

Integrated Master in Bioengineering

The Role of Vine Cane Compounds in Preventing Skin Aging by using Solid Lipid Nanoparticles

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Resumo

Hoje em dia, para atrasar os sinais de envelhecimento, cada vez mais se recorre a tratamentos que utilizam compostos naturais devido às suas propriedades benéficas. Estes compostos são conhecidos pelo seu poder antioxidante, que consegue controlar os níveis de radicais livres nas células, que são os principais responsáveis pelo envelhecimento. No entanto, estes compostos apresentam uma baixa biodisponibilidade e uma grande dificuldade em atravessar a barreira superficial da pele. Novas abordagens estão a ser desenvolvidas para ultrapassar estes problemas, sendo que uma delas consiste em encapsular estes princípios ativos em nanopartículas lipídicas.

Neste projeto, três compostos naturais (epigallocatechin gallate (EGCG), resveratrol e miricetina) presentes no extrato da cana do vinho foram encapsulados em nanopartículas lipídicas sólidas para criar uma formulação antienvelhecimento para uso cutâneo. Para selecionar os parâmetros de produção ideais e os excipientes usados, um processo de otimização foi realizado antes da produção das nanopartículas. O lípido selecionado foi o compritol® HD5 ATO e o emulsificante foi o pluronic-F127, que permitiram obter nanopartículas com aproximadamente 200 nm. Um estudo de estabilidade demonstrou que após 30 dias, as nanopartículas com composto mantiveram aproximadamente as mesmas características do dia seguinte à sua produção. Para além disso, estas também foram caracterizadas por microscopia de transmissão eletrónica, tendo-se observado um formato arredondado e um tamanho próximo do esperado. As eficiências de encapsulação foram acima de $(90 \pm 5)\%$; os valores da capacidade de carga foram próximos do valor teórico e os perfis de libertação *in vitro* demonstraram uma libertação prolongada. A atividade antioxidante foi testada e as misturas de EGCG e resveratrol e dos três compostos juntos permitiram obter um efeito sinérgico, demonstrando-se um resultado bastante promissor. Para além disso, estudou-se a estabilidade das nanopartículas a longo prazo, no qual se demonstraram estáveis e promissoras. Por fim, foram realizados testes de citotoxicidade *in vitro*, utilizando queratinócitos humanos imortalizados. A concentração de 50 µg/mL foi a escolhida para testar a atividade antioxidante, uma vez que nesta concentração não houve citotoxicidade significativa. De seguida, avaliou-se a atividade antioxidante das formulações contra o stress oxidativo, sendo que a mistura EGCG e resveratrol apresentou o maior efeito protetor das células. Concluindo, a encapsulação de EGCG, resveratrol e miricetina em nanopartículas lipídicas demonstrou ser uma forma viável de transportar estes compostos e melhorar sua biodisponibilidade, podendo promover a sua libertação prolongada na pele e tirando partido suas propriedades benéficas para retardar o processo de envelhecimento.

Palavras chave: envelhecimento da pele, radicais livres, compostos naturais, nanopartículas lipídicas sólidas.

Abstract

Aging is an inevitable process and has become a concerning topic among the population. Nowadays, people want to delay the signs of aging and increasingly rely on treatments that use natural compounds due to their beneficial properties. These natural compounds (NC) are known for their high antioxidant activity that controls the levels of free radicals in cells, which are the primary cause of aging. Despite this, these compounds present a low bioavailability and a great difficulty in crossing the skin's barriers. New approaches are being developed to overcome these problems and one of them is to encapsulate the NC in lipid nanoparticles, such as solid lipid nanoparticles (SLN).

In this project, three natural compounds (epigallocatechin gallate (EGCG), resveratrol and myricetin) present in the vine cane extract were encapsulated in solid lipid nanoparticles to create an anti-aging formulation for cutaneous use. To select the optimum process parameters and the excipients used to produce the nanoparticles, an optimization study was carried out before nanoparticle production. The selected lipid was Compritol® HD5 ATO and the emulsifier was pluronic-F127, which led to nanoparticles with approximately 200 nm. The nanoparticles loaded with NC maintained approximately the same initial characteristics after a stability study performed for 30 days. In addition, the nanoparticles were also characterized using transmission electron microscopy (TEM), being observed a rounded shape and a size close to the expected. The encapsulation efficiencies were over $(90 \pm 5)\%$; the loading capacity values were close to the theoretical value and the *in vitro* release profiles showed a prolonged release. Furthermore, the antioxidant activity was tested and the mixture of EGCG and resveratrol and the three compounds together provided a synergic effect, demonstrating a very promising result. An accelerated stability test was made, and the nanoparticles revealed a long-term stability. Finally, *in vitro* cytotoxicity tests were performed, using immortalized human keratinocytes. The 50 µg/mL concentration of NC-loaded nanoparticles was the one chosen to perform the antioxidant assay since there was no significant cytotoxicity at this concentration. Then, the antioxidant activity of the formulations against t-BHP-induced oxidative stress was evaluated, and the mixture of EGCG and resveratrol was the one with the highest protection against induced oxidative stress. Concluding, encapsulating EGCG, resveratrol and myricetin in lipid nanoparticles demonstrated to be an effective method for carrying these antioxidants and improving their bioavailability, delivering them slowly into the skin layers and, taking advantage of their beneficial properties to delay the aging process.

Keywords: skin aging, free radicals, natural compounds, solid lipid nanoparticles.

Declaration

Declara, sob compromisso de honra, que este trabalho é original e que todas as contribuições não originais foram devidamente referenciadas com identificação da fonte.

24/09/2021

Adriana Pereira.....

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Abbreviations, Acronyms and Symbols

List of abbreviations and acronyms

Abs	Absorbance
C	Concentration
CAGR	Compound Annual Growth Rate
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic acid
DCFH-DA	2',7'-Dichlorