature, it is perceivable that lipid nanoparticles have been exploited to a much greater extent for topical and dermal drug delivery, in comparison to transdermal application. Further search reveals that SLN have been better explored as carriers than NLC for systemic drug delivery via skin, in the present decade. To this end, the investigations have been limited to *in vitro* evaluation of drug permeation and retention through skin, while *in vivo* pharmacokinetic studies have been overlooked. It must be emphasized that *in vivo* evaluations of transdermal delivery systems are imperative to arrive at any conclusion, regarding their efficacy and clinical utility.

Guo et al. formulated ivermection (IVM) SLN employing hot homogenization technique, which was followed by ultrasonication, in order to reduce their size [129]. The obtained SLN were almost spherical in shape and displayed good stability. Delayed release was demonstrated from IVM-SLN and there was no burst release, owing to effective entrapment of the drug. Cumulative drug permeation across rat skin from SLN was found remarkably enhanced when compared with IVM suspension. This study demonstrated that IVM-SLN is an efficient carrier for transdermal application to avoid extended systemic distribution, thereby reducing the drug toxicity.

Lee et al. developed and investigated a thermo-responsive hydrogel embedding curcumin (Cur) loaded SLN for transdermal application [130]. Ultrasonication-homogenization was utilized for encapsulation of Cur with SLN, which were further introduced into Pluronic F68 and F127 (10:90 ratio) and xanthan gum thermo-responsive hydrogel. The prepared hydrogels gel in contact with the skin (at 29.3ºC). Xanthan gum played an important role, providing skin adhesiveness. Physicochemical evaluation of prepared nanogels was carried out for polydispersity index, particle size and morphological properties. The cumulative amount of curcumin that penetrated the skin was remarkably higher than its ethanolic solution.

Gaur et al. crafted curcumin SLN using emulsion solvent evaporation. Besides physicochemical evaluation, the prepared formulation was evaluated for *in vitro* drug release, pharmacokinetic parameters and anti-inflammatory effect [131]. Selected SLN were also assessed for stability. The prepared SLN were spherical in shape, with mean particle size

ranging from 102-156 nm (with negative zeta potential). Among the three types of curcumin SLN fabricated, ceramide-2: palmitic acid showed the highest encapsulation efficiency. The drug release presented the following order: stearic acid-SLN > glyceryl monostearate-SLN > ceramide-2: palmitic acid-SLN. The selected optimized formulation displayed good stability and drug permeation (through human skin). Bioavailability enhancement for the optimized nanoformulation was enhanced up to 68.12%. Further, C_{max} of the chosen formulation showed the highest value. Lastly, this formulation afforded high edema inhibition (90.75%) in 6 hours. This study showed that the nature of lipid plays a key role in designing improved SLN based delivery system, having optimum transdermal permeation.

Very recently Mendes et al. developed and characterized donepezil (DPB) NLC gel for transdermal application [132]. Drug loaded NLC were produced using the microemulsion technique. Excipients were chosen on the basis of their *in vitro* skin permeation potential. Stearic acid was chosen as a solid lipid, oleic acid as a liquid lipid, whereas lecithin and sodium taurodeoxycholate as surfactant and co-surfactant, respectively. Skin permeation of DPB was enhanced, as revealed from *in vitro* permeation assays, which was attributed to excipients used, as well as lipid nanocarriers. DPB-NLC gel was presented as an interesting formulation for improving Alzheimer's disease treatment.

Chauhan and Sharma developed NLC based transdermal carrier of rivastigmine for improvement of bioavailability [133]. For optimization of NLC, Box-Behnken design was employed. The optimized NLC formulations were engineered using castor oil (4% w/w), Span 80 (1.8% w/w/) and Tween 80 (3% w/w), and subsequently characterized. In this attempt, after routine characterization of NLC, these were loaded in transdermal patches. Results of *in vitro* release behaviour showed drug release in a sustained fashion, in comparison to commercial Exelen® patch. The results of pharmacokinetic studies presented higher C_{max} and $AUC_{0\text{-}72}$ values in plasma treated with NLC transdermal patches, in comparison to conventional patches. The findings of this work validated the potential of NLC transdermal patch for bioavailability enhancement of rivastigmine in dementia.

Yue et al. produced hyaluronic acid (HA) modified NLC for transdermal delivery of bupivacaine (BPV) and assessed their *in vitro* and *in vivo* performance [134]. Firstly, HA and linoleic acid conjugated PEG (propylene glycol) was prepared (HA-PEG-LOA) and the complex was then added to NLC during the course of production. Besides physicochemical characterization, *in vitro* skin permeation, drug release and *in vivo* therapeutic activity were also carried out. The prepared NLC were of small size (150 nm), with zeta potential -40 mV. BPV-NLC showed very high encapsulation efficiency i.e. 90%. *In vitro* release assay reported

sustained profile for 72 hours. BPV-NLC and HA-BPV-NLC exhibited 1.6 and 2.5-fold enhancement in percutaneous penetration, when compared to free BPV. Results demonstrated the efficacy of HA modified BPV-NLC for prolonging and improving anaesthetic action of the drug.

Despite being an attractive and fascinating approach, the use of lipid nanoparticles is still restricted to the cosmetic market.

6.5 EXPERT OPINION

Looking at the landscape of SLN and NLC, a few aspects standout. Being lipoidal systems, lipid nanoparticles have been most preferentially exploited for lipophilic and poorly water-soluble drugs. Nevertheless, some studies have successfully demonstrated their suitability for the delivery of hydrophilic moieties. On the other hand, studies have been undertaken to compare the inherent characteristics of SLN and NLC, using the same drug. The exploitation of SLN and NLC for co-delivery of drug moieties for better therapeutic outcomes, with recognised synergistic effects. Although, hydrogels represent a vehicle of choice for loading SLN and NLC, a few reports have described the use of ointments, lotions, emulgels, patches and other films as semi-solids most suitable for skin application. SLN and NLC can be used for topical, dermal and transdermal drug delivery. Tailoring their composition in terms of lipids and surfactants, optimized by factorial design approaches, site-specific targeting can be achieved with important therapeutic outcomes. Besides, the carriers are by definition biocompatible while their composition limits the risk of toxicity and irritation when applied onto the skin. Several skin lipids are used as raw materials in the composition of SLN and NLC thereby acting as penetration enhancers. Improving skin permeation and penetration of drugs, less drug may be needed to exhibit its therapeutic effect, further reducing eventual adverse side effects. With the expansion of knowledge and technical competences in the field, lipid nanoparticles have won the cosmetic market. However, the clinical application remains a challenge of its own, which comes up against issues such as the complexity of regulatory requirements. In addition, there is still little *in vivo* knowledge about the ability of these nanosystems to permeate biological membranes, distribute the drug in the skin strata and deposit themselves in the body's tissues [135]. Many companies are working with these nanoparticles, and it is only a matter of time before they reach the pharmaceutical market.

Table 6.3. Examples of drugs incorporated in SLN (since 2010).

Drugs	Category	Excipients Used	Purpose	References
Aceclofenac	NSAID	Glyceryl monostearate, Carbopol 934, soya lecithin, Tween 80	Development of SLN loaded hydrogel for topical administration of aceclofenac	[136]
N-Acetyl-D-Glucosamine	Anti-hyperpigmentation	Cetyl palmitate, phosphatidylcholine, PEG-25 hydrogenated Castor oil, Sasol, Areosil 200, hydrogenated Gliceryl palmate, Arlacel P-135	Formulation of N-acetyl-D-Glucosamine SLN for topical delivery in order to improve dermal properties in skin disorders	[137]
Aconitine	Analgesic	Transcotol P, Compritol® 888 ATO, polyethylene glycol- 35, castor oil, ethyl oleate	Improvement in safety and skin permeability of aconitine SLN via transdermal route	[138]
Acyclovir	Antiviral	Compritol® 888 ATO, soya lecithin	Development of acyclovir SLN for enhanced dermal delivery	[139]
Adapalene	Anti-acne	Tristearin, hydrogenated soya phosphatidylcholine, Triton X-100	For effective topical delivery of adapalene in acne	[140]
		Stearic acid, cetyl palmitate, tristearin, Brij 78, Pluronic F68, Tween 80, Span 20, Sodium dodecyl sulphate, glyceryl monostearate, Compritol® 888 ATO, Precirol ATO 5, glyceryl monooleate, Carbopol 980 NF, Carbopol Ultrez 10 NF, Pemulen TR-1	Enhancement of efficacy and improve skin tolerability of topical adapalene embedded gel	[115]
Amphotericin B	Antifungal	Compritol® 888 ATO, Precirol ATO 5, Poloxamer F-127, Poloxamer F-68, stearic acid, glycerol, Tween 80, sodium carboxymethyl cellulose	Design of amphotericin B SLN for improvement of therapeutic antifungal activity	[141]
Articaine	Local anaesthetic	Poly(ε-caprolactone), capric/caprylic triglycerides, glyceryl tripalmitate, propylene glycol, polyvinyl alcohol, methylparaben	Enhancement of chemical stability of articaine in topical nanocarrier loaded hydrogel	[142]
Avanafil	For erectile dysfunction	Cholesterol, Compritol® 888 ATO, Tween 80, castor oil	Formulation and optimization avanafil SLN and SLN-loaded hydrogel film for transdermal delivery	[143]
Benzoyl peroxide	Anti-acne	Precirol ATO 5, Tween 80, Carbopol 934 NF	Benzoyl peroxide SLN to reduce side effects associated with drug for acne treatment	[144]

Betamethasone 17-valerate	Corticosteroid	Cetyl palmitate, glycerol distearate, glycerol tripalmitate, liquid paraffin	Elucidation of the effect of corticosteroid on skin barrier and drug penetration	[145]
Caffeine	Anticancer	Xanthan gum, Softisan 100, Pluronic® F-68	Development of SLN of hydrophilic drug caffeine for topical administration	[146]
Capsaicin	Diabetic neuropathy	Tripalmitin, caprylic/capric/myristic/stearic triglyceride, Miglyol® 812, Poloxamer 188,Tween 80, xanthan gum	Evaluation of the influence of crystallinity and lipid matrix on physicochemical characteristics and skin permeation of capsaicin SLN	[45]
Coenzyme Q10	Antioxidant	Compritol® 888 ATO, Precifac ATO, Labrasol, stearyl alcohol, stearic acid, Span 60 , Tween 80, Tween 20, beeswax, cetyl alcohol	Investigation of the dermal penetration of Coenzyme Q10 SLN cream for hydration and anti-wrinkle property	[147]
		Compritol® 888 ATO, Lebrasol®, Poloxamer® 188, Tween 80, carbopol 974P	Development of coenzyme Q10 SLN loaded gels for enhanced dermal delivery	[148]
Colchicine	Anti-gout	Glyceryl monostearate, Tween 20, Sodium lauryl sulphate	Formulation and evaluation of colchicine SLN based transdermal patch for management of gout	[149]
Curcumin	Anti-inflammatory	Ceramide 2, Glyceryl monostearate, stearic acid, palmitic acid, Tween 80	Formulation and evaluation of curcumin SLN (ceramide-palmitic acid complex) for physical features and *ex vivo* permeation	[131]
	Anti-inflammatory, antioxidant, antimicrobial, antitumor	Pluronic F68, xanthan gum, Tween 80, soy bean lecithin	Development and investigation of curcumin thermoresponsive SLN gel for transdermal delivery	[150]
Cyclosporin A	Immunosuppressant	Lipocire™ DM, Pluronic® F-127, oleic acid	Production of stable, safe and improved Cyclosporin A nanocarrier for topical delivery	[151]
Diclofenac	NSAID	Epikuron 200, polyethylene glycol 400, Pluronic F68, Pluronic F127, Precirol ATO 5, Precirol ATO 888, Dynasan 114, Dynasan 118, Glycerol monostearate, stearic acid, Tween 80, Tween 60, Tween 20, glycerol, sorbitol	Preparation, characterization and *in vitro* evaluation of diclofenac sodium SLN for transdermal delivery	[152]

Doxorubicin	Anti-cancer	Stearic acid, lecithin, taurodeoxycholate sodium	Investigation of influence of iontophoresis on skin penetration from doxorubicin SLN	[153]
		Poloxamer 407, Precirol ATO 5, triethylamine, phosphoric acid	Investigation of potential of doxorubicin SLN for topical administration against skin cancer	[154]
Eugenol	Antifungal	Poloxamer 188, Compritol® 888 ATO, stearic acid	Formulation of eugenol SLN loaded hydrogels for epidermal targeting in skin fungal infections.	[155]
Flucinolone acetonide	Corticosteroid anti-inflammatory	Compritol® 888 ATO, soya lecithin, Poloxamer 188	Fabrication, optimization and evaluation of potential of flucinolone acetonide SLN for prolonged release and targeted delivery via topical route	[156]
Fluconazole	Antifungal	Compritol® 888 ATO, phosphatidylcholine, Pluronic F-68, Sephadex G-50	Design of fluconazole SLN for its topical delivery against candidiasis	[157]
		Poloxamer 407, carbopol 934, Compritol® 888 ATO, Pricerol ATO5	Improvement in efficacy of Fluconazole SLN topical gel for Pityriasis Versicolor	[158]
		Compritol® 888 ATO, oleic acid, phosphatidylcholine, pluronic F-68, sephadex G-50	Improvement of dermal delivery of fluconazole via SLN and their evaluation for cutaneous candidiasis	[159]
Genistein	Anticancer and antiproliferative	Tween 80, Span 85, glyceryl behenate, Miglyol® 812 N	Preparation of genistein SLN for its delivery to deeper skin layers	[160]
Griseofulvin	Antifungal	Tween 80, Rhodamine 123, Compritol® 888 ATO, Carbopol 980 NF, Phospholipon 90G	Fabrication of griseofulvin SLN for dermal application	[161]
Halobetasol propionate	Corticosteroid anti-inflammatory	Glycerol monostearate, Tween 80, methyl and propyl paraben	Development of halobetasol propionate SLN for skin targeting via topical route	[113]
Hydroquinone	Anti-hyperpigmentation agent	Precirol® ATO 5, Poloxamer 407, Span 20, Carbopol 934	Encapsulation of hydroquinone in SLN in order to improve its stability, skin penetration and reduce systemic absorption	[162]
Idebenone	Ubiquinone derivative having antioxidant activity	Brij 58, Brij 98, cetyl palmitate, Poloxamer 188, glyceryl oleate	For targeting idebenone to the upper layers of skin via topical delivery	[163]
		Glyceryl oleate, cetyl palmitate, methylisothiazolinone, Brij 98, methylchloroisothiazolinone, triethanolamine	Formulation of idebenone ester with pyroglutamic acid SLN to improve topical efficacy	[164]

Isotretinoin	Anti-acne	Phosphatidylcholine, Compritol® 888 ATO, butylated hydroxy toluene, tocopherol	Development of optimized SLN for isotretinoin to reduce dermal irritation and enhance therapeutic performance of drug	[165]
Ivermection	Antiparasitic	Palmitic acid, polyvinyl alcohol, polyglycerol fatty acid ester	Ivermection SLN were proposed for transdermal delivery to avoid systemic toxicity	[166]
Lornoxicam	NSAID	Compritol® 888 ATO, Lanette O, Pluronic F68, oleic acid, xanthan gum	Preparation of SLN and NLC gels for inflammatory and painful conditions of skin	[167]
Meloxicam	NSAID	Cetyl palmitate, propylene glycol, Tween 80, polyethylene glycol 400, Carbopol 940	Investigation of potential of meloxicam SLN gel for dermal application	[168]
Metformin	Anti-diabetic	Tween 60, cholesterol, Span 60, beeswax	For enhancement of skin delivery of metformin via SLN topical gel	[169]
Miconazole nitrate	Antifungal	Soya lecithin, carbopol 934, tristearin, Tween 80	Investigation of miconazole nitrate SLN hydrogel for topical delivery in fungal infections	[170]
Mometasone furoate	Glucocorticosteroid Anti-psoriasis	Glycerol monostearate, Compritol® 888 ATO, cetyl palmitate, Syncrowax-HRC and HGL, stearic acid, Tween 80, Carbopol 974p	Fabrication of mometasone furoate SLN for topical delivery to address the short coming conventional formulation of this corticosteroid	[171]
Naproxen	NSAID	Tween 80, Span 80, glyceryl mono stearate	Preparation of naproxen SLN to improve skin permeation and to explore influence of hydrophilic-lipophilic balance modifications on nanolipidic carriers	[172]
Piroxicam	NSAID	Brij 35, Brij 72, triethanolamine, chloroform, acetic acid, cholesterol and stearic acid, Carbopol	Preparation and assessment of piroxicam SLN gel to enhance its skin permeation for topical application	[173]
Resveratrol	Anti- inflammatory and antiproliferative	Precirol ATO 5, carbopol 940, Compritol® 888 ATO, Tween 20, Lebrasol	Resveratrol SLN engrossed gel for skin targeting in contact dermatitis	[174]
	Anti-tyrosinase activity	Stearic acid, soy phosphatidylcholine, poloxamer 407, polysorbate 80	Preparation of trans-resveratrol SLN for skin delivery and their *in vitro* evaluation for hyperpigmentation	[175]
Retinoic Acid	Anti-acne	Cholesterol, Brij 58, stearylamine, butylated hydroxytoluene (BHT),	Evaluation of application of retinoic acid loaded SLN for topical treatment of acne	[176]

		methyl-paraben, Compritol® 888 ATO, Vitanol A, hydroxyethyl cellulose		
Retinoic acid and Lauric acid	Retinoids, Anti-microbial	Propylene glycol, Compritol® 888 ATO, Brij 58, cholesterol, stearylamine, Butylated hydroxy toluene	Development of SLN for retinoic acid and lauric acid and evaluation of their antibacterial potential	[177]
Retinyl palmitate	Anti-wrinkle	Precirol® ATO5, Gelucire® 50/13, dicetyl phosphate, Carbomer® 940	Improvement of surface modified SLN loaded gel for enhancement of skin distribution of retinyl palmitate for skin aging	[93]
Safranal	Sunscreen and moisturing agent	Glyceryl monostearate, Tween 80	Loading of safranal in SLN and their evaluatation for sunscreen potential for topical delivery	[178]
Sesamol	Antioxidant and anticancer	Glyceryl monostearate, sodium deoxycholate, phosphatidylcholine	Fabrication of sesamol SLN for skin cancer	[179]
Silybin	Antioxidant and anti-inflammatory	Tween 20, Tween 80, Span 20, Span 80, Triton X-100, cetyl palmitate, stearic acid, Compritol® 888 ATO, Glyceryl monostearate, Precirol ATO5, Carbopol 940	Preparation and evaluation of silybin SLN gel for irritant contact dermatitis	[180]
Spironolactone	Anti-acne potential	Tween 80, Span 80, Span 60, stearic acid, dichloromethane, Highly Ordered Pyrolytic Graphite (HOPG)	Formulation of spironolactone SLN and their exploration for dermal delivery	[181]
Tacrolimus	Immunosuppressive macrolide	Cocoglyceride, Poloxamer 188, stearic acid, soybean lecithin, Brij® 93, Brij® 58	Improvement in penetration and retention of tacrolimus thermosensitive SLN in skin layers	[182]
Terbinafine	Antifungal	Glyceryl behenate, glyceryl palmitostearate, Pluronic F-127	Development of terbinafine hydrochloride SLN for controlled release via topical application	[183]
		Cremophor® EL, RH40, and RH60, Gelucire® 39/01, Gelucire® 44/14, glyceryl behenate (Compritol® 888 ATO), and glyceryl palmitostearate, Tween 80, Tween 60, Tween 40, Tween 20, propylenglycol	Terbinafine SLN as topical delivery system to resolve issues of longer treatment time and frequent delivery	[184]
Tretinoin	Metabolite of vitamin A having antiacne potential	Myristyl myristate, chitosan	Preparation and evaluation of tretinoin SLN with and without chitosan for acne	[185]
Triamcinolone acetonide	Glucocorticosteroid	Compritol® 888 ATO, precirol ATO 5, soya	Entrapment of triamcinolone acetonide in SLN for topical	[186]

	lecithin, poloxamer, glycerol monostearate, stearic acid	application in order to alleviate its systemic side effects	

Table 6.4. Examples of drugs incorporated in NLC (since 2010).

Drugs	Category	Excipients Used	Purpose	References
Acitretin	Anti-psoriatic agent	Oleic acid, Tween 80, Precirol ATO 5	Fabrication and evaluation of acitretin NLC for topical treatment of psoriasis	[52]
Adapalene and Vitamin C	Retinoid, Antioxidant	Phospholipid, tristearin, Triton X-100	Preparation and evaluation of topical gel of adapalene and vitamin C	[118]
Artemether	Antimalarial	Gelucire® 43/01, Compritol® 888 ATO, Transcutol® P, Phospholipon® 85 G, polysorbate 80 and 20, Macrogol 4000, sorbitol, Pluronic F68, Span 60	Fabrication of artemether NLC for topical delivery	[187]
Betamethasone dipropionate	Glucocorticoid	Oleic acid, Tween 80, Span 80, liquid paraffin, stearyl alcohol, iso-propyl alcohol, isopropyl palmitate, Precirol ATO 5, Carbopol 971	Investigation of betamethasone dipropionate NLC ointment for atopic dermatitis	[188]
Bupivacaine	Anaesthetic	Polysorbate 80, dimethylaminopyridine, fetal bovine serum, soya lecithin, Compritol® 888 ATO, Precirol® ATO 5	Design of Hyaluronic acid modified bupivacaine NLC for effective transdermal local anaesthetic delivery	[189]
Calcipotriol and Methotrexate	Anti-psoriatic, Anticancer	Precirol ATO5, Myverol™ 18-04K, Pluronic F68	Evaluation of combination of calcipotriol and methotrexate in NLC for topical management of psoriasis	[190]
Clobetasol propionate	Corticosteroid	Oleic acid, sodium taurodeoxycholate, low molecular weight chitosan, stearic acid, propylene glycol	Clobetasol propionate NLC for epidermal targeting	[95]
Coenzyme Q10	Antioxidant	Cetyl palmitate, Labrasol, carbomer	Coenzyme Q10 NLC for epidermal targeting	[191]
Diclofenac sodium	NSAID	Glyceryl monostearate, lanolin PEG-75, Phospholipon 90G, Precirol ATO 5, Tween 80, Cremophor RH 40, polyvinyl alcohol, carboxymethyl cellulose sodium, cetyl alcohol, cetosteryl alcohol, propylene glycol	Fabrication and evaluation of diclofenac sodium NLC gel for transdermal drug delivery	[192]
Difluctolone valerate	Corticosteroid	Precirol® ATO5, Labrasol®, Labrafil® M1944CS, Capryol™ 90, tristearin, Poloxamer® 407,	NLC work as reservoir for difluctolone valerate targeting via topical delivery	[193]

		isopropyl myristate, stearic acid		
Diphencyprone	For alopecia areata	Cetyl palmitate, Pluronic F68, hydrogenated soybean phosphatidylcholine	For improving skin absorption of diphencyprone and its follicular targeting	[194]
Docetaxel and nicotinamide	Anti-cancer, penetration enhancer	Egg lecithin, glycerine monostearate, capric glyceride	Development and evaluation of docetaxel-nicotinamide complex NLC to enhance skin permeation	[195]
Donepezil	Cholinesterase inhibitor	Oleic acid, stearic acid, sodium taurodeoxycholate hydrate, soy lecithin, glycerol monooleate	NLC gel for transdermal application of donepezil	[196]
Enoxaparin	Anticoagulant	Tristearin, oleic acid, tween 80, Carbopol 934	Investigation of NLC as a vehicle for topical delivery of enoxaparin	[197]
Flucinolone	Corticosteroid	Compritol® 888 ATO, polysorbate 80, Miglyol® 812	Design and evaluation of topical flucinolone acetonide NLC for psoriasis	[156]
Flurbiprofen	Anti-arthritic drug/NSAID	Soya lecithin, coconut oil, soybean oil, olive oil, castor oil	Evaluation of potential for transdermal delivery of flurbiprofen NLC	[198]
		Compritol® 888 ATO, Miglyol® 812, lecithin, Poloxamer 188, sodium deoxycholate, Tween 80, carbopol 940	Design of flurbiprofen NLC gel for topical application	[199]
Ketoprofen	NSAID	Glycerol, β-cyclodextrin, Compritol® 888 ATO, Lutrol® F68	Ketoprofen cyclodextrin complex loaded NLC improved therapeutic efficacy via topical delivery	[200]
Lansoprazole	For stomach infection	Glyceryl monostearate, stearylamine, Pluronic F68, Sodium dodecyl sulfate	Fabrication of lansoprazole NLC for transdermal application	[201]
Lidocaine	Anaesthetic	Cetyl ester wax, propylene glycol USP, Carbomer 940 NF, Tween 80	Lidocaine incorporated in NLC aiming to localization of drug and its controlled delivery	[202]
Lycopene	Antioxidant	Orange wax, rice bran oil, Eumuglin SG, mineral oil	Characterization of lycopene NLC for topical application	[203]
Meloxicam	NSAID	Cetyl palmitate, Caprylic acid, propylene glycol (PG), Tween 80, polyethylene glycol 400 (PEG 400), triethanolamine, Carbopol 940	Meloxicam NLC gel for improving transdermal delivery	[204]
Miconazole	Antifungal	Glyceryl monostearate, olive oil, coconut oil, mustard oil, lavender oil	Development of miconazole nitrate ultra-small NLC for topical delivery against athlete's foot	[205]
Minoxidil	For alopecia	Tristearin, oleic acid, cholesterol, Tween 80, soya lecithin, Pluronic F-	Minoxidil NLC gel for alopecia	[206]

		68, Triton X-100, carbopol 934		
Nimesulide	NSAID	Poloxamer 188, isopropyl alcohol, methanol, ethanol, soya lecithin, oleic acid, stearic acid	Fabrication, optimization of topically applied nimesulide NLC	[117]
Phenylethyl resorcinol	Tyrosinase inhibitor	Glycerol monostearate, olive oil, behenic acid, palmitic acid, stearic acid, Dynasan, Compritol® 888 ATO, Precirol ATO 5, Labrasol, Miglyol® 812, mineral oil, oleic acid, olive oil, polyvinyl alcohol, lecithin, Tween 80	Enhancement of skin whitening property of phenylethyl resorcinol by loading in NLC	[207]
Pioglitazone	Anti-hyperglycemic agent	Apifil, Labrasol, Carbopol, Tween 80	Design of pioglitazone NLC for its bioavailability enhancement via transdermal route for diabetes	[208]
Podophyllotoxin	Antimitotic Agent	Cremophor RH 40, Compritol® 888 ATO, Labrasol, soybean phosphatidylcholine	Fabrication of podophyllotoxin NLC for skin targeting	[209]
Quercetin	Flavonoid having anti-cancer, anti-oxidant activity	Soya lecithin, glyceryl monostearate, stearic acid	NLC encapsulating quercetin for topical administration	[210]
Retinyl retinoate	Anti-aging	Canola oil, Compritol® 888 ATO, Precirol® 5 ATO, Labrafil M 1944 CS, Miglyol® 840, oleic acid, soybean oil, Tween 80	NLC topical formulation entrapping retinyl retinoate for antiaging and anti-wrinkle effects	[211]
Rivastigmine	Cholinesterase inhibitor	Castor oil, Capmul MCM, soya oil, olive oil, palm oil, peanut oil, Tween 80, Span 80, Eudragit E-100, glyceryl monostearate, polyvinyl pyrrolidone, diethyl phthalate	Development of rivastigmine NLC loaded transdermal formulation for bioavailability enhancement	[212]
Ropivacaine	Anaesthetic	Soya lecithin, glyceryl monostearate, stearic acid	Evaluation of ropivacaine NLC for transdermal application	[213]
Salicylic acid	NSAID with antifungal activity	Compritol® 888 ATO, Miglyol® 812, Cremophor RH 60	Development and optimization of salicylic acid NLC for dermal use	[214]
Sildenafil citrate	Phosphodiesterase type 5 inhibitor	Cetyl palmitate, glycerol monolinoleate, hydrogenated castor oil, Span 85, propylene glycol, Tween 80	Transdermal permeation of NLC and SLN loaded with sildenafil citrate	[215]
Spironolactone	Diuretic having antiandrogenic properties	Tween 80, Transcutol® P, Compritol® 888 ATO	Follicular targeting of spironolactone NLC in alopecia	[216]

Tacrolimus	Immunosuppressive macrolide	Soybean lecithin, propylene glycol monocaprylate, glyceryl palmitostearate, Butylated hydroxytoluene, polysorbate 80	NLC lotion for improving dermal application of tacrolimus	[217]
Terbinafine	Antifungal	Precirol ATO 5, Compritol® 888 ATO, glyceryl monostearate, Tween 20, Tween 80, castor oil, oleic acid, Span 80	Investigation of terbinafine hydrochloride NLC for fungal infection via topical application	[218]
Tripterine	Anti-inflammatory/ anticancer	Glyceryl behenate, isopropyl myristate (IPM), Pluronic F68, Precirol ATO-5, soybean lecithin	Evaluation of surface charge of tripterine NLC on permeation and *in vivo* performance	[219]
Voriconazole	Antifungal	Compritol® 888 ATO, Miglyol® 812 N, Tween® 80, Span® 85	*In vitro* evaluation of the impact of the follicular pathway on epidermal deposition	[220]
		Oleic acid, Tween 80, polyethylene glycol	Fabrication of voriconazole NLC gel for skin targeting and alleviating adverse effects of drug	[116]
		Precirol® ATO 5, Labrafil M 1944 CS, Tween 80, Carbopol 940 NF	NLC hydrogel of voriconazole for mycotic infection via topical administration	[221]

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CHAPTER 7. NLC LOADED BY *LIPPIA SIDOIDES* ESSENTIAL OIL: OPTIMIZATION, CHARACTERIZATION, AND BIOLOGICAL ACTIVITY

7.1 INTRODUCTION

Essential oils (EOs) have aroused great interest, particularly due to their wide range of confirmed biological activity, such as antifungal, antibacterial, insecticide, antiviral, antioxidant, among others. Its use as an active ingredient brings several benefits, and maybe the main advantage relates to the ability to combat the resistance often presented by microorganisms against the antibiotics available in the market. However, due to their high volatility and sensitivity, they need to be incorporated into protective systems.

There is considerable interest in utilizing lipid systems in the food, cosmetic and pharmaceutical industries to encapsulate and deliver lipophilic functional agents, such as vitamins, flavors, colors, and preservatives. The second generation of lipid delivery system, the nanostructured lipid carrier (NLC), are innovative strategies for the encapsulation of EOs, improving their physicochemical properties and increasing their potential for use. Due to their advantages over other lipid systems are the possibility to encapsulate chemically different drugs and be produced in a sterilized environment, the avoidance of organic solvents in their production, and the possibility to obtain a tunable release profile (1).

The influence of the encapsulation system, lipid components, emulsifier type and other excipients should be evaluated to optimize system stability and retention of bioactive compounds. Design of experiments (DoE) is a very useful tool to identify critical process parameters and optimize the process conditions (2). The presented research aimed to use design of experiment approach to develop essential oil (EO) encapsulated NLC, to establish a better understanding of the of the lipid matrix used in the encapsulation and analyze the effect and interactions of each component with the EO. It will be used in the study *Lippia sidoides* EO, which has demonstrated potential of application in the pharmaceutical, food and cosmeceutical sectors due to its considerable antimicrobial activity. The NLC formulations were produced, characterized, and evaluated for antifungal activity for both yeast and filamentous fungus.

7.2 MATERIAL AND METHODS

7.2.1 Material

Lippia sidoides EO was purchased from Pronat (Produtos Naturais LTDA, Horizonte – CE, Brazil). Methanol and oleic acid were purchased from Labsynth (Brazil). Thymol, sodium dodecyl sulphate, and acetonitrile HPLC grade were purchased from Sigma-Aldrich (St Louis, USA). Poloxamer® 188 was purchased from Basf (Brazil). Carnauba wax was kindly donated by Foncepi (Brazil), Beeswax, cupuaçu butter, cocoa butter and stearic acid were purchased from Via Farma (Brazil), Compritol® 888 ATO was purchased from Brasquim (Brazil), Precirol ATO5 and Apfil CG were a gift from Gattefossé (France), Quillaja saponaria extract was kindly donated by Desert King International (San Diego, USA).

7.2.2 Methods

7.2.2.1 NLC development studies: screening of lipids

The solubility of *Lippia sidoides* EO in different solid lipids was performed according to SOUTO *et al.* (2005), evaluated by mixing the EO in different concentrations (40%, 50% and 60% of EO in solid lipid - m/m) with the lipid and melting the mixture at 90 ºC. The solubility was determined visually after 1 h at 90 ºC, and after solidification of the lipid at room temperature (25 ºC) analyzing the appearance of the mixture applied on filter paper. The tests were made in triplicate.

7.2.2.2 Development and optimization of NLC production using experimental design

With the purposes to optimize the production of NLC containing *L. sidoides* EO and investigate the relationship between NLC composition, we proposed an experimental design using solid lipid (X1), surfactant (X2), and surfactant concentration (X3) level as independent factors in a full factorial design 2x3x3 (Table 7.1).

Table 7.1. Full factorial design 2x3x3

Run	Factors* Lipid system (X1)	Surfactant (X2)	Surfactant concentration** (X3)
1	-1	P	20
2	-1	P	40
3	-1	P	60
4	-1	Q	20
5	-1	Q	40
6	-1	Q	60
7	-1	SDS	20
8	-1	SDS	40
9	-1	SDS	60
10	1	P	20
11	1	P	40
12	1	P	60
13	1	Q	20
14	1	Q	40
15	1	Q	60
16	1	SDS	20
17	1	SDS	40
18	1	SDS	60
19	-1	P	40
20	1	P	40
21	-1	Q	40
22	1	Q	40
23	-1	SDS	40
24	1	SDS	40

* Where: -1 = Compritol® 888 ATO; 1 = mixture (1:1) of carnauba wax + beeswax; P = Poloxamer 188; Q = Quillaja saponin; SDS = Sodium dodecyl sulfate. ** % related to the solid lipid

7.2.2.3 Preparation of NLC

NLC were prepared according to Baldim *et al.* (2019) with some modifications, by emulsification using high-speed homogenization followed by ultrasonication method. In brief, aqueous and oil phases were separately prepared in glass vials. Solid lipid was melted at 10 ºC above its melting point and mixed with the liquid lipid and the *L. sidoides* EO. Surfactant was dissolved in a 10 mM phosphate buffer solution at different ratio, as indicated in Table 7.2, and heated to the same temperature as the lipid phase. The aqueous phase was gently and homogeneously dispersed into the lipid phase, and homogenised by a high-speed stirrer (UltraTurrax T18, IKA-Wilmington, NC, USA) at 14,000 rpm/min for 3 min. The oil-in-water nanostructured lipid system formed was then sonicated by an ultrasonic sonicator VCX-750 (SONICS Vibracell, Newtown, USA), equipped with a 13 mm diameter probe, in a constant-temperature water bath (85 °C). The magnitude of the ultra-sonication was at 45% amplitude,

at a frequency of 20 kHz, lasting 5 cycles of 2 min on and 1 min off to form the nanoparticles. The formulation was rapidly cooled by immersing the beaker into icy water (0 °C).

Table 7.2. *Lippia sidoides* EO-containing NLC. Quantities expressed as % w/w

NLC	*Quillaja*	Poloxamer	SDS	Compritol® 888 ATO	CA+ CC	Oleic acid	EO	Phosphate buffer (qsp)
F1	x	0.7	x	2.6	x	0.9	0.9	45.0
F2	x	1.4	x	2.6	x	0.9	0.9	44.3
F3	x	2.1	x	2.6	x	0.9	0.9	43.6
F4	0.7	x	x	2.6	x	0.9	0.9	45.0
F5	1.4	x	x	2.6	x	0.9	0.9	44.3
F6	2.1	x	x	2.6	x	0.9	0.9	43.6
F7	x	x	0.7	2.6	x	0.9	0.9	45.0
F8	x	x	1.4	2.6	x	0.9	0.9	44.3
F9	x	x	2.1	2.6	x	0.9	0.9	43.6
F10	x	0.7	x	x	2.6	0.9	0.9	45.0
F11	x	1.4	x	x	2.6	0.9	0.9	44.3
F12	x	2.1	x	x	2.6	0.9	0.9	43.6
F13	0.7	x	x	x	2.6	0.9	0.9	45.0
F14	1.4	x	x	x	2.6	0.9	0.9	44.3
F15	2.1	x	x	x	2.6	0.9	0.9	43.6
F16	x	x	0.7	x	2.6	0.9	0.9	45.0
F17	x	x	1.4	x	2.6	0.9	0.9	44.3
F18	x	x	2.1	x	2.6	0.9	0.9	43.6
F19	x	1.4	x	2.6	x	0.9	0.9	44.3
F20	x	1.4	x	x	2.6	0.9	0.9	44.3
F21	1.4	x	x	2.6	x	0.9	0.9	44.3
F22	1.4	x	x	x	2.6	0.9	0.9	44.3
F23	x	x	1.4	2.6	x	0.9	0.9	44.3
F24	x	x	1.4	x	2.6	0.9	0.9	44.3

* Where: CA+CC = carnauba wax + beeswax; SDS = sodium dodecyl sulfate; EO = essential oil

7.2.2.4 Determination of pH and electrical conductivity

The determinations were made in triplicate, the pH measurements were made by a pH meter (model 827 pH Lab, using electrode unitrode PT100, Metrohm, Switzerland), and electrical conductivity with an electrical conductivity meter (model 856, Metrohm, Switzerland).

7.2.2.5 Particle size, polydispersity index, and ζ-potential

The dynamic light scattering was used to measure particle size, with measurements made in a Zetasizer Nano – ZS90 (Malvern, UK). Samples were diluted 1:200 using a 10 mM phosphate buffer solution at pH 7, to prevent multiple scattering effects. In addition to the mean hydrodynamic radius of the particles, the equipment also reports the polydispersity index (PI), which ranges from 0 (monodisperse) to 1 (very broad distribution). ζ-potential was also determined by micro electrophoresis, using the same instrument. All the measurements were carried out in triplicate, at 25°C.

7.2.2.6 Rheological properties

The rheological behaviour of samples was measured using a Brookfield Rheometer (Model LV-DV III, USA), concentric cylinders operating the Reocalc® software (in duplicate). To perform the readings, a program was selected with a speed increase/reduction of 20 rpm, a reading range from 0 to 120 (increasing and decreasing the speed), a 15-second ramp and SC4-18 spindle.

7.2.2.7 Thymol retention

The major component present *in L. sidoides* essential oil (thymol), was quantified in the lipid formulations by high performance liquid chromatography coupled to a diode arrangement detector (HPLC-DAD). The method of sample preparation consisted of diluting the samples in acetonitrile (4.0 µL/mL), homogenising the mixture in ultrasound bath, and keeping the solution under magnetic stirring for 30 minutes. After the extraction, the samples were centrifuged for 5 min at 5000 rpm and the supernatant were filtered and analysed by HPLC. The chromatographic conditions were based on the method proposed by Benelli, Souza and Oliveira (2013) and Leal and co-workers (2003), with some modifications. A calibration curve was made with thymol concentrations varying from 50 to 800 µg/mL. Analyses were performed in a HPLC (model Prominence LC-20A series and a LC-6A double pump - Shimadzu Corporation, Kyoto, Japan) using a C-18 column (Shimadzu Shim-Pack CLC(M) 4.6mm x 25 cm, 5µm, 100Å) at 30°C, with a volume of sample injected of 20 µL. The mobile phase was a gradient of water (A) and acetonitrile (B). The acetonitrile concentration was changed as follows: 0–2 min, 10% B; 2–7 min, linear increase of B to 78%; 7–17 min, 78% B; 17– 20 min, linear increase of B to 100%; 20–23 min, 100% of B; 23–26 min, linear decrease of B to 10%, 23–32 min, 10% of B. The chromatograms were obtained at a wavelength of 276 nm.

7.2.2.8 Thermal analysis

Thermogravimetric analysis (TGA)

Aiming to analyse the thermal decomposition of essential oil in samples, we made TGA analysis, carried out in a thermal analyser model TGA-50 (Shimadzu, Japan), under N2 atmosphere, with a heating rate of 10 ºC/min in the temperature range from 0 ºC to 600 ºC (5).

Differential scanning calorimetry (DSC)

DSC thermograms of NLCs samples were obtained to determine the glass transition temperature of components and their relationship to particle structure. Analyzes were performed in a DSC equipment (TA Instruments Q2000), with a cooling system. The analyzes used 3 mg samples sealed in aluminum pans and a heating ramp from 20 °C to 120 °C, with a heating rate of 5 °C/min, N_2 dynamic atmosphere and a flow rate of 50 mL/min (KHERADMANDNIA et al., 2010).

7.2.2.9 Antifungal susceptibility tests

A screening test was performed by agar diffusion assay, to evaluate the growth inhibition of the yeast *Candida albicans* (ATCC 64548) and the filamentous fungi *Fusarium solani* (ATCC 36031) both by EOs and NLCs. The tests were conducted according to the Clinical Laboratory Standards Institute (CLSI) reference method for broth dilution antifungal susceptibility testing of yeasts (M27-A3 (6), and of filamentous fungi (M38-A2 (7)). The screening for antifungal activity was performed by agar diffusion assay to evaluate the growth inhibition for both *C. albicans* and *F. solani* against the EO, NLC formulations and for the isolated compounds of the formulations. The medium used was RPMI-1640 (Life Technologies, Grand Island, EUA) with L-glutamine (0.3 g/L) buffered to pH 7.0 with MOPS (USB Corporation, Cleveland, OH, USA), supplemented with D-glucose (2.0 g/L) for *C. albicans* and RPMI-1640 buffered with 0.165M 3-(N-morpholino) propane sulfonic acid (MOPS), pH 7.0 for *F. solani*. The cell suspension was prepared in saline, with an optical density equivalent to 0.5 McFarland standard, and diluted in RPMI to obtain a final concentration of $1–5\times10^3$ colony-forming units per milliliter (CFU/mL) for *C. albicans* and 1×10^4 microconidia per mL for *F. solani*. A drop of 10 µL of each NLC formulations and 5 µL of the pure EO were placed on the top surface of the inoculated agar plate. The plates were incubated at 37 °C for 24 h for *C. albicans* and 48 h of incubation at 28 °C for *F. solani*. The results were evaluated according to the presence of absence of inhibition zones.

7.2.2.10 Statistical analysis

Data normality was checked using the Shapiro-Wilk test. The lipid system statistical analysis was performed using the Mann-Whitney test (a nominal variable that divides the population into two categories), while the surfactant properties were statistically analyzed by Kruskall-Wallis test (a nominal variable that divides the population into three categories). For both analyses, a p value of <0.05 was considered significant.

7.3 RESULTS AND DISCUSSION

7.3.1 Screening of lipids

Screening tests were essential to determine the best lipids to compose the NLC formulations, as well as their concentrations. The best selected lipids to compose the lipid matrix were: Compritol 888 ATO and a mixture of beeswax and carnauba wax in the proportion of 1:1 for each wax. The proportions between the components will be those listed in the subsequent item (3.2).

7.3.2 Characterization of NLC

The dynamic light scattering technique was used to characterize the NLC for particle size and polydispersion index (PDI). ζ-potential measurements were determined by micro electrophoresis. The polydispersion index (PDI) reveals the quality of the dispersion and varies from values less than 0.1, for adequate and good quality measurements, to values close to 1, for poor quality samples that do not present colloidal size or present very high polydispersion. The ζ-potential characterizes the surface charge of particles and provides information about the repulsion forces between the NLC particles. Values above 30 mV, in module, are desired, once they are related to greater stability, favoring the repulsion of particles from each other and preventing flocculation and coalescence of the particles (8). Figure 7.1 presents the results of particle, polydispersity index (PDI), and ζ-potential of the NLC, which falls between 168 nm to 1206 nm, 0.111 to 0.895, and -94,3 mV and -8,38 mV, respectively.

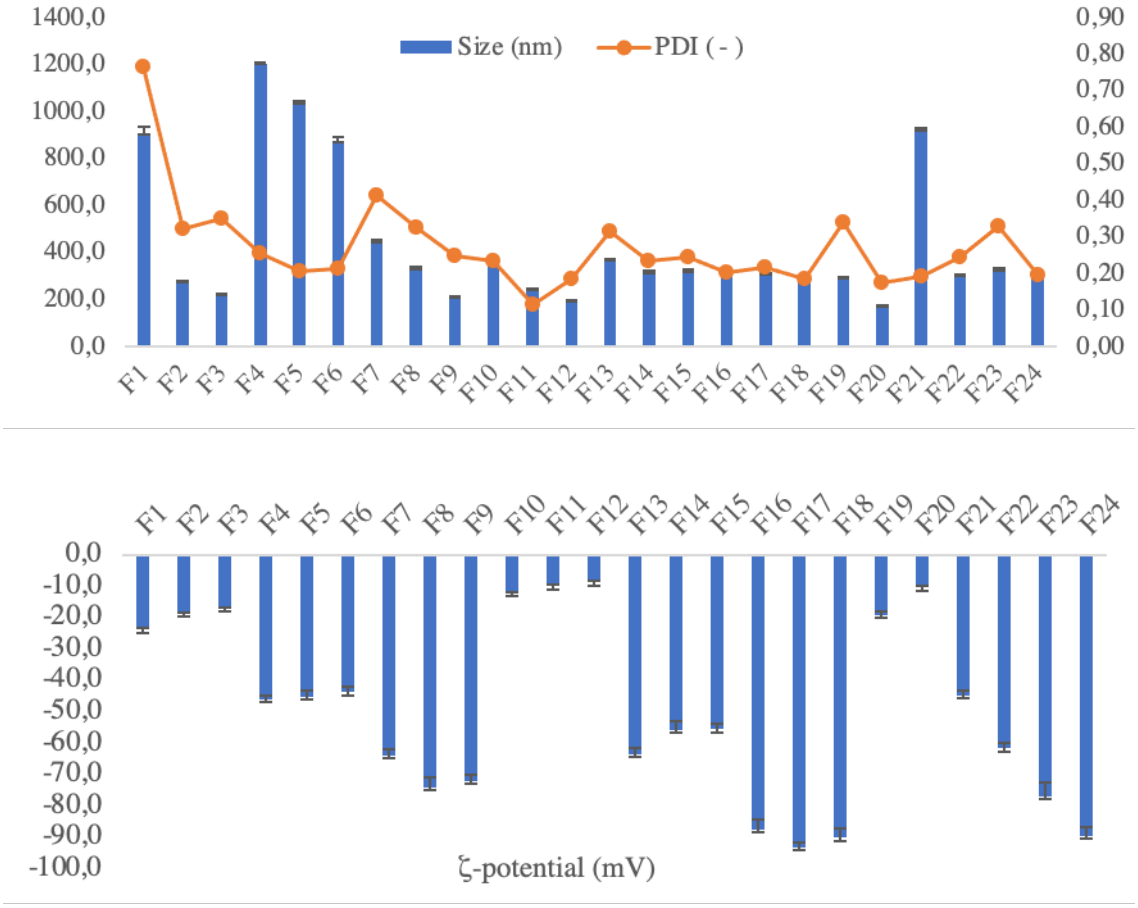

Figure 7.1. Particle size (nm), polydispersity index (PDI), and ζ-potential (mV) of NLC loaded with *L. sidoides* essential oil

Regarding electrical conductivity, basically, when a steady state has high conductivity values, water represents the continuous phase, while values close to zero mean that the continuous phase is oil (9). The values varied widely between the NLC (between 134.38 µS/cm and 5119.68 µS/cm). The pH values also varied among the NLC formulations, ranging from 4.52 to 6.76. The measurements are shown in Figure 7.2.

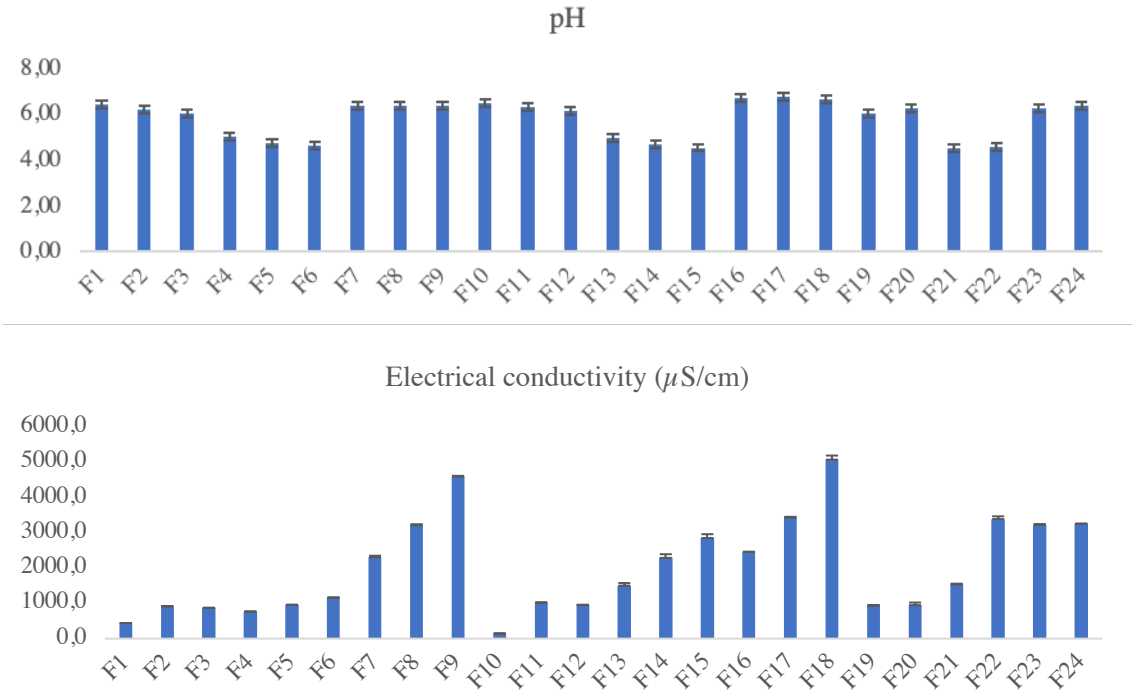

Figure 7.2. pH and electrical conductivity of NLC loaded with *L. sidoides* essential oil

Figure 7.3 presents the retention of thymol (percentage) for the different NLC. All formulations retained a high percentage of thymol, with values ranging from 77.42% to 100%.

These initial analyzes demonstrate that most of the response variables analyzed showed high dispersion (high standard deviation in comparison to the mean). The exceptions were pH and thymol retention, which are less dispersed. None of the variables presented normal distribution by the Shapiro-Wilk test. We summarized the exploratory statistical results on Table 7.3.

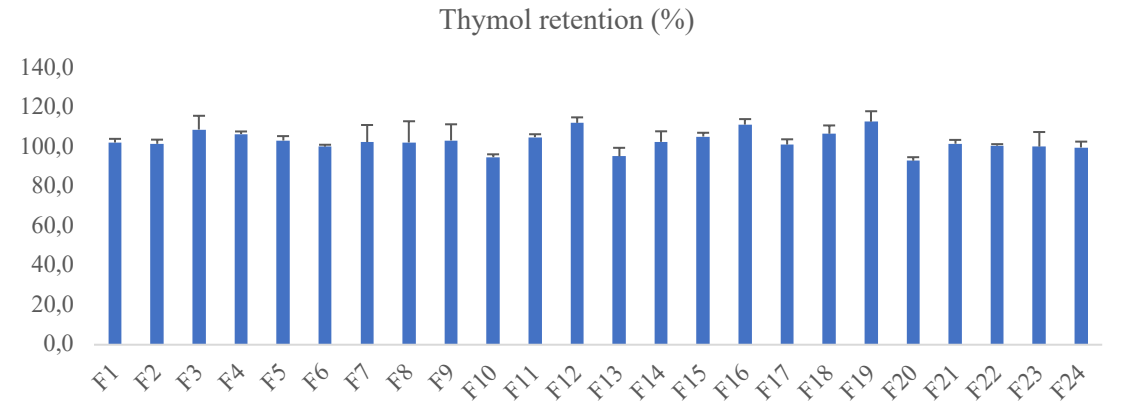

Figure 7.3. Thymol retention of NLC loaded with *L. sidoides* essential oil

Table 7.3. Exploratory analysis of NLC characterization

Factor	Characteristic	Statistical
Particle size (nm)	Min-Max	168-1206
	Mean/Sd	436.64 / 294.09
PDI	Min-Max	0.111-0.895
	Mean/Sd	0.27 / 0.13
ζ-potential (mV)	Min-Max	-94.3 - -8.38
	Mean/Sd	-49.65 / 28.65
pH (-)	Min-Max	4.52-6.76
	Mean/Sd	5.82 / 0.81
Electrical conductivity (μS/cm)	Min-Max	134.38-5119.68
	Mean/Sd	2003.66 / 1338.6
Thymol retention (%)	Min-Max	77.42-100
	Mean/Sd	88.72 / 5.39

*Where: Sd = Standard deviation

7.3.3 Influence of lipid system

The responses particle size, PDI and electrical conductivity were significantly influenced by the composition of lipid system, in which the -1 lipid system (Compritol® 888 ATO) presented higher values for particle size and PDI and lower values for electrical conductivity at the 5% significance level. These results can be seen in Table 7.4 and the boxplot of Figure 7.4.

Table 7.4. Influence of lipid system (Compritol® 888 ATO or a mixture 1:1 of carnauba wax and beeswax) on the characterization parameters of NLC loaded by *L. sidoides* essential oil

Factor	Characteristic	-1	1	p-value
Particle size (nm)	Min-Max	212-1206	168-361.2	0.004d*
	1Q-3Q	287.3-920.82	277.95-317.88	
	Mean / Median	585.26 / 391.55	288.03 / 302.65	
	N (NA's)	36 (0)	36 (0)	
	Shapiro-Wilk	<0.001	<0.001	
PDI (-)	Min-Max	0.14-0.9	0.11-0.37	<0.001d*
	1Q-3Q	0.24-0.37	0.18-0.24	
	Mean / Median	0.32 / 0.28	0.22 / 0.21	
	N (NA's)	36 (0)	36 (0)	
	Shapiro-Wilk	<0.001	0.025	
ζ-potential (mV)	Min-Max	-81.8–16.7	-94.3–8.38	0.383d
	1Q-3Q	-67.03–22.5	-88.38–11.55	
	Mean / Median	-46 / -44.8	-53.29 / -58.95	
	N (NA's)	36 (0)	36 (0)	
	Shapiro-Wilk	0.002	<0.001	
pH (-)	Min-Max	4.52-6.48	4.55-6.76	0.197d
	1Q-3Q	4.96-6.37	4.89-6.55	
	Mean / Median	5.76 / 6.12	5.88 / 6.27	
	N (NA's)	36 (0)	36 (0)	
	Shapiro-Wilk	<0.001	<0.001	
Electrical conductivity (μS/cm)	Min-Max	439.15-4594.99	134.38-5119.68	0.018d*
	1Q-3Q	900.41-2554.35	1009.48-3283.52	
	Mean / Median	1737.84 / 1052	2269.49 / 2373.34	
	N (NA's)	36 (0)	36 (0)	
	Shapiro-Wilk	<0.001	0.026	
Thymol retention (%)	Min-Max	77.42-100	77.68-97.94	0.308d
	1Q-3Q	86.61-92.73	83.99-92.27	
	Mean / Median	89.35 / 89.4	88.09 / 88.24	
	N (NA's)	36 (0)	36 (0)	
	Shapiro-Wilk	0.414	0.59	

Calculated p-values for the Mann-Whitney test of equality of distributions between the two types of lipid system. Where: -1 = Compritol® 888 ATO; +1 and a mixture 1:1 of carnauba wax and beeswax

* Statistically significant at p<0.05

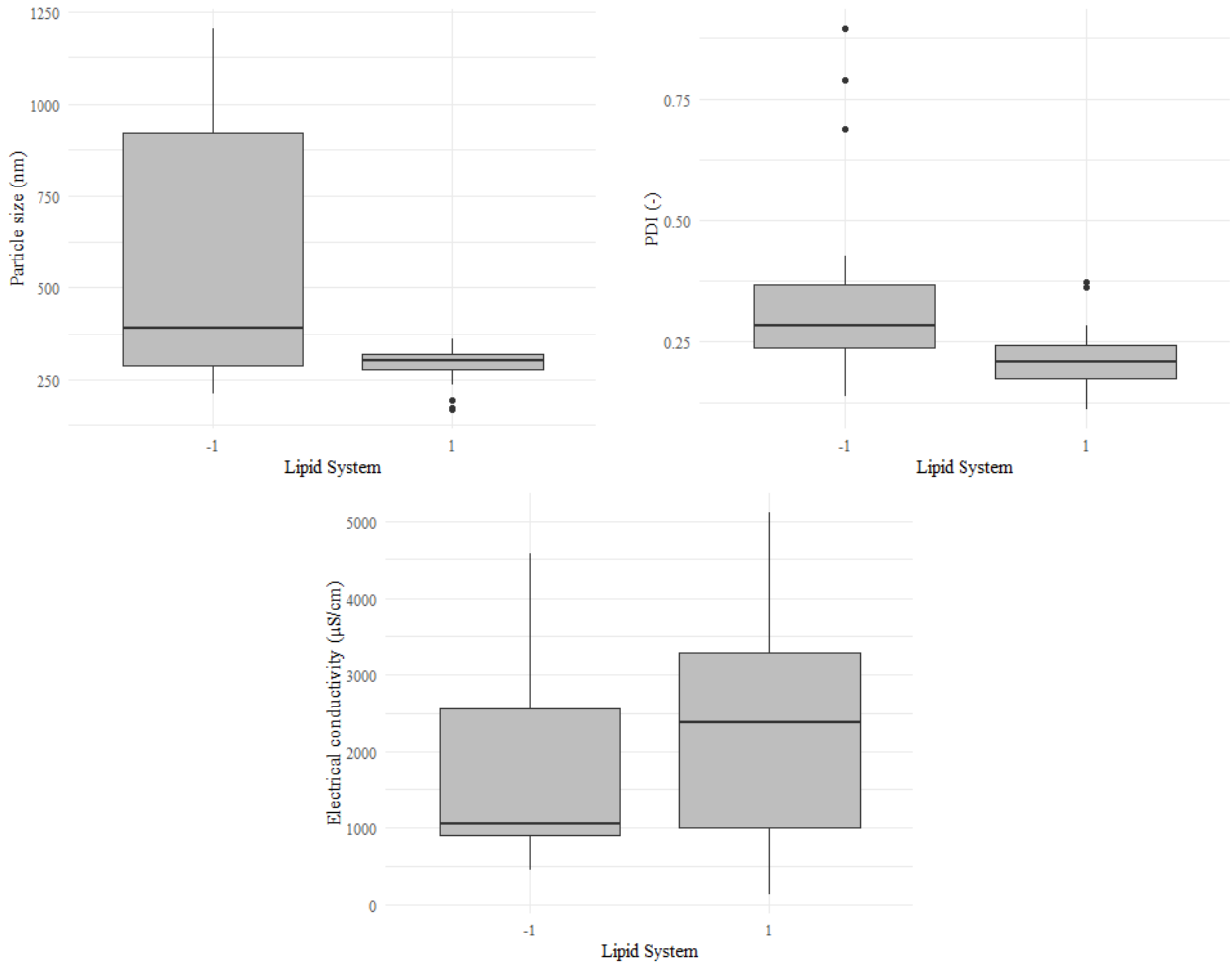

Figure 7.4. Mann-Whitney box plots representing the significant influence of lipid system on particle size, PDI and electrical conductivity (* Where: -1 = Compritol® 888 ATO; 1 = mixture (1:1) of carnauba wax + beeswax).

7.3.4 Influence of surfactants

Particle size, ζ-potential, pH and electrical conductivity were the variables significantly influenced by the surfactant system of NLC. Dunn's tests with Bonferroni correction were conducted to assess which distributions differed. The results show that NLC containing Quillaja saponin as surfactant presented the highest values of particle size, lower pH value and intermediate electrical conductivity and ζ-potential values, in module (lower than SDS and higher than Poloxamer 188) at the 5% significance level. The SDS containing NLC showed the highest electrical conductivity and Z-potential (in module) values, which can be attributed to the strong negative charges of this surfactant. SDS based NLC also presented higher pH values (around 6.5), favoring the formation of more anionic carboxylic groups (—COO^-) (10) and justifies the higher electrical conductivity and Z-potential than the others. These results are summarized on Table 7.5 and Figure 7.5.

Table 7.5. Influence of surfactant system (Poloxamer 188, Quillaja saponin and SDS) on characterization variables of NLC loaded by *L. sidoides* essential oil

Factor	Characteristic	Poloxamer 188	Quillaja	SDS	p-value
Particle size (nm)	Min-Max	168-934.2	291.5-1206	212-453.3	<0.001f*
	1Q-3Q	213.02-306.78	312.1-948.05	299.42-323.03	
	Mean / Median	332.58 / 254.4	661.06 / 602.3	316.29 / 307.3	
	N (NA's)	24 (0)	24 (0)	24 (0)	
	Shapiro-Wilk	<0.001	<0.001	<0.001	
PDI (-)	Min-Max	0.11-0.9	0.14-0.37	0.17-0.43	0.753f
	1Q-3Q	0.17-0.36	0.2-0.26	0.2-0.3	
	Mean / Median	0.31 / 0.26	0.24 / 0.24	0.26 / 0.24	
	N (NA's)	24 (0)	24 (0)	24 (0)	
	Shapiro-Wilk	<0.001	0.099	0.009	
ζ-potential (mV)	Min-Max	-24.2–8.38	-65.6–41.9	-94.3–61.7	<0.001f*
	1Q-3Q	-18.85–10.47	-58.53–44.8	-90.7–74.28	
	Mean / Median	-15.03 / -14.35	-51.98 / -50.15	-81.93 / -82.95	
	N (NA's)	24 (0)	24 (0)	24 (0)	
	Shapiro-Wilk	0.016	0.01	0.047	
pH (-)	Min-Max	6.04-6.49	4.52-5.04	6.24-6.76	<0.001f*
	1Q-3Q	6.1-6.34	4.55-4.8	6.37-6.74	
	Mean / Median	6.24 / 6.22	4.71 / 4.65	6.5 / 6.39	
	N (NA's)	24 (0)	24 (0)	24 (0)	
	Shapiro-Wilk	0.011	<0.001	<0.001	
Electrical conductivity (μS/cm)	Min-Max	134.38-1019.57	749.97-3454.74	2253.52-5119.68	<0.001f*
	1Q-3Q	760.86-958.9	1099.42-2415.69	3009.92-3724.86	
	Mean / Median	779.87 / 919.33	1789.91 / 1495.12	3441.21 / 3233.31	
	N (NA's)	24 (0)	24 (0)	24 (0)	
	Shapiro-Wilk	<0.001	0.01	0.003	
Thymol retention (%)	Min-Max	78.63-100	77.68-93.05	77.42-97.42	0.448f
	1Q-3Q	84.68-94.53	85.9-90.28	84.94-93.22	
	Mean / Median	89.37 / 88.88	87.76 / 87.3	89.02 / 90.09	
	N (NA's)	24 (0)	24 (0)	24 (0)	
	Shapiro-Wilk	0.253	0.178	0.102	

Calculated p-values for the Kruskall-Wallis test of equality of distributions between the three types of surfactants.
* Statistically significant at $p<0.05$

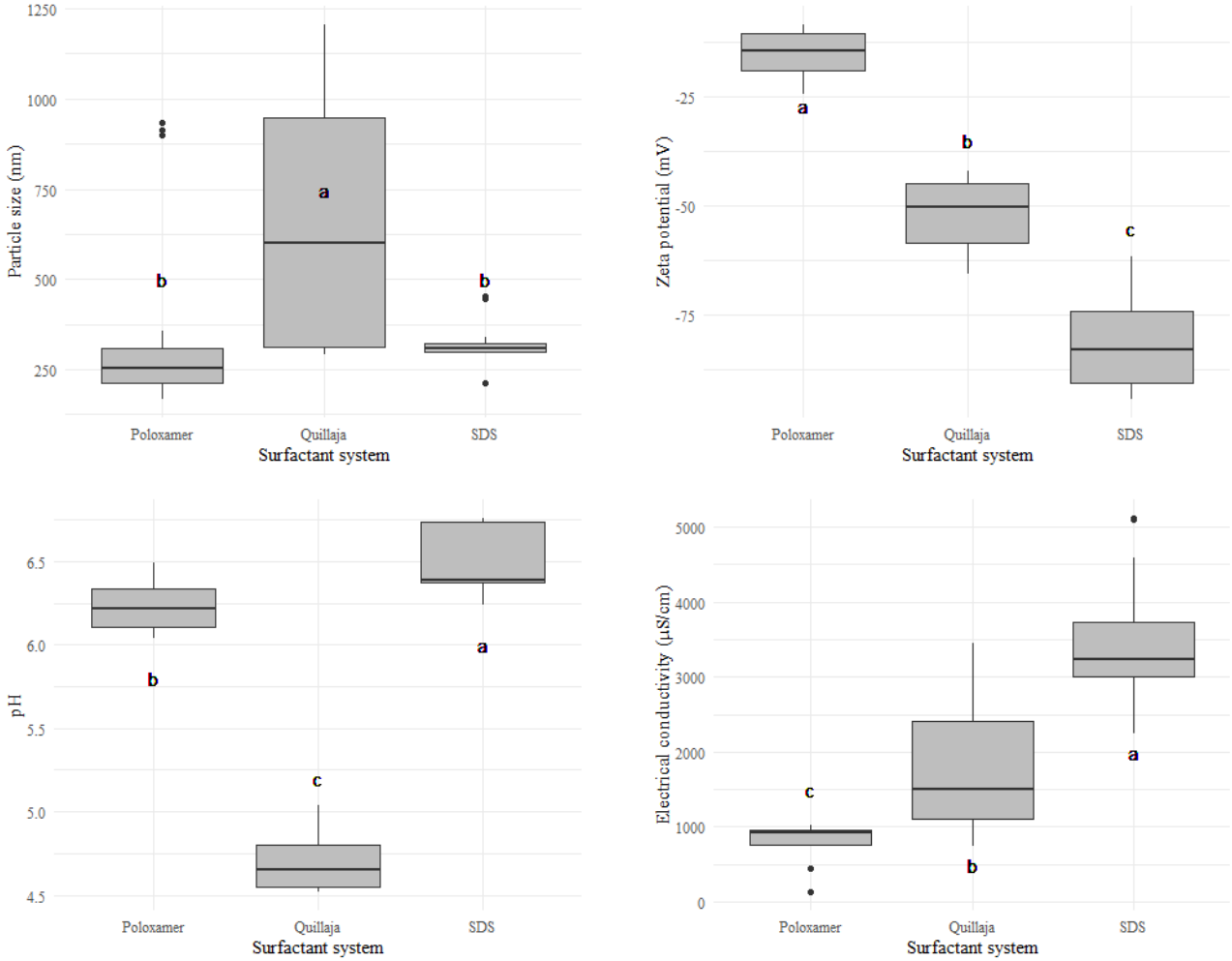

Figure 7.5. Kruskall-Wallis box plots representing the significant influence of surfactant system on particle size, ζ-potential, pH and electrical conductivity (* Different letters indicate a statistically significant difference at $p<0.05$).

7.3.5 Influence of surfactant concentration

Particle size, PDI and electrical conductivity were parameters significantly influenced by the surfactant concentration (Table 7.6). When performing the Dunn test, we concluded that the concentration -1 (20% of surfactant in comparison to the solid lipid) differed in particle size (by generating larger particles in comparison to the others), electrical conductivity (presenting smaller values than the others) and, for PDI, it differed only from level 1 (60% of surfactant in comparison to the solid lipid) to 5% significance level. Higher values of electrical conductivity due to the increase in the concentration of SDS in the samples was already expected, since a higher concentration of anionic surfactant is related to the increase in electrical conductivity (11). This result can be seen in the boxplot charts below:

Table 7.6. Influence of surfactant concentration on the characterization parameters of NLC loaded by *L. sidoides* essential oil

Factor	Characteristic	-1	0	1	p-value
Particle size (nm)	Min-Max	317.1-1206	168-1038	193.5-890.3	<0.001f*
	1Q-3Q	352.15-910.32	287.3-317.47	212.27-317.42	
	Mean / Median	599.13 / 403.25	397.94 / 302.65	351.56 / 260.25	
	N (NA's)	18 (0)	36 (0)	18 (0)	
	Shapiro-Wilk	<0.001	<0.001	<0.001	
PDI (-)	Min-Max	0.18-0.9	0.11-0.38	0.17-0.38	0.037f*
	1Q-3Q	0.22-0.41	0.19-0.28	0.18-0.25	
	Mean / Median	0.37 / 0.28	0.24 / 0.24	0.24 / 0.24	
	N (NA's)	18 (0)	36 (0)	18 (0)	
	Shapiro-Wilk	0.001	0.587	0.017	
ζ-potential (mV)	Min-Max	-90.5–11.8	-94.3–9.54	-92.9–8.38	0.804f
	1Q-3Q	-65.2–23.82	-77.73–18.95	-72.8–17.3	
	Mean / Median	-49.42 / -54.4	-50.59 / -50.15	-47.98 / -49.1	
	N (NA's)	18 (0)	36 (0)	18 (0)	
	Shapiro-Wilk	0.109	0.003	0.095	
pH (-)	Min-Max	4.96-6.75	4.52-6.76	4.55-6.75	0.058f
	1Q-3Q	5.04-6.49	4.72-6.31	4.63-6.4	
	Mean / Median	6.01 / 6.42	5.75 / 6.22	5.75 / 6.08	
	N (NA's)	18 (0)	36 (0)	18 (0)	
	Shapiro-Wilk	<0.001	<0.001	0.001	
Electrical conductivity (µS/cm)	Min-Max	134.38-2452.65	910.03-3454.74	867.98-5119.68	0.008f*
	1Q-3Q	439.33-2264.43	967.84-3233.3	954.52-4585.01	
	Mean / Median	1253.97 / 1102.29	2091.3 / 1846.88	2578.09 / 1948.69	
	N (NA's)	18 (0)	36 (0)	18 (0)	
	Shapiro-Wilk	0.01	<0.001	<0.001	
Thymol retention (%)	Min-Max	77.68-97.42	77.42-100	80.86-97.94	0.055f
	1Q-3Q	82.88-92.73	85.3-90.28	87.34-94.14	
	Mean / Median	87.9 / 88.88	87.82 / 88.5	91.33 / 92.49	
	N (NA's)	18 (0)	36 (0)	18 (0)	
	Shapiro-Wilk	0.474	0.274	0.379	

Calculated p-values for the Kruskall-Wallis test of equality of distributions between the three concentrations.
* Statistically significant at $p<0.05$

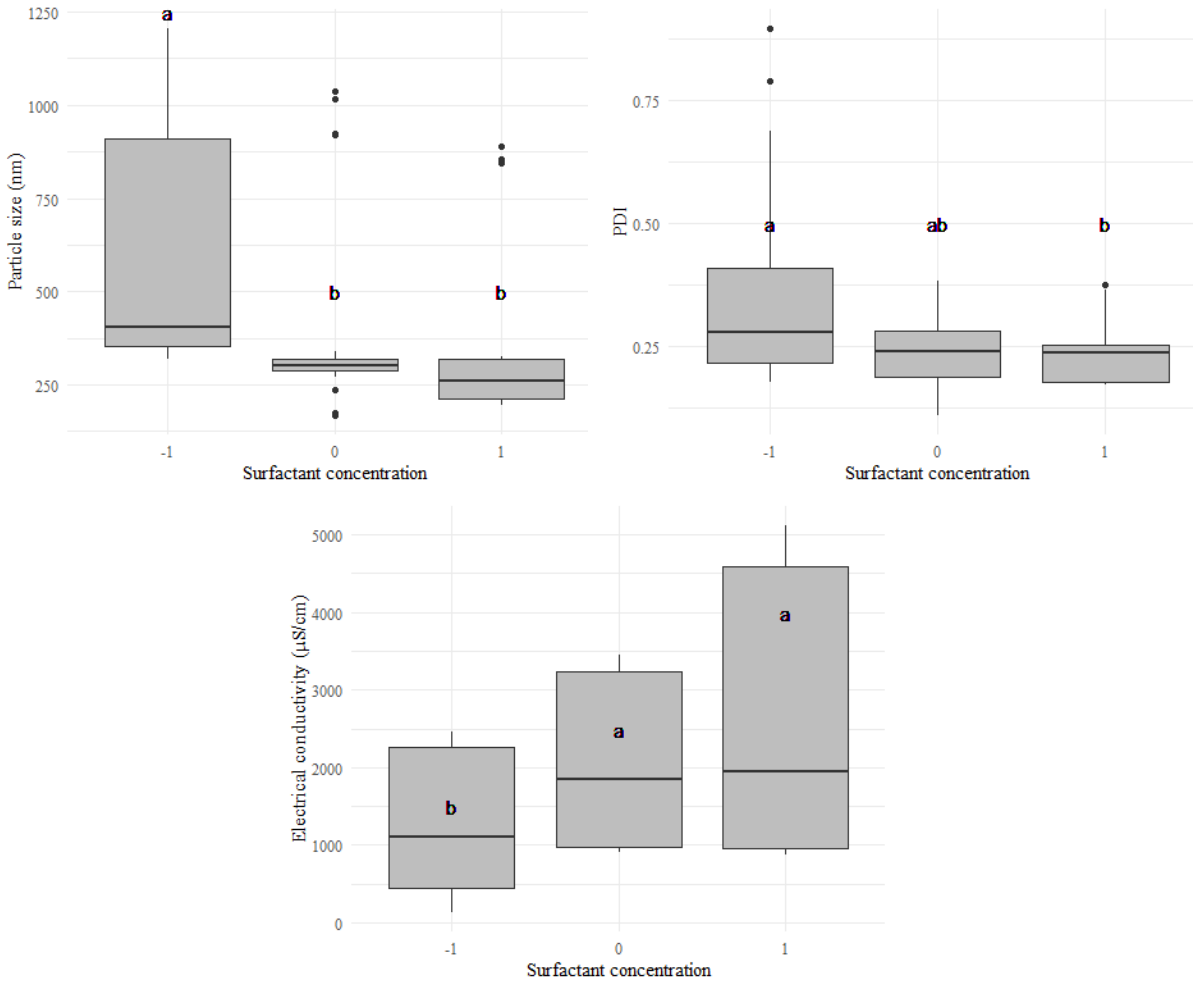

Figure 7.6. Kruskall-Wallis box plots representing the significant influence of surfactant concentration on particle size, PDI and electrical conductivity (* Different letters indicate a statistically significant difference at $p<0.05$).

7.3.6 Global comparison: regression model

Using as a reference the level -1 for the lipid system (Compritol® 888 ATO), the surfactant Poloxamer 188 and the surfactant concentration level of -1 (20% of surfactant in comparison to the solid lipid), we conclude that (Table 7.7):

Table 7.7. Evaluation of the proposed regression model

	Particle size (nm)	**PDI (-)**	**ζ-potential (mV)**
(Intercept)	643.69***	0.46***	-11.17***
Lipid system = 1	-297.23***	-0.11***	-7.29***
Surfactant = Quillaja saponin	328.48***	-0.07*	-36.94***
Surfactant = SDS	-16.30	-0.05	-66.90***
Surfactant concentration = 0	-201.19***	-0.13***	-1.17
Surfactant concentration = 1	-247.58***	-0.13***	1.44

Regression analysis coefficients: ***significant at 0.1%, *significant at 5%

• The lipid system 1 (mixture 1:1 of carnauba wax + beeswax) is associated with a decrease of 297 nm in particle size and 0.11 in PDI, but also a decrease of 7.29 mV (in module) on ζ-potential.
• Changing the surfactant Poloxamer 188 by Quillaja saponin, results in an increase in particle size by 328.48 nm, a 0.07 decrease in PDI and a 37 mV (in module) decrease in ζ-potential.
• The surfactants Poloxamer 188 and SDS did not provide a statistically significant difference for particle size and PDI, however SDS is associated with a 66.9 mV (in module) decrease in ζ-potential compared to Poloxamer.
• Regarding the concentration of surfactant, there was a reduction of 201.19 nm in particle size and 0.13 in PDI for level 0 (40% of surfactant in comparison to the solid lipid) in relation to level -1, but the ζ-potential did not show a statistically significant change.
• There is a decrease of 247.58 nm in particle size and 0.13 in PDI for the level 1 in concentration (60% of surfactant in comparison to the solid lipid) relative to the -1 level, but the ζ-potential did not show a statistically significant difference.

By adjusting the regression models, it was possible to estimate the expected particle size, PDI and ζ-potential for each set of lipid system, surfactant, and concentration (Table 7.8). Comparing the results of the adjusted model, the best NLC formulation would be composed by a mixture of carnauba wax and beeswax (1:1), SDS as surfactant, at the concentration of 60% of the total solid lipid. This combination of components provides the smallest and least dispersed particles, with the greatest ζ-potential, tending to the greatest stability.

Table 7.8. Estimated characterization parameters after adjusting the regression models

Lipid system	Surfactant	Surfactant concentration	Particle size (nm)	PDI (-)	ζ-potential (mV)
-1	Poloxamer	-1	643.69	0.46	-11.17
1	Poloxamer	-1	346.46	0.35	-18.45
-1	Quillaja	-1	972.17	0.39	-48.11
1	Quillaja	-1	674.94	0.28	-55.39
-1	SDS	-1	627.39	0.41	-78.06
1	SDS	-1	330.16	0.31	-85.35
-1	Poloxamer	0	442.50	0.33	-12.33
1	Poloxamer	0	145.27	0.22	-19.62
-1	Quillaja	0	770.98	0.26	-49.28
1	Quillaja	0	473.75	0.15	-56.56
-1	SDS	0	426.20	0.28	-79.23
1	SDS	0	128.97	0.18	-86.52
-1	Poloxamer	1	396.11	0.33	-9.72
1	Poloxamer	1	98.88	0.22	-17.01
-1	Quillaja	1	724.59	0.26	-46.67
1	Quillaja	1	427.36	0.15	-53.95
-1	SDS	1	379.81	0.28	-76.62
1	SDS	1	82.58	0.18	-83.91

7.3.7 Rheological behavior of NLC

The rheological properties of the NLC formulations are very important parameters for determining the topical formulation potential. Their viscoelastic parameters are not dependent on the applied shear rate (12,13), which indicates a non-Newtonian rheological behavior. Measurements rheological behavior after 24 hours showed that all the formulations analyzed presented pseudoplastic behavior, and generally with low degree or absence of thixotropy. The variation of the shear stress seems to be more related to the lipid composition of NLCs and the interactions between the components of lipid matrix than to the isolated concentration of the surfactant, as NLC with the different surfactant concentration presented similar shear stress profiles. The analysis also showed that the NLC prepared with a mixture of carnauba wax and beeswax (from F10 to F18, and the repetitions F20, F22 and F24) presented higher apparent viscosity in comparison to those produced with Compritol® 888 ATO. This difference could be explained by the differences in the formation and structuration of lipid microstructure of the NLC (14).

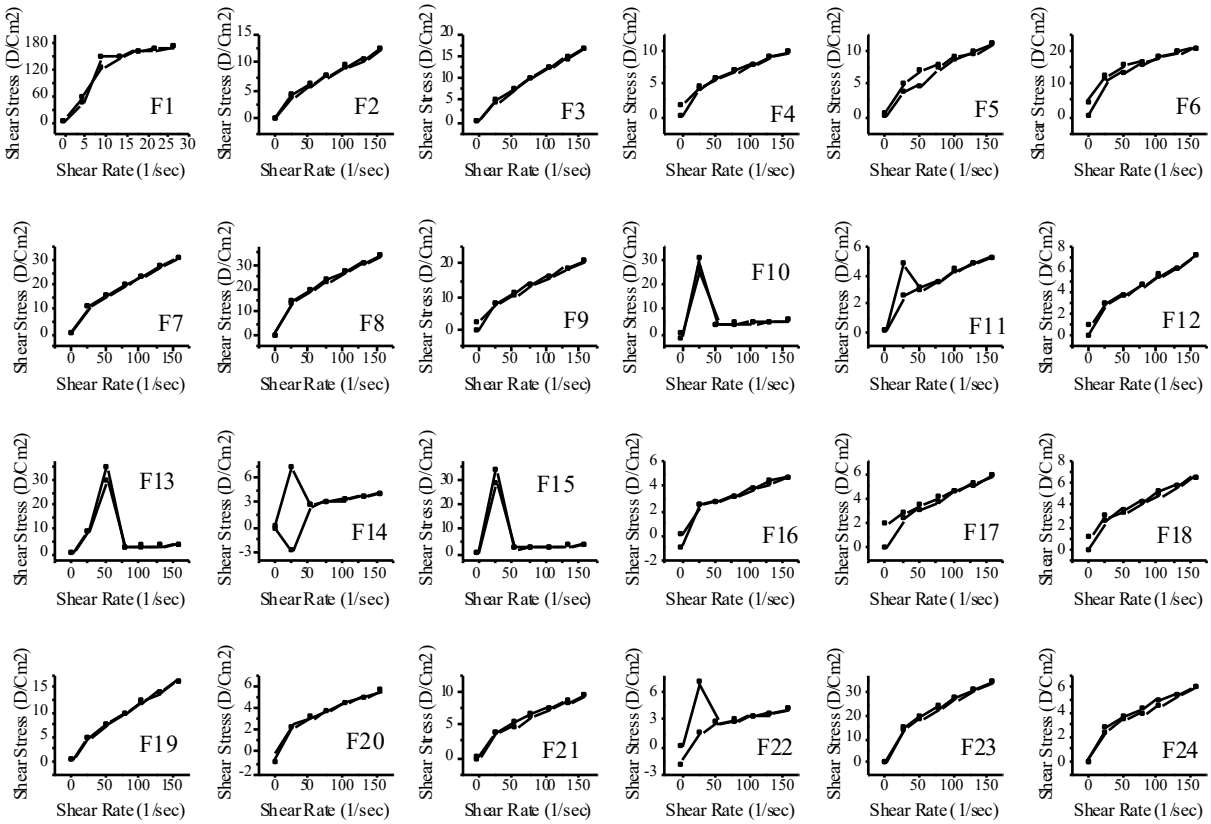

Figure 7.7. Rheological behavior of NLCs containing *L. sidoides* essential oil.

7.3.8 Antifungal activity

The antifungal activity was evaluated both for the NLC formulations and for the isolated components of the formulations. Figures 7.8 and 7.9 shows the experimental results of the screening for antifungal activity, performed by agar diffusion method against *Candida albicans* and *Fusarium solani*, respectively. The antifungal activity of *L. sidoides* essential oil is already well known (4,15–18). As expected, *L. sidoides* EO showed high antifungal activity against both *C. albicans* and *F. solani*. For both fungi the constituents isolated from the NLC formulations did not show antifungal activity, as well as the NLC containing Poloxamer 188 as a surfactant (F1, F2, F3, F10, F11, F12, F19, and F20), which were then discarded from the future activities of the study. Although all the other NLCs showed antifungal activity against both fungi, the formulations containing SDS as a surfactant (F7, F8, F9, F16, F17, F18, F23, and F24) showed remarkable activity, with a very distinct inhibition halo, both for the yeast and for the filamentous fungus. This activity may be related to the anionic charge of this surfactant, which is more explored in this study. These antifungal profiles are very promising for the continuation of the study, whose objective is to obtain nanoparticles capable of fighting resistant microorganisms. From this stage of the study onwards, we continued with only formulations

containing SDS and Quillaja saponin as surfactants (F4, F5, F6, F7, F8, F9, F13, F14, F15, F16, F17, F18, 21, 22, F23, and F24).

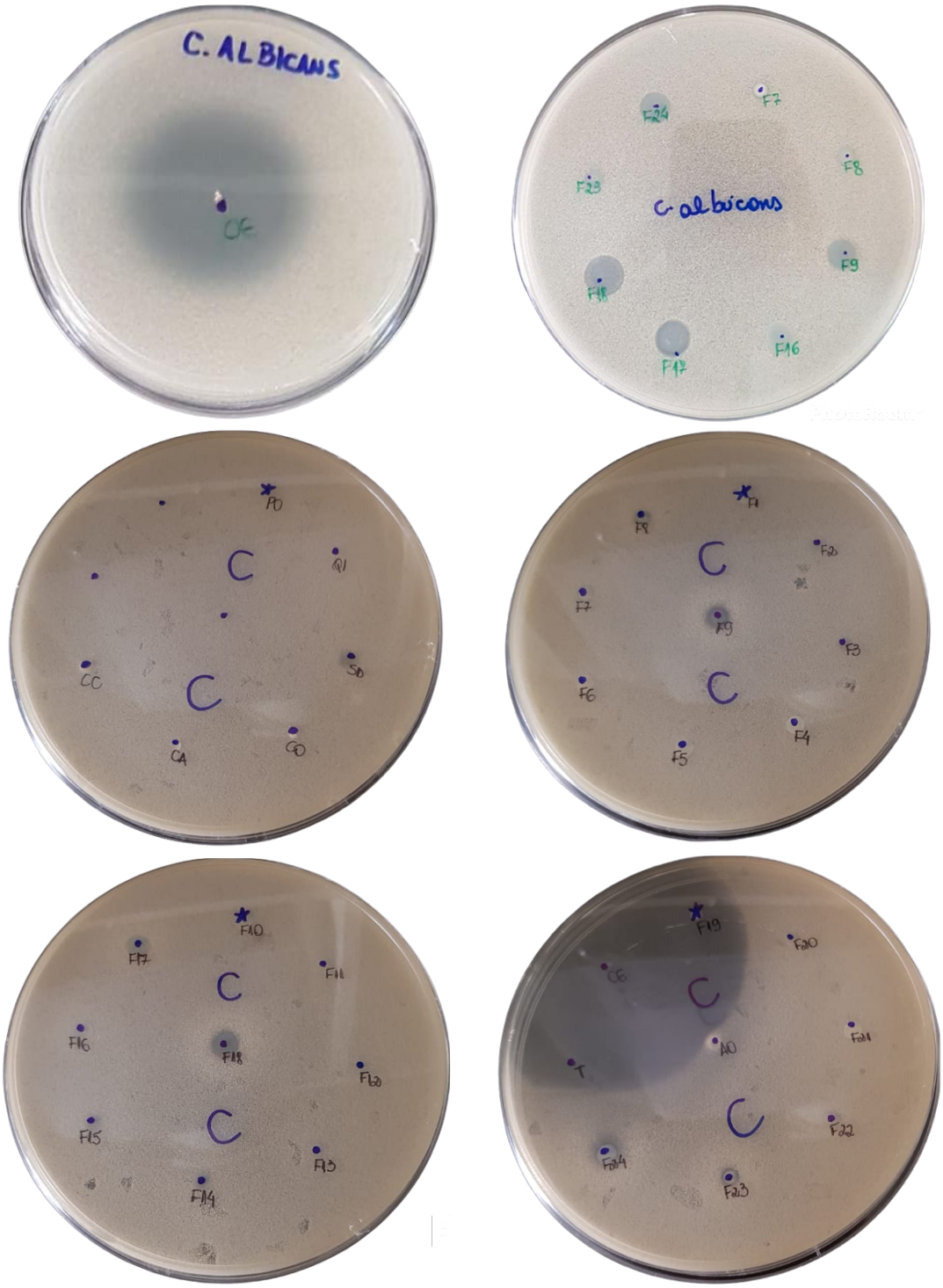

Figure 7.8. Antifungal sensibility screening of *Candida albicans* to *L. sidoides* essential oil (EO), NLC formulations loaded by the EO, and NLC isolated constituents determined by agar diffusion method. * Where: CO = Compritol® 888 ATO; CA = Carnauba wax; CC = Beeswax; PO = Poloxamer 188; Ql = Quillaja saponin; SD = Sodium dodecyl sulfate.

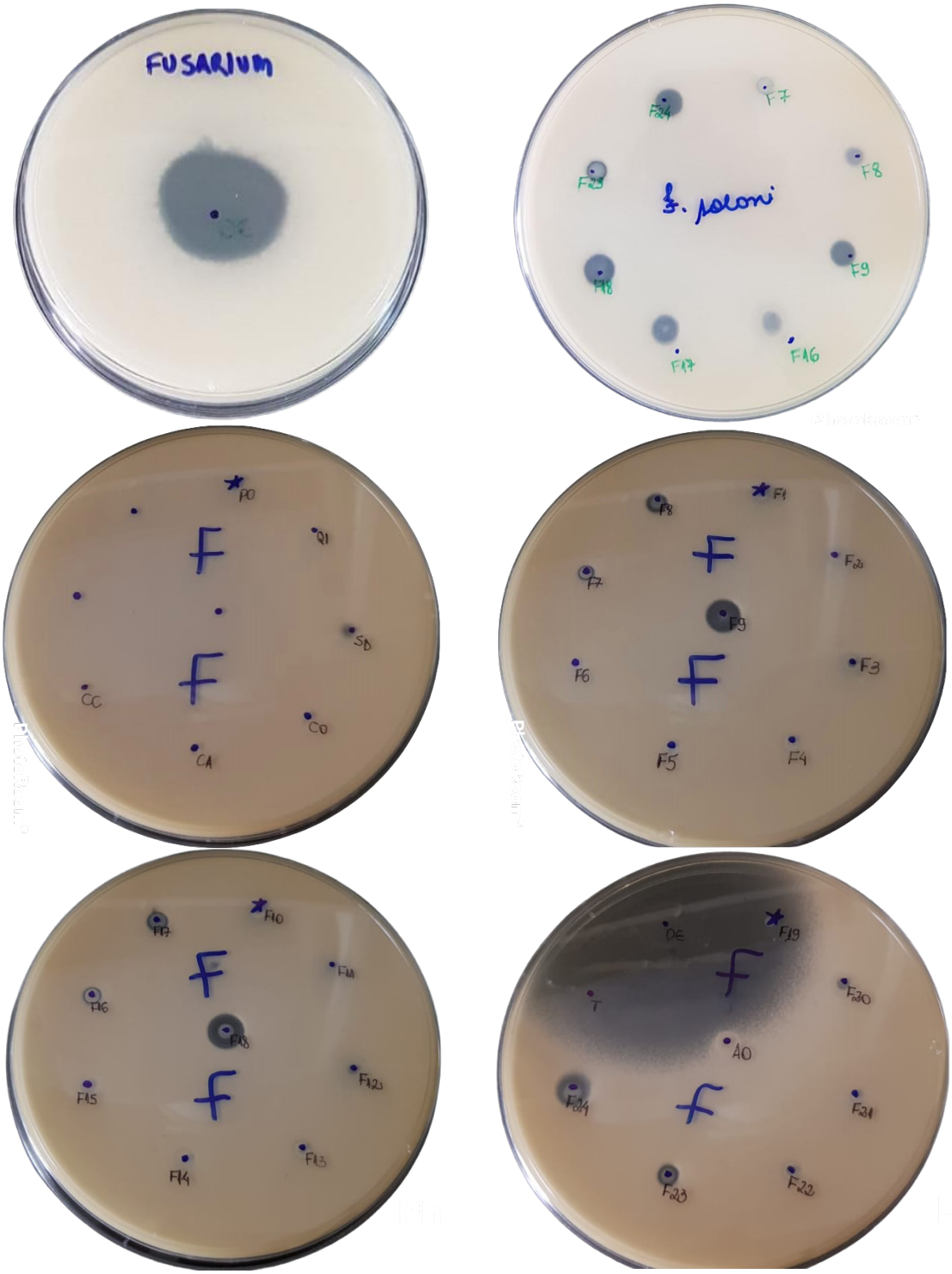

Figure 7.9. Antifungal sensibility screening of *Fusarium solani* to *L. sidoides* essential oil (EO), NLC formulations loaded by the EO, and NLC isolated constituents determined by agar diffusion method. * Where: CO = Compritol® 888 ATO; CA = Carnauba wax; CC = Beeswax; PO = Poloxamer 188; Ql = Quillaja saponin; SD = Sodium dodecyl sulfate.

7.3.9 Thermal analysis:

7.3.9.1 Thermogravimetric analysis:

The production of NLC containing *Lippia sidoides* essential oil is carried out at high temperatures (around 85 ºC to 90 ºC), a condition that can lead to the destruction of sensitive compounds, as essential oils. Aiming to assess the chemical stability of both nanoparticles and essential oil, we performed the thermal analysis of these materials by thermogravimetric analysis. *Lippia sidoides* essential oil presented a small loss of mass (less than 5%) up to 85 ºC (Figure 7.10). However, above 120 ºC the essential oil mass loss was fast and accentuated. All nanoparticle formulations showed the same mass loss profile: a more pronounced loss (around 86% to 94%) between 125 °C and 140 °C, followed by successive intervals of small mass loss between 150 °C and 600 °C, which may be associated with the release of essential oil contained in nanoparticles and its consecutive volatilization (19).

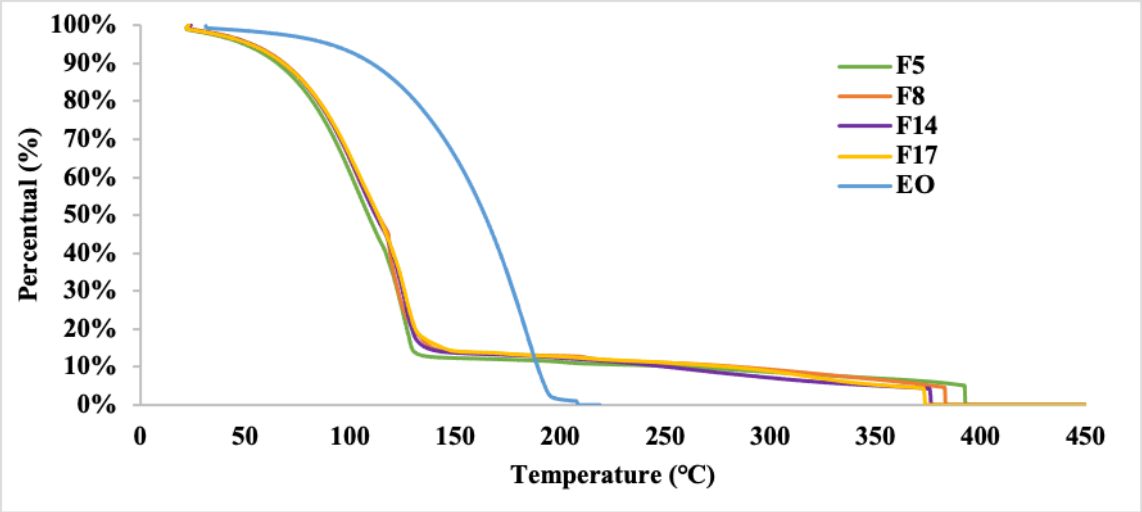

Figure 7.10. Thermogravimetric curves of NLC containing *Lippia sidoides* essential oil (F5, F8, F14, F17) and pure *L. sidoides* essential oil (EO).

7.3.9.2 Differential scanning calorimetry:

Thermal analyses were performed both for the NLC formulations (containing SDS and Quillaja saponin as surfactants) and for the isolated components of the formulations, as well as their physical mixture (mixture of excipients, without going through any process). The DSC was performed to determine the glass transition temperature and to obtain information about the crystal structure of lipid nanoparticles. Table 7.9 brings together the melting temperatures shown for NLCs and for the isolated components of the lipid matrix, and Figure 7.11 shows the fusion profile of these samples. NLC containing different concentrations of Quillaja saponin

showed distinct melting peaks and higher saponin concentration also resulted in higher melting temperatures. F4 and F13, both containing a lower concentration of saponin, had only 2 endothermic peaks, while the other formulations, containing 1.4% and 2.1% of Quillaja saponin, presented 3 melting peaks. A similar behavior was observed for NLC containing sodium dodecyl sulfate as surfactant: in general, higher concentrations of the surfactant resulted in higher melting temperatures at the endothermic peaks. All formulations containing Compritol® 888 ATO had more endothermic peaks than Compritol® 888 ATO alone, which has only one endothermic peak around 72 °C. This behavior is related to the greater polymorphism presented by these nanoparticles when compared to isolated Compritol® 888 ATO. Both beeswax and carnauba wax presented three endothermic peaks, at different temperatures, and their association promotes the appearance of a fourth endothermic peak. This polymorphism was also observed in NLC containing the association of these waxes as a lipid matrix.

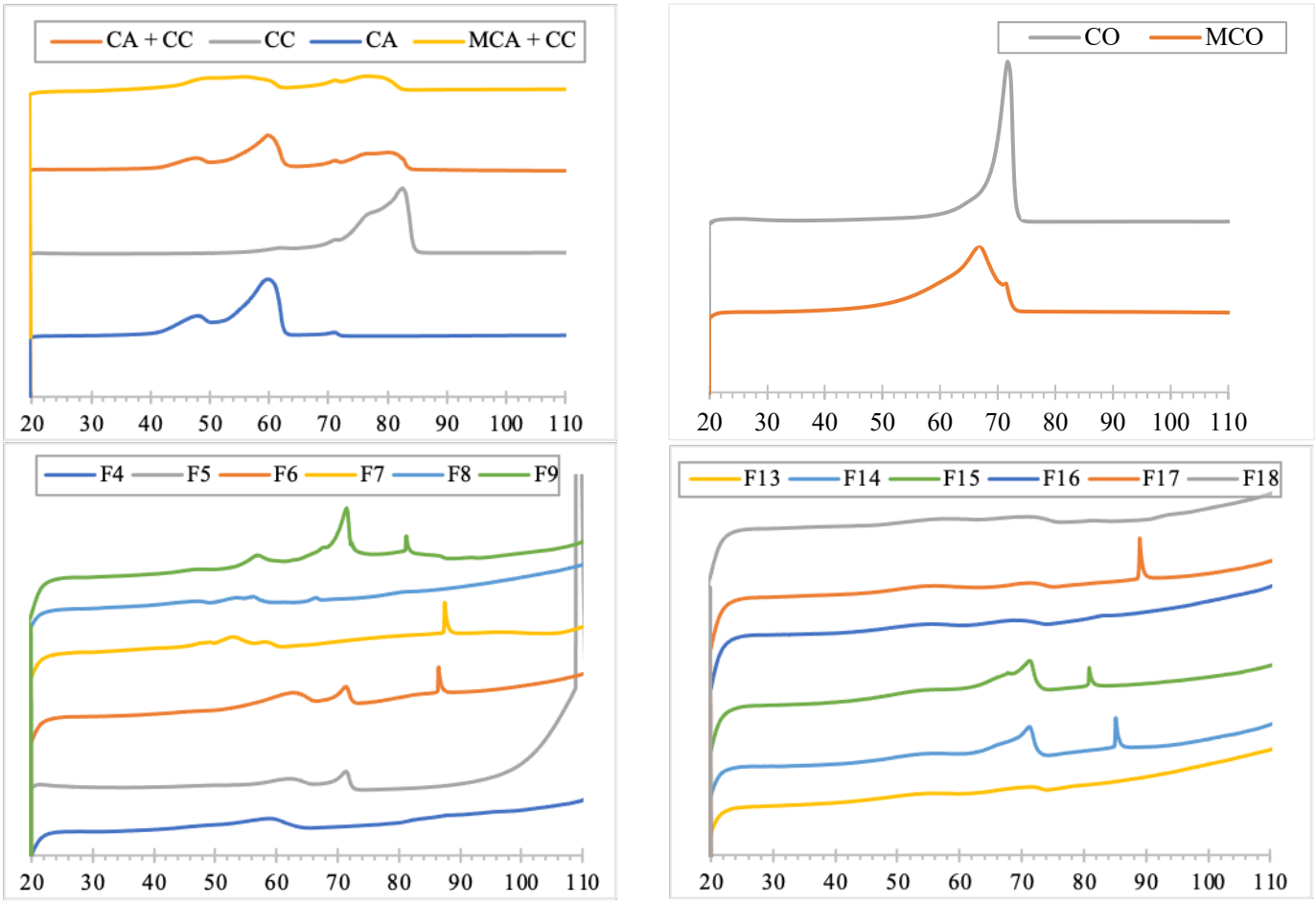

Figure 7.11. Thermal behavior (DSC) of fusion of NLCs containing essential oil of *Lippia sidoides* and lipid matrix components under heating at a rate of 5 ºC/min. * Where: CO = Compritol® 888 ATO; CA = Carnauba wax; CC = Beeswax; PO = Poloxamer 188; Ql = Quillaja saponin; SD = Sodium dodecyl sulfate; M = physical mixture.

Table 7.9. Thermal fusion behavior (DSC) of NLC containing essential oil of *L. sidoides* and lipid matrix components under heating at a rate of 5 ºC/min.

NLC	Lipid system	Surfactant concentration (%)	Peak 1 (ºC)	Peak 2 (ºC)	Peak 3 (ºC)	Peak 4 (ºC)
F4	Compritol	0,7 Quill.	58,8	87,7	-	-
F5	Compritol	1,4 Quill.	71,3	108,4	114,0	-
F6	Compritol	2,1 Quill.	62,4	71,4	86,4	-
F7	Compritol	0,7 SDS	48,8	53,3	58,3	-
F8	Compritol	1,4 SDS	46,2	53,8	56,2	66,5
F9	Compritol	2,1 SDS	46,7	56,7	67,5	84,7
F13	BW + CW	0,7 Quill.	53,2	70,8	-	-
F14	BW + CW	1,4 Quill.	53,9	71,2	85,1	-
F15	BW + CW	2,1 Quill.	53,1	71,3	80,9	-
F16	BW + CW	0,7 SDS	53,7	68,6	82,8	116,4
F17	BW + CW	1,4 SDS	54,4	71,7	89,0	-
F18	BW + CW	2,1 SDS	56,3	71,5	93,0	116,7
Beeswax	-	-	49,7	60,2	71,3	-
Carnauba wax	-	-	61,8	71,3	82,8	-
BW + CW	-	-	47,8	60,0	71,3	81,0
Compritol	-	-	71,8	-	-	-

***** Compritol: Compritol® 888 ATO; BW + CW: mixture of beeswax + carnaúba wax (1:1); Quill.: Quillaja saponin; SDS: sodium dodecyl sulfate

7.4 CONCLUSIONS

The study was found to be suitable for production of NLC loaded by *L. sidoides* essential oil using DOE approach. Our findings demonstrated that selected matrix components significantly influence critical quality attributes of the NLC. The adjustment of the statistical model provided an optimal NLC composition: composed by a mixture of carnauba wax and beeswax (1:1), SDS as surfactant, at the concentration of 60% of the total solid lipid. This combination of components provides the smallest and mono dispersed particles, with the greatest ζ-potential, tending to the greatest stability. Furthermore, NLC composed by SDS and Quillaja saponin presented a lipid matrix with polymorphic structure and remarkable antifungal activity. These results are relevant indicating possible applications as a “natural” active component in pharmaceutical antifungal preparations or as a food preservative.

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CHAPTER 8. NANOSTRUCTURED LIPID CARRIERS LOADED WITH *LIPPIA SIDOIDES* ESSENTIAL OIL AS A STRATEGY TO COMBAT THE MULTIDRUG-RESISTANT *CANDIDA AURIS*

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8.1 INTRODUCTION

Since 2009, when it was first isolated in Japan [1], *Candida auris* became a major concern to medical mycology, emerging across the globe to more than 40 countries, being easily transmissible and associated with high mortality rates [2–4]. In addition to widespread as a multiple drug-resistant pathogen, a number of other attributes make this fungus a major matter of concern, including the easy spreading in health-care facilities, and the ability to persist for days to weeks both in the human host and on inanimate surfaces [5–7]. Moreover, *C. auris* diagnostics in resource-limited countries is yet another challenge, as it involves complex and expensive equipment, such as MALDI-TOF MS [4]. In addition, of particular concern is that the COVID-19 pandemic may provide the ideal conditions for new outbreaks of *C. auris* infection in ICUs. Part of the current mortality of patients with COVID-19 may be related to *C. auris* infection [8,9].

The treatment of resistant fungal infections is currently a difficult task due to the limited therapeutic options on the market. *C. auris* presents an even more challenging treatment profile, as it can develop different resistance mechanisms, such as drug target mutation and overexpression, activation of stress response pathways, biofilm formation, and alterations in drug efflux and uptake [10]. Lipid nanoparticles are biocompatible encapsulation systems which are attracting widespread interest due to the possibility to expand the range of molecules to be clinically used, with limited toxicological risk [17]. The encapsulation of EOs in lipid nanoparticles is a strategy to modulate their release properties and enhance their stability and antimicrobial activity [18,19]. Undoubtedly, this technology has the potential to combat multidrug-resistant antimicrobial strains, including *C. auris*.

An overview in the current literature shows several studies concerning the EOs encapsulation in nanostructured lipid carrier (NLC). Saporito et al. [20] investigated the antimicrobial activity and wound healing properties of lipid nanoparticles loaded by eucalyptus EO. The nanoparticles exhibited good antimicrobial properties against *Staphylococcus aureus* and *Streptococcus pyogenes*, and wound healing properties toward fibroblasts. Baldim et al. [21] encapsulated *Lippia sidoides* EO into lipid nanoparticles and obtained a promising antimicrobial system, with potent activity against *Candida albicans*.

The potent antimicrobial properties of *L. sidoides* EO and its major constituent, thymol, are well described in the literature [21–25]. In an attempt to investigate the extent of *C. auris* resistance we proposed the NLC as a delivery system to encapsulate *L. sidoides* EO. We opted for this type of lipid nanosystem due to some of its peculiarities, such as high loading capacity, thermodynamic stability, and high biocompatibility with human tissues. Lipid matrices were based on Compritol® 888 ATO or a 1:1 (w/w) mixture of Beeswax (BW) and Carnauba wax (CW) as solid lipid, while oleic acid was used as liquid lipid. The anionic surfactant Sodium dodecyl sulphate (SDS) was selected to stabilize nanoparticles and to prevent their aggregation. The systems were prepared by hot emulsification using high-speed homogenization followed by sonication. Optimized formulations were produced and characterized by the determination of the particle size, polydispersity index and ζ-potential; evaluation of the in vivo toxicity using *Galleria mellonella* larvae model, and antifungal activity against the multidrug-resistant *C. auris*. We believe that the technological platform proposed here, and the results obtained, are of great relevance and open perspectives for future proposals for novel EOs' based antifungal. At the best of our knowledge, such products are not commercially available yet.

8.2 MATERIALS AND METHODS

8.2.1 Material

L. sidoides EO was purchased from Pronat (Produtos Naturais LTDA, Brazil). Methanol and oleic acid were purchased from Labsynth (Brazil). Thymol, sodium dodecyl sulfate, and acetonitrile HPLC grade were purchased from Sigma-Aldrich (St Louis, USA). Carnauba wax, with a melting range from 80 °C to 86 °C, was kindly donated by Foncepi (Brazil). Beeswax, with a melting range from 61 °C to 65 °C, was purchased from Via Farma (Brazil). The Compritol® 888 ATO (Gattefossé), with melting range from 65 °C to 77 °C, was purchased from Brasquim (Brazil).

8.2.2 *Lippia sidoides* essential oil characterisation by GC-MS

The main constituents of *L. sidoides* EO were identified by gas chromatography coupled to mass spectroscopy (Shimadzu model GC-MS QP-2010, Japan), using capillary column EN5-MS (30m x 0.25mm x 0.25µm), H_2 as carrier gas (flow rate of 1.0 mL/min). The temperature was programmed from 60 °C to 240 °C, at 3 °C/min. Injection temperature was 240 °C, flow rate of 1.30 mL/min of H_2; detector temperature of 260 °C and split ratio 1:50. EO was diluted in methanol (0.5 mg/mL) and the injected volume was 1 µL (Fernandes, 2008). The main constituents of the EO were identified by comparison of their mass spectra with the ones published in the Wiley electronic library (WILEY7.LIB), and Kovats index (IK) relative to a series of alkanes (C9-C20) with the ones reported in Adams (2007) [22].

8.2.3 Preparation of the nanostructured lipid carrier systems

The nanostructured lipid carrier systems loaded with *L. sidoides* EO were prepared by hot emulsification using high-speed homogenization followed by ultrasonication (US), according to Soleimanian et al. (2018) [22] with some modifications. The solid lipid was melted at 10 °C above its melting point and mixed with the oleic acid (liquid lipid) and the *L. sidoides* EO. Sodium dodecyl sulphate (SDS) was dissolved in a 10 mM pH 7.0 phosphate buffer saline (PBS) at different ratio, as indicated in Table 8.1, and heated to the same temperature as the lipid phase. The aqueous phase was gently and homogeneously dispersed into the lipid phase, and homogenised by a high-speed stirrer (UltraTurrax T18, IKA-Wilmington, NC, USA) at 14,000 rpm/min for 3 min. The oil-in-water nanostructured lipid system formed was then sonicated by an ultrasonic sonicator VCX-750 (SONICS Vibracell, Newtown, USA), equipped with a 13 mm diameter probe, in a constant-temperature water bath (85 °C). The magnitude of the US was fixed at 45% amplitude at the frequency of 20 kHz, lasting for 5 cycles of 2 minutes ON and 1 minute OFF.

Table 8.1. *Lippia sidoides* EO-containing NLC prepared with variable surfactant and lipid content. Quantities are expressed as % w/w.

Form.	Surfactant	Lipid matrix			EO	10 mM pH 7.0 PBS
		Solid lipid		Liquid lipid		
	SDS	Compritol® 888 ATO	BW + CW	Oleic acid		
F7	1.4	5.2	0	1.8	1.8	89.8
F8	2.8	5.2	0	1.8	1.8	88.4
F9	4.2	5.2	0	1.8	1.8	87.0
F16	1.4	0	5.2	1.8	1.8	89.8
F17	2.8	0	5.2	1.8	1.8	88.4
F18	4.2	0	5.2	1.8	1.8	87.0
F23	2.8	5.2	0	1.8	1.8	88.4
F24	2.8	0	5.2	1.8	1.8	88.4

*BW: beeswax; CW: carnauba wax; EO: *L. sidoide*s essential oil

8.2.4 Droplet size, polydispersity index, and ζ-potential

The dynamic light scattering was used to measure the size and the polydispersity index of the EO-loaded NLCs. The measurements were made in a Zetasizer Nano – ZS90 (Malvern, UK). Samples were diluted 1:200 using a 10 mM PBS at pH 7.0, to prevent multiple scattering effects. In addition to the mean hydrodynamic radius (z-average) of the particles, the equipment also reports the polydispersity index (PI), which ranges from 0 (monodisperse) to 1 (very broad distribution). ζ-potential was also determined by micro electrophoresis, in the same instrument. The measurements were carried out in triplicate, at 25 °C.

8.2.5 Thymol retention of liquid emulsions

Thymol, the major component present in *L. sidoides* EO was used as marker compound to evaluate the encapsulation process. The high-performance liquid chromatography coupled to a diode arrangement detector (HPLC-DAD) was used to quantify the thymol present in the EO-loaded NLCs systems. The chromatographic conditions were based on the method proposed by Benelli, Souza and Oliveira (2013) and Leal and co-workers (2003), with some modifications. Previously, a calibration curve was made with thymol concentrations varying from 50 to 800 µg/mL. Analyses were performed in a HPLC (model Prominence LC-20A series and a LC-6A double pump - Shimadzu Corporation, Kyoto, Japan) using a C-18 column (Shimadzu Shim-Pack CLC(M) 4.6 mm x 25 cm, 5 µm, 100Å) at 30 °C, with a volume of sample injected of 20 µL. The mobile phase was a gradient of water (A) and acetonitrile (B). The acetonitrile

concentration was changed as follows: 0–2 min, 10% B; 2–7 min, linear increase of B to 78%; 7–17 min, 78% B; 17– 20 min, linear increase of B to 100%; 20–23 min, 100% of B; 23–26 min, linear decrease of B to 10%, 23–32 min, 10% of B. The chromatograms were acquired at a wavelength of 276 nm. The method of sample preparation consisted of diluting the samples in acetonitrile (4.0 µL/mL), homogenizing the mixture in ultrasound bath, and keeping the solution under magnetic stirring for 30 minutes. After the extraction, the samples were centrifuged for 5 min at 5,000 rpm and the supernatant were filtered and 10 µL was injected in the HPLC.

8.2.6 Antifungal activity

The antifungal activity of *L. sidoides* EO and EO-loaded NLCs against the multidrug-resistant *C. auris* (CDC B11903) was performed both by a previous screening analysis, to determine the sensitivity, and by determining the minimum inhibitory concentrations (MIC) and minimum fungicidal concentration (MFC).

The screening for anti-*Candida* activity was performed by agar diffusion assay to evaluate the growth inhibition of *C. auris* by EOs and EO-loaded NLC formulations. The inoculum was prepared using 24-hour plate cultures of *C. auris* in Sabouraud Agar dextrose incubated at 37 °C. The colonies were suspended in 0.85% saline buffer in suspension of $1x10^6$ cells/mL. The RPMI1640 agar supplemented with glucose 2.0 g/L was inoculated with the *C. auris* suspension. A drop of 10 µL of each NLC formulations and 5 µL of the pure EO were placed on the top surface of the inoculated agar plate. The plates were incubated at 37 °C for 24 h and the results were evaluated by the presence or absence of inhibition zones.

Antifungal susceptibility testing was performed in accordance Clinical and Laboratory Standards Institute (CLSI) guidelines for broth microdilution test (protocol M27-A3) [32] against the multidrug-resistant *C. auris*, with modifications. The EO and EO-loaded NLC formulations concentration range was 9 - 1.76×10^{-2} mg/mL. The yeast inoculum was prepared in a final concentration of 4×10^5 CFU/mL and inoculated in RPMI-1640 (Life Technologies, Grand Island, EUA) with L-glutamine (0.3 g/L) buffered to pH 7.0 with MOPS (USB Corporation, Cleveland, OH, USA), supplemented with D-glucose (2.0 g/L). The plates were incubated at 37 °C and readings were taken at 24 h and 48 h of fungus development. The minimum inhibitory concentration (MIC) was considered as the lowest concentration able to inhibit fungal growth. For analysis of the results, geometric means (GM) and ranges of MICs were calculated. The minimum fungicidal concentration (MFC) of the EO and EO-loaded NLCs was evaluated by the inoculation of 10 µL of MIC well on Potato Dextrose Agar (PDA) media

culture (Becton, Dickinson Company, USA). The plates were incubated at 37 °C for 5 days and de CFU were counted. All experiments were carried out in duplicate.

8.2.7 In Vivo Toxicity Assay

The toxicity of EO-loaded NLCs was determined in a *Galleria mellonella* in vivo model [33]. Groups of 10 *G. mellonella* larvae were randomly divided and individually weighed prior to treatment (larvae weighing between 250−350 mg each were used). The tested concentrations were based on the MIC concentrations: sub-inhibitory (low), minimum inhibitory concentration (MIC) and supra inhibitory (high) (Table 8.4). The toxicity profile of pure EO and at a 1:2 (v/v) dilution was also determined. The artificial inoculation was performed by injecting 5 µL of each NLC in each concentration (low, MIC and high), using a Hamilton micro syringe for gas chromatography model 7000.5 KH, 10 µL. Doses were injected into the last right proleg of the larvae. Phosphate buffered saline (PBS) was used as a negative control for toxicity. Between injections, the micro syringe was rigorously washed 3 times with sodium hypochlorite, 70% ethanol, and autoclaved water. The larvae were then incubated at 37 °C, deprived of feed and direct lighting. Every 24 h, the larvae were removed from the prepupae to delay their metamorphosis. Survival was analyzed every 24 h, and the statistical analyses were performed by the Log-rank method (Mantel-Cox) were using the GraphPad Prism 5 software. The EO-loaded NLC formulations, as well as their respective concentrations tested, were considered toxic when they were able to kill at least 50 % of larvae in a period of 5 days post-injection, following the protocol of Gottardo et al. (2019) [36].

8.2.8 Statistical analysis

The statistical significance of the experimental data was determined by ANOVA followed by the post-hoc Tukey´s multiple comparison test ($p \leq 0.05$).

8.3 RESULTS AND DISCUSSION

8.3.1 *Lippia sidoides* essential oil characterisation

Twenty-six components were identified in the LSEO (Table 8.2). Thymol, the bioactive molecule associated to the biological activities of this EO [37], was confirmed as the major component of the oil (68.22%), followed by p-cymene (9.43%), trans-caryophyllene (7.72%), β-myrcene (2.84%), γ-terpinene (2.71%), α-terpinene (1.16%), and thymol methyl ether (0.97%). Altogether, these compounds represented 93.05% of the total detection area.

Table 8.2. Chemical composition of the *Lippia sidoides* EO

Compound	% [a]	Kovats Index [c]
α- Thujene	0.95	928
Bicyclo[3.1.1]hept-2-ene, 2,6,6-trimethyl	0.56	937
2-β-Pinene	0.16	980
β-Myrcene	2.84	989
1-Phellandrene	0.07	1007
E-β-Ocimene	0.16	1011
α-Terpinene	1.16	1018
Benzene, 1-methyl-4-(1-methylethyl)-	9.43	1025
Bornylene	0.64	1032
1,8-Cineole	0.53	1033
1,3,6-Octatriene, 3,7-dimethyl-, (Z)- (CAS)	0.11	1037
1,3,6-Octatriene, 3,7-dimethyl-, (E)- (CAS)	0.16	1048
γ-Terpinene	2.71	1060
Linalyl acetate	0.35	1099
2-(Chloromethyl)tetrahydropyran	0.15	1145
Bicyclo[3.1.0]hex-3-en-2-one, 4-methyl-	0.26	1170
3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)	0.68	1180
Thymol methyl ether	0.97	1231
Thymol	68.22	1297
α-Copaene	0.34	1377
trans-Caryophyllene	7.72	1420
Aromadendrene	0.46	1440
α- Caryophyllene	0.34	1455
Ledene	0.44	1493
δ-cadinene	0.10	1520
Caryophyllene oxide	0.43	1580

a Percentages were calculated based on normalised MS peak areas
b Kovats Index: retention index relative to a series of alkanes (C10–C22)

8.3.2.2 Characterization of NLCs

The polydispersity index (PI) is a parameter to determine the homogeneity of particle sizes and their dispersion quality. PI values range from 0 to 1, and the lower the value, the better the suspension colloidal quality [38]. ζ-potential characterises the surface charge of the NLC particles with information concerning the repulsion forces between the particles. Values above 30 mV (in module), favour the repulsion between the particles, prevent coalescence and contribute to increase the system stability [39]. The results of the NLCs' characterization are shown in Table 8.3.

The composition of the NLCs affected both particle size and PI. NLC composed of beeswax and carnauba wax presented smaller (301.2 to 318.7 nm) and more homogeneous (0.18 to 0.22) particles compared to those composed of Compritol® 888 (whose sizes varied between 213.1 and 445.5 and the PI between 0.25 and 0.41). The effect of surfactant concentration was significantly more pronounced in formulations composed of Compritol® 888 as solid lipid: higher concentration of SDS provided smaller and less polydisperse particles.

Table 8.3. Characterization of NLCs loaded by *Lippia sidoides* essential oil

NLC	Size (nm)	PI (-)	ζ-potential (mV)
F7	445.5 ± 8.7^{a}	0.41 ± 0.01^{a}	-63.8 ± 8.7^{a}
F8	328.0 ± 8.7^{b}	0.33 ± 0.04^{b}	$-74.0 \pm 10.7^{b.c}$
F9	213.1 ± 1.7^{f}	$0.25 \pm 0.01^{c.d}$	-72.0 ± 8.2^{b}
F16	318.7 ± 2.3^{c}	0.20 ± 0.00^{d}	-87.5 ± 2.8^{d}
F17	$307.8 \pm 3.0^{d.e}$	0.22 ± 0.03^{d}	-93.1 ± 2.7^{d}
F18	301.2 ± 4.8^{e}	0.18 ± 0.03^{d}	-90.1 ± 4.2^{d}
F23	$321.3 \pm 10.9^{b.c}$	0.33 ± 0.04^{b}	-77.0 ± 7.4^{c}
F24	301.5 ± 5.0^{e}	0.19 ± 0.02^{d}	-89.5 ± 3.0^{d}

Same letter means no significant difference according to Tukey´s multiple comparison test ($P < 0.05$)

SDS is an anionic surfactant, employed in this study to stabilize different hydrophobic matrices: a pure Compritol® 888 matrix and a mixture (1:1) of beeswax and carnauba wax. Surfactants are characterized by parameters such as CMC (critical micelle concentration) and HLB (hydrophilic-lipophilic balance) [40]. CMC is defined as the concentration of surfactants when the micelles spontaneously form, while HLB is a parameter to qualify the emulsification properties of a surfactant. Both of these definitions are closely related [41]. The micelle formation process depends on two factors: the electrostatic interactions between the charged head groups of the components; and the hydrophobic interactions between the hydrocarbon tails of components [42]. The more hydrophobic the chain, the lower the CMC, so micelles are formed at a lower concentration of surfactant. Upon reaching CMC, any further addition of surfactants will only increase the number of micelles, not interfering with particle size. Compritol® 888 ATO has an amphiphilic character, due to the presence of acylglycerols in the composition [43]. On the other hand, the mixture of beeswax and carnauba wax gives strong lipophilic properties to the nanoparticles and, therefore, contributes to reduce the CMC, which justifies the fact that the concentration of SDS has not influenced the particle size of these formulations.

The nanoparticle charge is one of the factors related to the physical stability of the system. All formulations presented negative ζ-potential values, in the range -63.8 to -93.1 mV, and this parameter was significantly affected by the composition of the formulations. High negative or positive electrical charge avoids the occurrence of aggregation, once the charges tend to repel each other [44]. For this study, NLC containing a combination of beeswax and carnauba wax had significantly higher values of ζ-potential (from -87.5 to -93.1 mV), which indicates greater electrostatic stability in comparison to nanoparticles composed of Compritol® 888 ATO as solid lipid (from -63.8 to -77.0 mV). The negative charge is related to the presence of negatively charged head groups of the anionic surfactant SDS exposed in the external region of the nanoparticles [45].

All EO-loaded NLC formulations retained a high percentage of the marker compound thymol, the major constituent of *L. sidoides* EO (Figure 8.1). The retention values varied from 90% to 100% and did not change significantly by increasing the concentration of surfactant nor by modifying the composition of the lipid matrix ($p < 0.05$). Some factors are closely related to a high loading capacity of NLCs, such as the solubility of the encapsulated material in the lipid matrix, type and concentration of the surfactant, type of lipid matrix and the ratio of solid and liquid lipids forming the core of NLC [17]. Yue et al. successfully loaded bupivacaine (BPV) into NLCs and reported very high encapsulation efficiency, i.e. 90% [46]. Baldim et al. developed a lipid nanosystem to encapsulate *L. sidoides* EO and obtained fairly high rates of thymol retention, in the range of 91-100% [21]. In our study, the high levels of thymol retention can be attributed to the lipophilic nature of the essential oil and NLCs that subsequently causes higher thymol partitioning into lipid matrix of NLC and lesser into the aqueous phase [47].

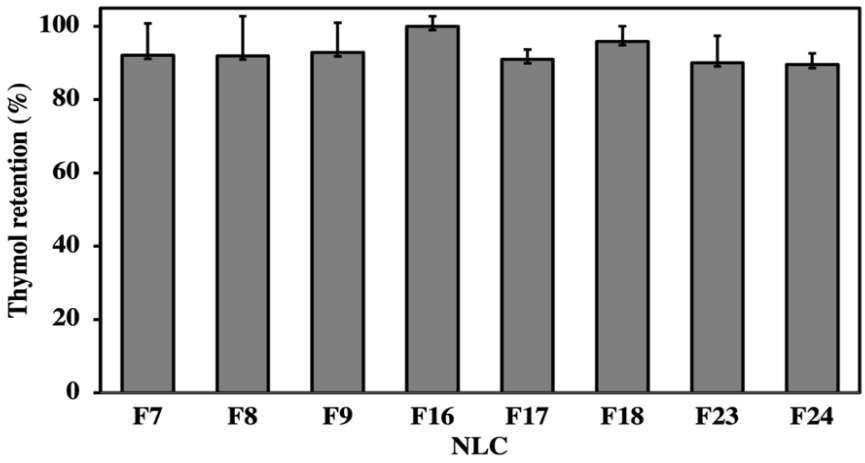

Figure 8.1. Thymol retention of NLC loaded by *Lippia sidoides* essential oil

8.3.5 Anti-*Candida* activity

The in vitro antifungal activity of *L. sidoides* EO-loaded NLCs against multidrug-resistant *C. auris* was firstly assayed by agar diffusion test. EO and EO-loaded NLC formulations showed inhibition zone. F9, F16, F17, F18, and F24 EO-loaded NLCs formulations exhibited larger inhibition zones (Figure 8.2).

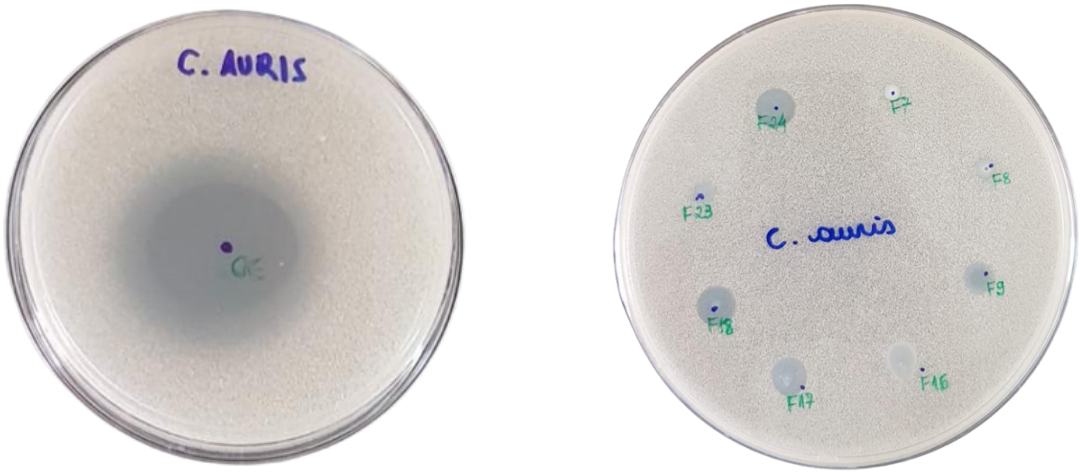

Figure 8.2. Agar diffusion test of *L. sidoides* essential oil (left) (5 µL) and EO-loaded NLC formulations (right) (10 µL) against clinically isolated strain of multidrug-resistant *C. auris*. The NLC compositions are described in the Table 8.1.

The broth microdilution assays determinate the MICs of *L. sidoides* EO and EO-loaded NLCs against *C. auris*. Table 8.4 gives a summary of the MIC and MFC for the EO and EO-loaded NLC formulations against *C. auris*. The highest anti-*Candida* activity was achieved with EO and EO-loaded NLCs containing higher concentration of SDS in the composition (F8, F9, F17, F18 and F24), which exhibited the lowest MIC values. *Candida auris* was inhibited by both EO and EO-loaded NLC with concentrations between 0.281 and 0.563 mg/ml. *L. sidoides* EO and its isolated major compound (thymol) have already been shown to be highly effective in ATCC strains of *C. albicans* [21]. However, in this previous study, both EO and thymol showed lower MIC against yeast than the EO-loaded lipid nanoparticles. Herein, F8, F9, F17, F18, and F24 formulations exhibited similar MIC values to that showed by the EO, showing the highest antifungal activities against *C. auris* in the tested samples. Despite having different combinations of solid lipids, these formulations contain the highest concentration of SDS in the composition. In addition, all tested formulations showed a fungicidal profile in the minimal inhibitory concentrations (Table 8.4).

The mechanism of antifungal action of *L. sidoides* EO is related to its functional groups. The main class of bioactives in the *L. sidoides* EO are terpenes, which are formed by a variety

of functional groups [48]. Although there is a limited understanding of the mechanisms of antifungal resistance of *C. auris*, a number of gene families encoding proteins associated with mechanisms of resistance have been identified [49]. Additionally, it has already been shown that the complex composition and the combination of EO constituents present synergistic effects, simultaneously attacking different targets of a microorganism cell [50,51]. On the other hand, the influence of anionic surfactants (as SDS) on enzyme activity through binding to enzymatic proteins has been extensively demonstrated [52]. Furthermore, the exposure of *C. auris* cells to SDS is related to the denaturation of cell wall proteins and lipid damage [6]. In our study, the concentration of SDS provided a positive effect on the antifungal activity, which may be related to the interaction of the anionic surfactant molecules with proteins and lipid content of the cell wall. Based on these observations we hypothesized that the synergy between the different EO constituents, which presents different functional groups, favoured the high antifungal activity of the samples. In addition, the higher the concentration of anionic surfactant in the samples, the greater the availability for the EO constituents to interact with the yeast cell wall, which explains the same MIC values for the free EO and the formulations F8, F9, F17, F18, and F24.

Table 8.4. Minimum inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of *L. sidoides* EO and EO-loaded NLC formulations against clinically isolated strain of multidrug-resistant *C. auris*

NLC	Geometric Mean MIC (mg/mL)	MIC range (mg/mL)	MFC (mg/mL)
L. sidoides EO	0.281	0.563 – 0.140	0.281
F7	0.563	1.125 – 0.281	0.563
F8	0.281	0.563 – 0.140	0.281
F9	0.281	0.563 – 0.140	0.281
F16	0.563	1.125 – 0.281	0.563
F17	0.281	0.563 – 0.140	0.281
F18	0.281	0.563 – 0.140	0.281
F23	0.563	1.125 – 0.281	0.563
F24	0.281	0.563 – 0.140	0.281
F8B [*,1,2]	>2.250	>2.250	>2.250
F17B [*,1,2]	>2.250	>2.250	>2.250
SDS [*]	0.281	0.281	0.281

* EO equivalent in the formulations at intermediary SDS concentration (F8 and F17) 1: Fungal Growth at all concentrations assayed 2: NLC without EO.

Despite the need for deeper studies about the mechanism of action, the interactions between nanoparticles and the fungal cell wall, and the modulation of *L. sidoides* EO release, it is undeniable that the MIC values obtained (between 0.281 and 0.563 mg/mL) evidenced that the NLCs here developed are a highly effective systems in combating *C. auris*.

8.3.6 In vivo toxicity

The *in vivo* toxicity assays of the EO-loaded NLCs were performed by the method of injection in *Galleria mellonella* larvae. A peculiarity of this model is its similarity with the immune and toxic responses of mammals. The larval hematocytes act as phagocytes and release proteins with close similarity to mammalian antibodies [36,53,54]. *L. sidoides* EO and EO-loaded NLC formulations were tested with the MIC concentrations against *C. auris* and additionally one concentration below (low) and one above (high) the MIC (Figure 8.3).

All but one EO-loaded NLC formulation with the concentration below (low) and MIC, did not kill the larvae. F18 EO-loaded NLC killed 60% of the larvae. The EO-loaded NLC formulations containing lower and intermediate concentrations of SDS in the composition (F7, F16, F17 and F24) and with the concentration above the MIC (high), also did not killed the larvae, which demonstrates that the toxicity profile may be related to high anionic surfactant content in the nanoparticles. Regarding the *L. sidoides* EO, the 1:2 (v/v) dilution killed 40% of the larvae, whereas the pure *L. sidoides* EO killed all the larvae 24 h post-injection. The negative toxicity control (PBS), as expected, was not able to kill any larvae, presenting a non-toxic profile (data not shown). Moreover, these findings reinforce the excellent performance of NLCs as highly biocompatible carrier systems.

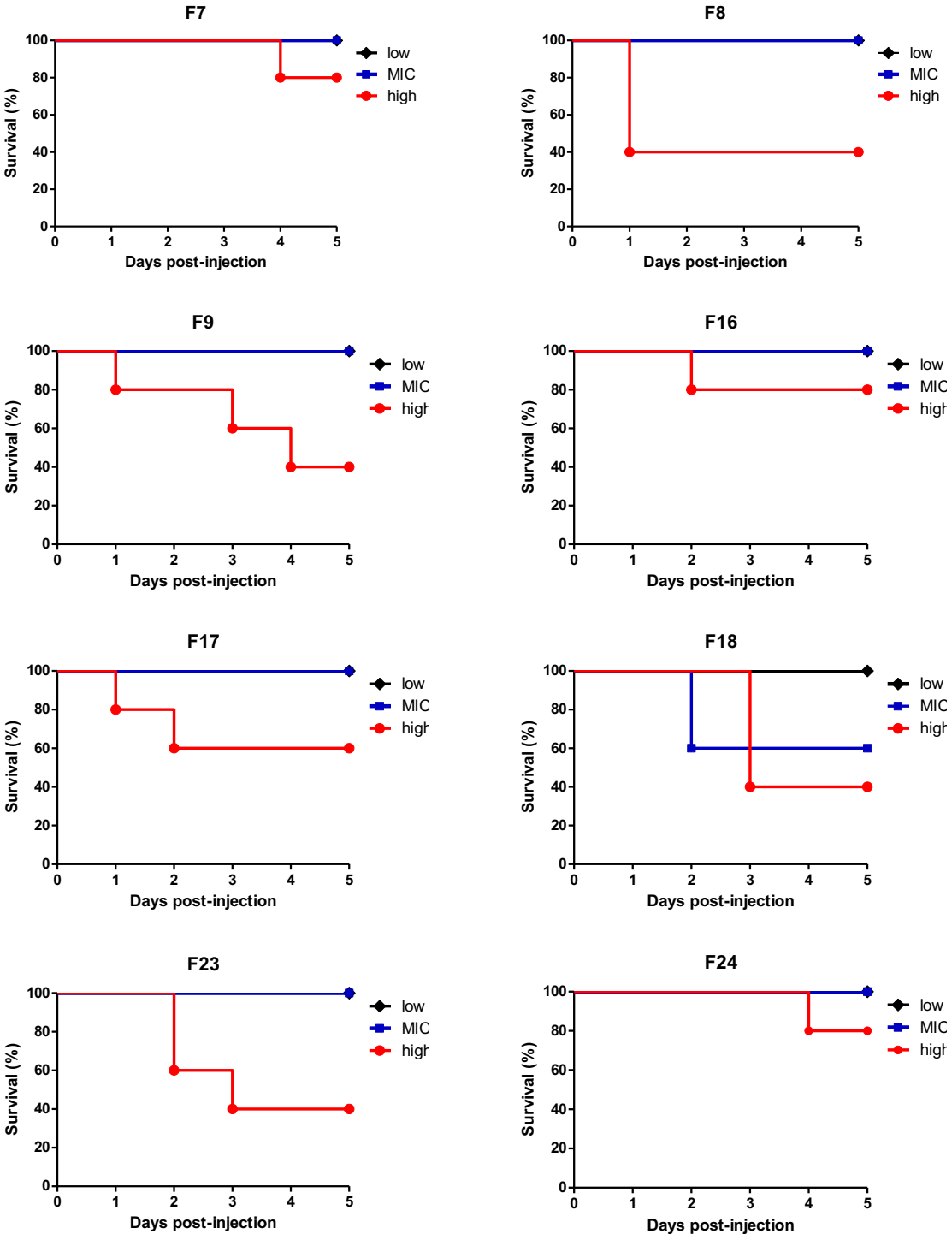

Figure 8.3. In vivo toxicity assay of *L. sidoides* OE-loaded NLC formulations in *G. mellonella* larvae. Low, dilution below the MIC; MIC, minimal inhibitory concentration; high, dilution above the MIC.

8.4 CONCLUSIONS

We have shown that *L. sidoides* EO-loaded NLCs can be successfully produced by hot emulsification method followed by ultra-sonication. The characterization of the NLC systems by different methods confirmed the formation of particles in the nanometric range, with high retention efficiency of *L. sidoides* EO. Regarding the composition of nanoparticles, the concentration of SDS was a key factor to the product properties: NLC containing high SDS content presented smaller and less polydisperse nanoparticles, with good thymol retention and strong activity against *C. auris*, comparable to *L. sidoides* EO antifungal activity. Furthermore, the in vivo nontoxic profile of EO-loaded NLC formulations observed for *G. mellonella* larvae suggest a close relationship with the enhanced biocompatibility of the lipid matrix. Finally, the high performance in anti-*C. auris* assays and low toxicity of EO-loaded NLC formulations highlights the high relevance and novelty of this study. To the best of our knowledge, this is one of the few successful strategies to effectively fight *C. auris*, and open perspectives for further studies with EO and EO-loaded NLCs as an effective approach to combat multidrug-resistant pathogens.

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CHAPTER 9. OPTIMIZATION OF FREEZE DRYING OF NLC LOADED BY *LIPPIA SIDOIDES* ESSENTIAL OIL USING EXPERIMENTAL DESIGN

9.1 INTRODUCTION

Encapsulating essential oils in NLC is an innovative strategy that makes it possible to preserve their characteristics, increase their stability, modulate their release, and even increase the range of application of these bioactives. However, the liquid formulation can present a certain degree of instability, both physicochemical and microbiological, which can arise from problems such as aggregation of particles and sedimentation, as well as contamination by microorganisms, causing a reduction of the product shelf life. Therefore, the drying of these formulations is a fundamental process to provide greater stability and longer shelf life for the product, which can be easily redispersed at the time of use.

It is well known that the lipid matrix, drying carriers types and concentrations and drying technique are parameters that strongly influence the characteristics presented by the particles, such as: bioactive retention, size, degree of polydispersion, and charge of the particles; and also physicochemical aspects related to the particulated solids, such as moisture content and water activity (1). Design of experiments (DoE) is a very useful tool to identify and understand critical parameters of production processes and optimize their conditions (2).

Different carbohydrates can be used as cryoprotectants in freeze drying of NLCs (3). They can protect the nanoparticle structure by serving as water substitutes, since the hydrogen bonding replace the position of water molecules (4). Trehalose is an efficient disaccharide to be used as cryoprotectant of lipid nanoparticles (5). It possess many advantages over other sugars, which includes: higher glass transition temperature, less hygroscopicity, and higher flexibility to form hydrogen bonds with nanoparticles during freeze drying, by the absence of internal hydrogen bond (4). Mannitol is a naturally occurring and non-reducing sugar alcohol usually used as bulking agent because it can form supporting structure for the active components by crystallizing during the slow freezing process (6). It is very stable in dry state and aqueous solutions, difficult to be oxidized, and does not react with the active components of a formulations (7). Mannitol is also classified as non-hygroscopic, as it does not absorb moisture below 75% relative humidity (8). In this study, both trehalose and mannitol were used as cryoprotective agents for freeze-drying of lipid nanoparticles.

The presented research was conducted to establish a better understanding of the influence of lipid matrix of NLC and cryoprotectants on the powder's properties. The objective was to identify the process conditions to achieve the better characteristics of particulate systems. The interaction between the factors (NLC matrix, cryoprotectant type and concentration) were therefore systematically examined by applying a $3x2^2$ full factorial design. The particle size as z-average, polydispersity index (PI), ζ-potential, moisture content (XP), water activity (A_w), and thymol retention were investigated as responses describing the quality of the resulting powders.

9.2 MATERIAL AND METHODS

9.2.1 Material

Lippia sidoides EO was purchased from Pronat (Produtos Naturais LTDA, Horizonte – CE, Brazil). Methanol and oleic acid were purchased from Labsynth (Brazil). Thymol, sodium dodecyl sulphate, and acetonitrile HPLC grade were purchased from Sigma-Aldrich (St Louis, USA). Carnauba wax was kindly donated by Foncepi (Brazil), Beeswax, Compritol® 888 ATO was purchased from Brasquim (Brazil), *Quillaja* saponaria extract was kindly donated by Desert King International (San Diego, USA). D(+) trehalose dihydrate and D-mannitol were purchased from Sigma-Aldrich (EUA).

9.2.2 Methods

9.2.2.1 NLC drying study using experimental design

We evaluate the drying performance of NLC by using a factorial experimental design of type $3x2^2$ with central point and a repetition. The NLC chosen to follow in the drying studies were the ones that presented better results on the study presented in Chapter 7. The chosen formulations were: F8, F17 and a more "natural based" composition F15. A experimental planning using a $3x2^2$ full factorial design composed of 3 variables which were set at 3-levels each was applied to evaluate the effects of processing factors on the quality of the lyophilized product, requiring a total of 18 experiments. The independent variables were NLC composition (X1), type of cryoprotectant (X2), both categorical variables, and percentage of cryoprotectants (X3), a quantitative variable with lower (−1), medium (0) and higher (+1) values. The percentage of cryoprotectant ranged from 5%, 7.5% and 10% (Table 9.1). The data were analyzed using the R software.

Table 9.1. Full factorial design $3x2^2$.

Run	FACTORS*		
	NLC (X1)	**Cryoprotectant (X2)**	**Cryoprotectant concentration (%)** (X3)**
1	F8	T	5
2	F17	T	5
3	F15	T	5
4	F8	T	10
5	F17	T	10
6	F15	T	10
7	F8	M	5
8	F17	M	5
9	F15	M	5
10	F8	M	10
11	F17	M	10
12	F15	M	10
13	F8	T+M	7.5
14	F17	T+M	7.5
15	F15	T+M	7.5
16	F8	T+M	7.5
17	F17	T+M	7.5
18	F15	T+M	7.5

* Where: T = Trehalose; M = Mannitol; T+M = Trehalose + mannitol (1:1)
** % related to the total solid content of NLC

The NLCs were prepared following the methodology previously described in section 7.2.2.2.1 (Chapter 7), by emulsification using high-speed homogenization followed by ultrasonication method. The cryoprotectant solution was previously prepared and added to the NLCs fresh formulations. Table 9.2 shows the NLCs base formulations used. The freeze-drying process was carried out in a Thermo Fisher Scientific model SNL108B equipment containing a 1.5 L Micromodulyo lyophilizer unit (305x330x432 mm), a stainless-steel condenser, a 1/4 hp compressor and 0.30 kW power, a LyoPump VLP195FD ultra-vacuum pump, lyophilization bottles and independent valves. The samples were frozen at -20 °C for 12 h in 50 mL falcon tubes, and later at -80 °C for another 4 h, and then lyophilized for 96 h.

Table 9.2. *Lippia sidoides* EO-containing NLC prior to addition of cryoprotectants. Quantities expressed as % w/w.

NLC	*Quillaja*	SDS	Compritol® 888 ATO	CA+CC	Oleic acid	Essential oil	Phosphate buffer (qsp)
F8	x	1.4	2.6	x	0.9	0.9	44.3
F15	2.1	x	x	2.6	0.9	0.9	43.6
F17	x	1.4	x	2.6	0.9	0.9	44.3

* Where: CA+CC = carnauba wax + beeswax; SDS = sodium dodecyl sulfate

9.2.2.2 Particle size, polydispersity index, and ζ-potential

Particle size and polydispersity index (PI) were measured by dynamic light scattering (Dynamic Light Scattering) in a Zetasizer Nano – ZS90 (Malvern, UK), equipped with a 90° scattering angle. Samples were diluted in a 10 mM phosphate buffer solution at pH 7 1:200 (v/v). The ζ-potential values were obtained with the same equipment and calculated from the average of electrophoretic mobility values using the Smoluchowski equation (9). All the measurements were carried out in triplicate, at 25°C.

9.2.2.3 Moisture content (XP) and water activity (Aw) of dry NLC

The moisture content of dry NLC was determined by the Karl Fischer titration method, using a Karl Fischer 870 Titrino Plus equipment (Methrom, Switzerland), previously calibrated with water. Water activity analyzes were performed in the Aqua Lab 4TEV water activity meter (Decagon Devices, Pullman, USA), using the capacitive electrode. Analyses were performed in triplicate and results were expressed as mean and standard deviation.

9.2.2.4 Thymol retention

The major component present in *L. sidoides* essential oil (thymol) was used as marker and was quantified in the liquid (after addition of cryoprotectants) and dried NLC lipid formulations by high performance liquid chromatography coupled to a diode arrangement detector (HPLC-DAD). The samples were prepared by diluting them in acetonitrile (4.0 μL/mL), homogenizing them in an ultrasound bath and using magnetic stirring (for 30 minutes) to extract the active. Then, they were subjected to centrifugation (5 min at 5000 rpm) and the supernatant was filtered and analysed by HPLC. The chromatographic conditions were based on the method proposed by Benelli, Souza and Oliveira (2013) (10,11) and Leal and co-workers (2003), as previously described in section 7.2.2.4 of Chapter 7.

9.2.2.5 Statistical analysis

Data normality was checked using the Shapiro-Wilk test. To check if at least one NLC base formulation differs from the others we applied the Kruskall-Wallis test. After a significant result in the Kruskall-Wallis test, there is at least one group that differs from the others. To find out which groups differ from each other, the Dunn test was used to compare all pairs of groups. The association between the studied variables was tested by the Spearman correlation, a non-parametric test that varies between -1 and 1 and does not depend on the magnitude of the numbers, but on the order in which they occur. Negative sign of the correlation indicates an

indirect association between the variables, and direct association for the positive sign. The farther from 0, the stronger the correlation. The null hypothesis of the test is that the correlation value is equal to 0 (there is no correlation). P-values less than 0.05 indicate that the correlation found is in fact far enough from 0 and significant. For a more detailed evaluation, the classification by Hinkle, Wiersma and Jurs (2003) (12) consider: 0.90 to 1.00 “Very high” correlation; 0.70 to 0.90 "High"; 0.50 to 0.70 "Moderate"; 0.30 to 0.50 "Low"; 0.10 to 0.30 “Small”.

9.3 RESULTS AND DISCUSSION

9.3.1 NLC drying process

Right after the drying process, the powders were visually evaluated for appearance and colour. Figure 9.1 shows pictures of the resulting lyophilized products, according to the conditions presented in Table 9.1. Visually, the type and concentration of cryoprotectant did not influence the appearance of the powders, but rather the composition of the lipid matrix of the NLC-based formulation. The powders whose NLC-based formulation is composed of a combination of beeswax and carnauba wax, and sodium dodecyl sulfate (F17) are those that showed a brownish colour and greater formation of lumps. The powders formed by the NLC base formulation containing Compritol® 888 ATO and sodium dodecyl sulfate (F8) presented a whiter and more airy appearance, with the formation of flakes instead of lumps. The NLC-based formulation composed by a combination of beeswax and carnauba wax and *Quillaja* saponin as a surfactant (F15), provided a powder with an intermediate aspect of colour and lumps in comparison with the other samples.

Figure 9.1. Visual appearance of the dried NLC loaded by *L. sidoides* essential oil. The samples differed in the composition of the lipid matrix of the NLC-based formulation and in the type and concentration of cryoprotectant, according Table 9.1.

9.3.2 Descriptive analysis and correlation

We started with an exploratory analysis of the results in conjunction with the Spearman correlation (represented by the letter "g" next to the p-value) performed to test the association between the variables under study and each of the factors in the formula. These data are summarized on Table 9.3. As expected, powder formulations presented larger (from 446.77 to 3565.25 nm) and more polydisperse (between 0.21 and 0.97) particles when compared to the liquid ones (ranging from 246.33 to 702.3 nm and between 0.16 and 0.34 for PI). Both thymol retention and ζ-potential presented more homogeneous values for liquid formulations than for powders.

Table 9.3. Descriptive analysis and Spearman correlation analysis between variables. N=18

Factor	Min/Max	1Q/3Q	Mean / Median	Sd	Tre corr.	Man corr.	[Cryo] corr.
XP (%)	0.32/3.57	1.01/1.53	1.36/1.29	0.79	0.04g (ρ = 0.49)*	0.025g (ρ = -0.53)*	0.533g (ρ = -0.16)
A_w (-)	0.16/0.54	0.18/0.25	0.27/0.23	0.13	0.027g (ρ = -0.52)*	0.016g (ρ = 0.56)*	0.533g (ρ = 0.16)
Particle size – powder (nm)	446.77/ 3565.25	728.09/2277.06	1629.75/ 1423.38	1015.23	0.288g (ρ = 0.26)	0.282g (ρ = -0.27)	0.959g (ρ = -0.01)
Particle size – liquid (nm)	246.33/ 702.3	258.11/ 654.27	404.82/ 289.93	94.67	0.98g (ρ = -0.01)	0.97g (ρ = -0.01)	0.796g (ρ = -0.07)
PI – powder (-)	0.21/0.97	0.46/0.85	0.64/0.59	0.23	0.021g (ρ = -0.54)*	0.029g (ρ = 0.51)*	0.679g (ρ = -0.1)
PI – liquid (-)	0.16/0.34	0.18/0.27	0.23/0.22	0.06	0.664g (ρ = 0.11)	0.814g (ρ = -0.06)	0.418g (ρ = 0.2)
ζ-potential – powder (mV)	-80.87/-33.93	-68.68/ -44.15	-55.02/-49.08	15.77	0.949g (ρ = -0.02)	0.869g (ρ = 0.04)	0.679g (ρ = 0.1)
ζ-potential – liquid (mV)	-65.6/-41.2	-56.38/ -42.76	-49.98/ -48.58	7.44	0.839g (ρ = 0.05)	0.98g (ρ = -0.01)	0.466g (ρ = 0.18)
Thymol retention – powder (%)	39.3/100	46.5/65.6	56.99/ 53.96	14.84	0.231g (ρ = 0.3)	0.333g (ρ = -0.24)	0.374g (ρ = 0.22)
Thymol retention – liquid (%)	60.9/83.6	72.7/78.2	75.36/ 77.67	5.32	0.829g (ρ = 0.05)	0.531g (ρ = -0.16)	0.083g (ρ = -0.42)

Where: XP = moisture content; A_w = water activity; Sd = standard deviation; Tre corr. = trehalose correlation; Man corr. = mannitol correlation; [Cryo] corr. = cryoprotectant concentration correlation. * Statistically significant at $p<0.05$

The results preented in Table 3 show that the parameters significantly affected ($p<0.05$) by the type of cryoprotectant were XP, A_w and PI (for the lyophilized samples). On one hand we noted considerable and negative correlations between trehalose and PI (for powder samples), trehalose and A_w, and between mannitol and XP. In other words, the increase in the concentration of trehalose leads to a powder with less polydisperse particles and less water activity; and increasing the concentration of mannitol reduces the moisture content of the powders. Both results point to excellent and desirable characteristics for the dried powders. On the other hand, mannitol presented moderate or considerable positive correlation both with A_w and PI (for powder samples), as well as trehalose presented positive correlation with XP, that is, the increase of trehalose concentration leads to an increase on XP, as well as increasing the mannitol concentration there is also an increase in both Aw and PI. The other correlations were not considered statistically significant. These correlations can be seen through the scatter plots in Figure 9.2.

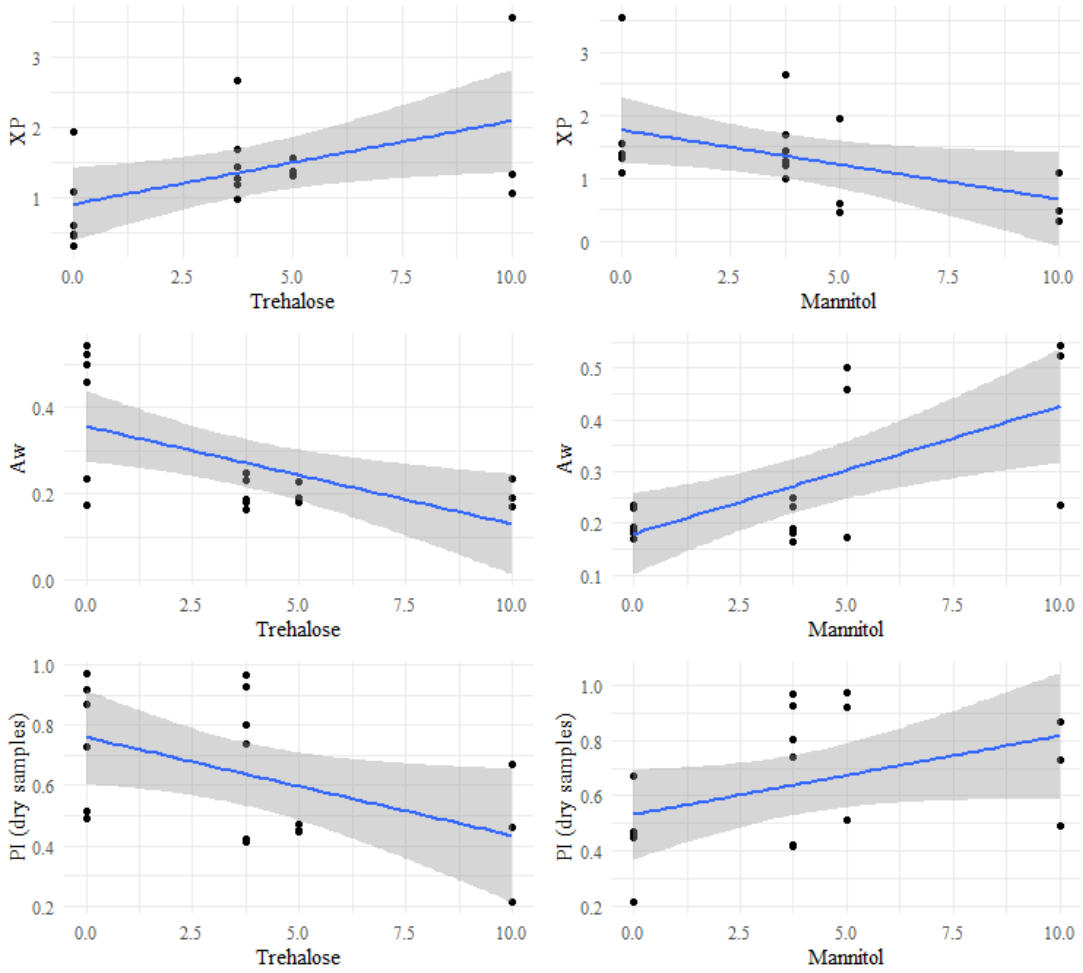

Figure 9.2. Scatter plot of Spearman correlation between type and concentration of cryoprotectant and the analyzed factors. Where: XP = moisture content; A_w = water activity; PI = polydispersity index.

A_w and XP are important parameters related to the chemical and biological stability of powders. Both should be maintained at safe levels to prevent degradation reactions and microorganism growth (13). Values of less than 0.5 for Aw are usually considered safe to avoid microbial growth. XP depends on the composition of the formulation and drying parameters (13,14). From the results (Table 9.3), the Aw and XP of the powders were in the range of 0.16-0.54 and 0.32-3.57 respectively. This wide variation in XP values is more related to the composition of the lipid matrix of NLC-based formulations than the cryoprotectant, as can be seen in Table 9.4.

9.3.3 Association of variables with the NLC base formulation

To evaluate the influence of NLC base formulations we applied the Kruskall-Wallis test (represented by the letter "f" next to the p-value). The results are shown on Table 9.4. Except for Aw, particle size of powder and thymol retention of liquid samples, all other analysed variables were influenced by the NLC base formulation used. Thus, we applied the Dunn test to assess which NLC base formulation had the greatest influence on the evaluated factors. The base formulation composed of a combination of beeswax and carnauba wax, and sodium dodecyl sulfate as surfactant (F17) presented all parameters with higher values than the others, except for PI of the dry powder, which presented a lower value than the other two formulations, and for the thymol retention of powder samples, which was higher than F8 but equal to F15. These relationships can be evaluated by the letters in the boxplots in Figure 9.3.

Despite presenting a powder with more polydisperse particles, the all-natural NLC-based formulation (F15) composed by a combination of beeswax and carnauba wax as solid lipids, and *Quillaja* saponin as surfactant were the ones that presented a powder with better characteristics (XP, particle size, ζ-potential, and thymol retention).

Table 9.4. Comparative analysis using the Kruskall Wallis test to evaluate the influence of different NLC base formulations on the analyzed factors. N=18, with 6 experiments for each NLC base formulation.

Factor	Characteristic	F15	F17	F8	p-value
XP (%)	Min-Max	0.32-1.33	1.09-3.57	0.49-1.43	0.019f *
	1Q-3Q	0.65-1.31	1.59-2.48	0.7-1.31	
	Mean / Median	0.98 / 1.23	2.08 / 1.81	1.0 / 1.03	
	Shapiro-Wilk	0.021	0.594	0.471	
A_w (-)	Min-Max	0.16-0.54	0.17-0.24	0.17-0.52	0.431f
	1Q-3Q	0.19-0.43	0.18-0.23	0.21-0.41	
	Mean / Median	0.3 / 0.21	0.21 / 0.21	0.31 / 0.25	
	Shapiro-Wilk	0.027	0.022	0.132	
Size – powder (nm)	Min-Max	506.12-3302.5	446.77-2266.75	1005.6-3565.25	0.172f
	1Q-3Q	1030.06/2800.75	565.22-1636.17	1196.66-2379.5	
	Mean / Median	1820.65/1505.62	1084.38/643.18	1984.23/1924.75	
	Shapiro-Wilk	0.33	0.023	0.494	
Size – liquid (nm)	Min-Max	280.5-296.93	642.57-702.3	246.33-259.03	<0.001f *
	1Q-3Q	285.85-291.04	658.42-683.82	252.68-257.42	
	Mean / Median	288.87 / 289.93	671.08 / 668.95	254.52 / 256.2	
	Shapiro-Wilk	0.893	0.875	0.251	
PI – powder (-)	Min-Max	0.46-0.93	0.21-0.51	0.45-0.97	0.025f *
	1Q-3Q	0.55-0.91	0.42-0.48	0.69-0.91	
	Mean / Median	0.74 / 0.83	0.42 / 0.44	0.75 / 0.73	
	Shapiro-Wilk	0.04	0.086	0.437	
PI – liquid (-)	Min-Max	0.16-0.21	0.21-0.34	0.18-0.25	0.003f *
	1Q-3Q	0.17-0.18	0.28-0.33	0.21-0.23	
	Mean / Median	0.18 / 0.18	0.3 / 0.31	0.22 / 0.23	
	Shapiro-Wilk	0.407	0.314	0.413	
ζ-potential – powder (mV)	Min-Max	-80.87–68.13	-44.5–33.93	-52.47–46.33	<0.001f *
	1Q-3Q	-80.7–69.69	-43.92–37.39	-51.27–47.22	
	Mean / Median	-75.23 / -76.38	-40.58 / -42.48	-49.26 / -49.08	
	Shapiro-Wilk	0.044	0.126	0.322	
ζ-potential – liquid (mV)	Min-Max	-65.6–46.87	-45.9–41.2	-54.62–42.4	0.002f *
	1Q-3Q	-58.86–57.38	-42.88–42.19	-52.08–48.26	
	Mean / Median	-57.6 / -58.62	-42.86 / -42.48	-49.48 / -49.53	
	Shapiro-Wilk	0.209	0.117	0.774	
Thymol retention – powder (%)	Min-Max	50.35-72.08	53.66-100	39.29-47.37	0.002f *
	1Q-3Q	53.66-59.99	60.86-69.38	41.73-45.41	
	Mean / Median	57.92 / 55.18	69.7 / 68.16	43.34 / 42.91	
	Shapiro-Wilk	50.35-72.08	53.66-100	39.29-47.37	
Thymol retention – liquid (%)	Min-Max	73.48-83.61	60.89-79	69.92-80.28	0.16f
	1Q-3Q	77.65-79.7	69.52-76.55	73.58-78.16	
	Mean / Median	78.46 / 77.89	71.79 / 72.09	75.84 / 76.88	
	Shapiro-Wilk	0.724	0.649	0.701	

Where: XP = moisture content; A_w = water activity. * Statistically significant at $p<0.05$.

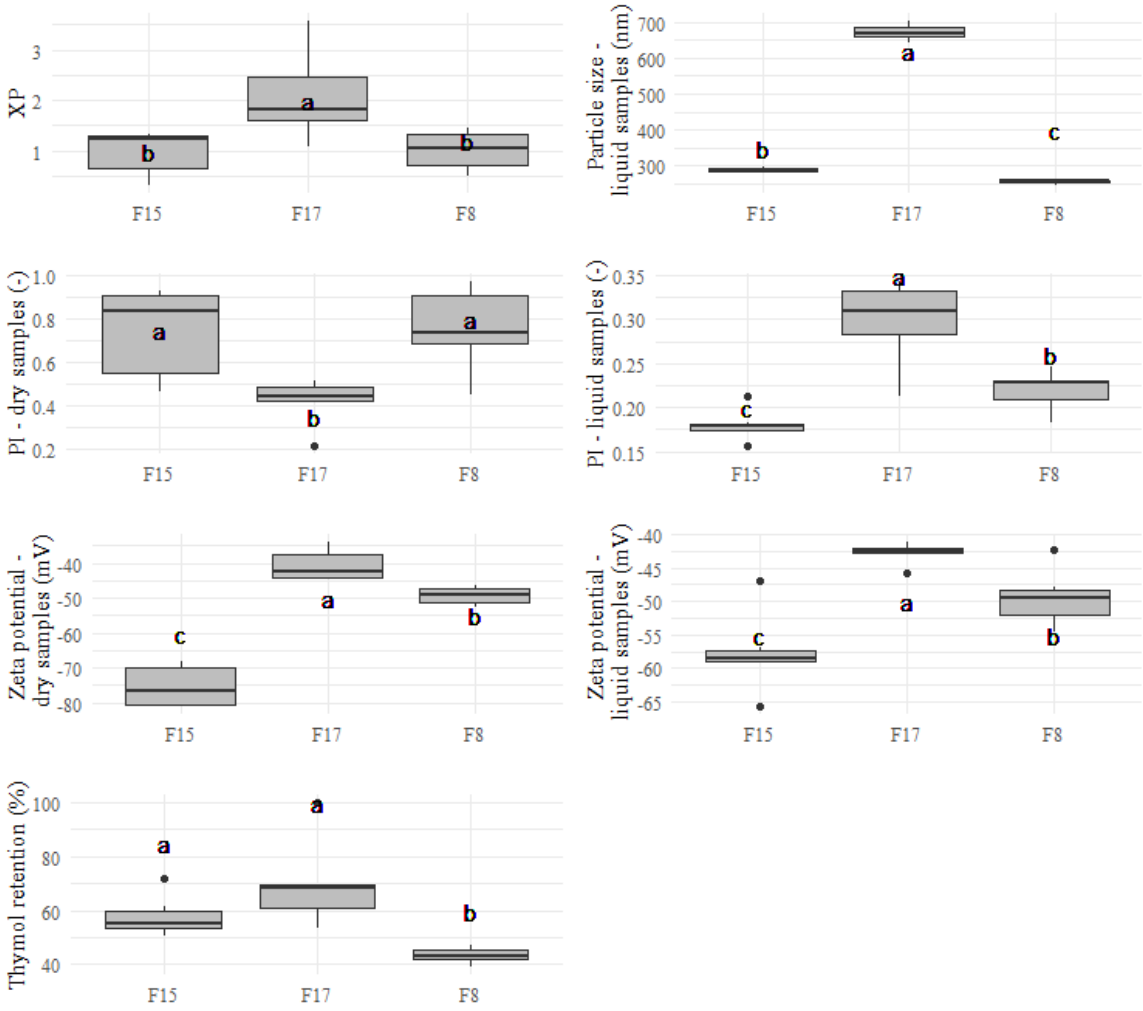

Figure 9.3. Dunn's test boxplots to compare the influence of NLC base formulations on the evaluated factors. F15 is composed by a combination of beeswax and carnauba wax and *Quillaja* saponin as a surfactant, F17 by a combination of beeswax and carnauba wax, with sodium dodecyl sulfate as surfactant, and F8 lipid matrix contains Compritol® 888 ATO and sodium dodecyl sulfate. Where: "a" is higher than "b"; "b" higher than "c".

9.4 CONCLUSIONS

Freeze-drying of lipid nanoparticles is a very complex process that requires a major investigation of the formulation and process conditions. We found that many parameters of the powders are strictly related to the characteristics presented by the initial lipid nanoparticles composition. The Design of Experiment (DoE) proved to be an effective approach to determine the variables exerting more significant effects on the lyophilized product properties. The adjustment of the statistical model demonstrated that freeze-drying is a very suitable technique for dehydration of heat sensitive compounds, as NLCs loaded with essential oil, resulting in a

dry powder with good thymol retention. In addition, low values of water activity and moisture content give to the product high stability to chemical and/or biochemical degradation reactions. These results revealed that product properties of NLC loaded with *Lippia sidoides* EO can be modified through the correct selection of the constituents of the NLC formulation, the cryoprotectant type used and its respective concentration. Additional studies are relevant, such as toxicity assays and evaluation of the promising biological activity of these dry systems, which have remarkable potential to be used in a wide range of products of the cosmetic, pharmaceutical, food or even agricultural industries.

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CHAPTER 10. REMARKS

The growing demand for more natural products has moved academia and industry in search of less toxic and polluting, ecologically correct and sustainable strategies. At the same time, the need to seek new actives able to overcome the resistance presented by various microorganisms is a global health concern. A rational approach to deal with antimicrobial resistance problems requires detailed knowledge of the different biological and non-biological factors that affect the development of resistance. The use of EOs as an antimicrobial agent, alone or in combination with conventional drugs, may enlarge the antimicrobial spectrum and increase the efficiency of conventional drugs; prevent the emergence of resistant mutants; provide high antimicrobial activity than expected from each antimicrobial agent individually. Thus, it could be possible to achieve a good therapeutic effect with relatively small doses compared to an isolated synthetic drug (JAFRI et al., 2020).

In this thesis we proposed the investigation of different strategies to encapsulate essential oil, generating innovative nanostructured systems aiming to be used to combat antimicrobial resistance. Among the proposed systems, the proliposomes, resulting from the drying process of EO-in-cyclodextrins-in-liposomes, formed a product rich in bioactives, stable, but with a complex and expensive production process. However, this proposed system made it possible to encapsulate not only two isolated bioactives, but also two essential oils, composed of numerous bioactives, from different classes, which is quite interesting when using combined therapy to overcome antimicrobial drug resistance.

A clear advantage in using NLC as carrier systems is the composition of their lipid matrix, which allows the use of ingredients generally recognized as safe (GRAS), decreasing the risk of acute and chronic toxicity (SOUTO, 2005). These advantages were endorsed by the NLC produced in this study. They loaded and retained a good amount of *L. sidoides* EO, which was able to fight microorganisms of clinical interest at relatively low and non-toxic doses, including the multidrug-resistant *Candida auris*, an emerging superbug fungus that is a threat to global health.

Freeze-drying technique preserves the stability of nanoparticles and is the best drying process to be used for heat sensitive compounds. The NLC powders obtained presented adequate thymol retention and ready redispersibility in aqueous medium to form nanoparticles with acceptable properties. These characteristics highlight the potential and possibility of using

this product as an active ingredient for various pharmaceutical, cosmetic, personal hygiene or even agricultural preparations.

CHAPTER 11. CONCLUSION

This thesis covered the entire process involved in developing novel lipid-based release systems loaded with essential oils, aiming to be used as a microbial agent effective against resistant microorganisms. We brought the combination between the formulation studies with the technologies and processes involved in producing these nanosystems.

EOs, especially *Lippia sidoides* EO, were successfully encapsulated in both types of nanoparticles studied: EO-in-cyclodextrins-in-liposomes and NLC. Stable systems with good retention of actives were developed, demonstrating good systems to encapsulate essential oils. In particular, NLC has proven to be a simpler technique to encapsulate essential oil.

Data confirm that NLC presents various beneficial properties in a relatively simple formulation. The inherent advantages of NLC, i.e., increased stability for labile compounds, as EOs, ease of manufacture, low toxicity, and high loading capacity, when compared to liposomes, were confirmed in this study. Furthermore, the results demonstrate that the developed systems can maintain the antimicrobial activity of the essential oil and, in some cases, even enhance it. This result is highly promising, especially for the combat of multidrug-resistant microorganisms. Relatively low doses of NLC were sufficient to show fungicidal activity against the emerging superbug multidrug-resistant Candida auris, with relatively low toxicity.

The lyophilization of the NLCs in the presence of cryoprotectants generate a powder with good retention of actives and good physicochemical properties, evidencing a product with high stability against biochemical/chemical degradation. The properties of the product obtained can be engineered through the correct selection of the formulation components.

Results obtained in this thesis provides strong evidence and a relevant scientific basis for using the innovative lipid nanoparticles loaded with essential oils as antimicrobial agents and a toll to combat multidrug-resistant pathogens, with high potential of application in pharmaceutical or personal care products, as well as in other sectors such as in food and agricultural.

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APPENDIX

Book Chapters

1. Iara Baldim, Wanderley P. Oliveira, Rekha Rao, Singh Raghuvir, Sheefali Mahant, Francisco M. Gama, Eliana B. Souto, **Biofate and cellular interactions of lipid nanoparticles**, Editor(s): Prashant Kesharwani, Kamalinder K. Singh, Nanoparticle Therapeutics, Academic Press, Pages 211-246, 2022. ISBN 9780128207574. https://doi.org/10.1016/B978-0-12-820757-4.00015-6

2. Iara Baldim, Adriana M. Ribeiro, João Dias-Ferreira, Wanderley P. Oliveira, Francisco M. Gama, Eliana B. Souto, **Drug Delivery Systems for Targeting Blood Brain Barrier**, Editor(s): Neelesh Kumar Mehra, Arvind Gulbake, Micro- and Nanotechnologies-Based Product Development, CRC Press, Pages 257-268, 2021. ISBN 9781003043164

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Articles Published in Scientific Journals

1. Baldim, I.; Rosa, D.M.; Souza, C.R.F.; Da Ana, R.; Durazzo, A.; Lucarini, M.; Santini, A.; Souto, E.B.; Oliveira, W.P. Factors Affecting the Retention Efficiency and Physicochemical Properties of Spray Dried Lipid Nanoparticles Loaded with *Lippia sidoides* Essential Oil. *Biomolecules* **2020**, 10, 693.

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4. Souza, C.R.F.; Baldim, I.; Bankole, V.O.; da Ana, R.; Durazzo, A.; Lucarini, M.; Cicero, N.; Santini, A.; Souto, E.B.; Oliveira, W.P. Spouted Bed Dried *Rosmarinus officinalis* Extract: A Novel Approach for Physicochemical Properties and Antioxidant Activity. *Agriculture* **2020**, 10, 349.

APPENDIX

Consent of the publishers for the publication of book chapter/articles in the Thesis

1. Baldim, I.; Souza, C.R.F.; Oliveira, W.P. *Encapsulation of essential oils in lipid-based nanosystems*, In: **Phytotechnology: A Sustainable Platform for the Development of Herbal Products**. Chapter 8. Oliveira, W.P. (Ed.). ISBN: 9781003225416, CRC Press. Boca-Raton, FL, 197 – 230, 2021 (DOI: 10.1201/9781003225416-10)

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Number of words	14804
Page numbers	197-230
Number of pages	34
Identifier / First few words	8.1 INTRODUCTION The growing interest of consumers for sustainable "green products" has contributed to the increased interest in using plant-derived products and impacted their production methods.
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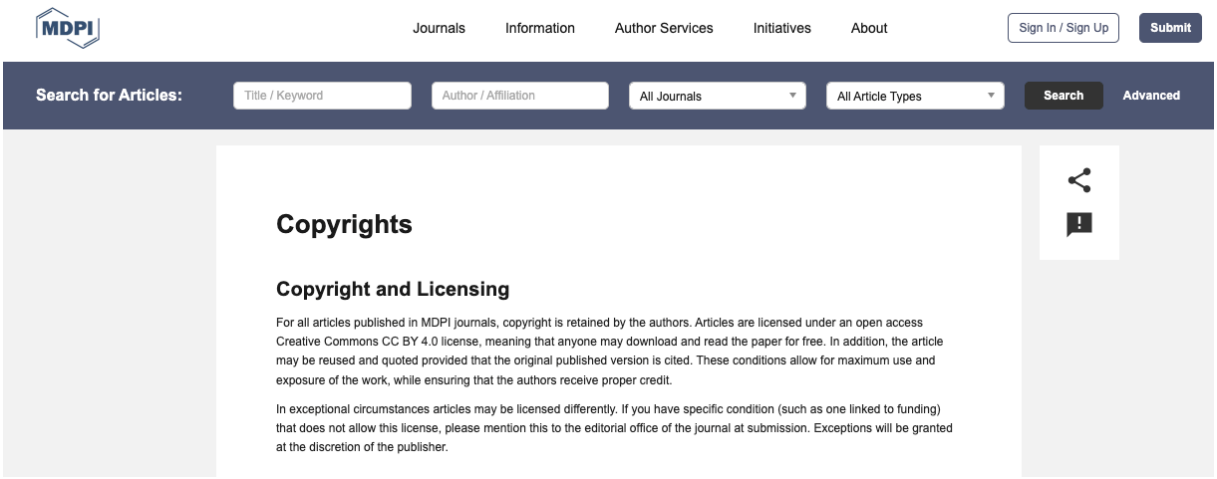
2. Baldim, I.; Oliveira, A.M.; Souto, E.B.; Oliveira, W.P. Cyclodextrins-in-Liposomes: A Promising Delivery System for *Lippia sidoides* and *Syzygium aromaticum* Essential Oils. **Life** 2022, 12, 95. DOI: 10.3390/life12010095



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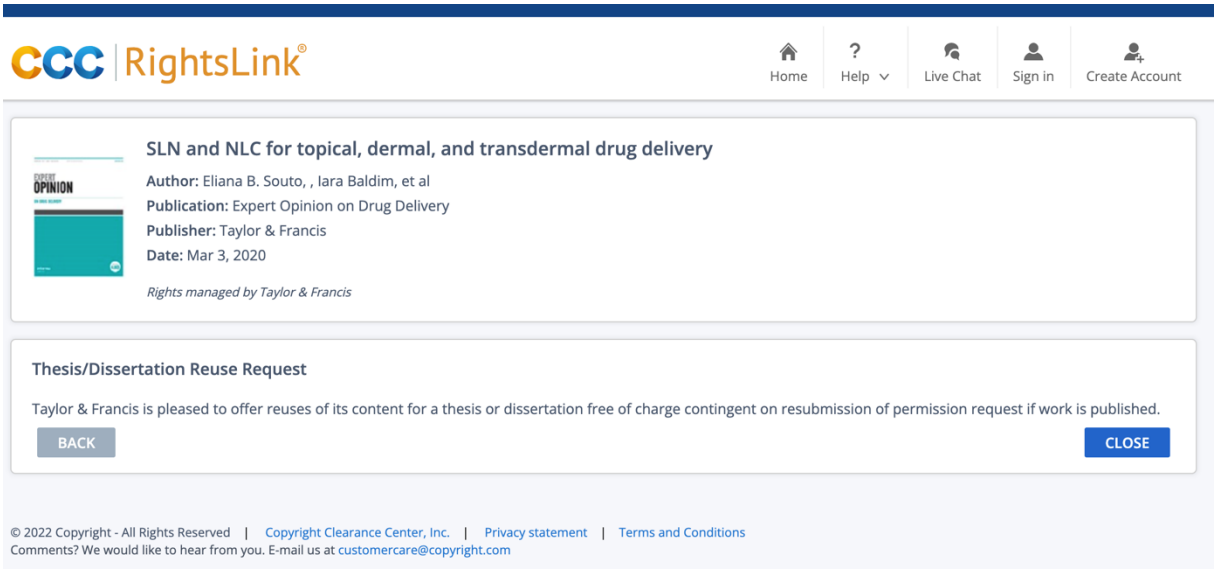

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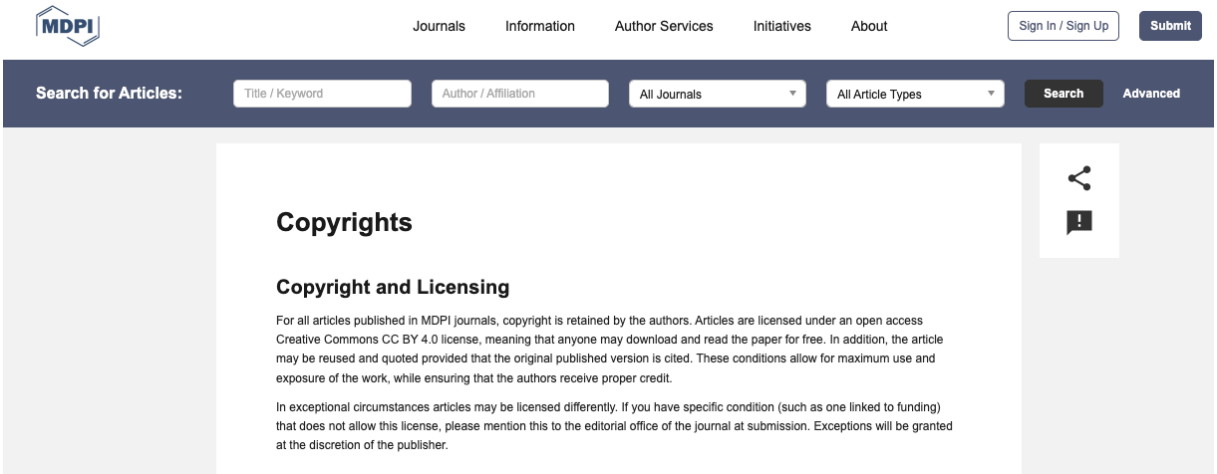


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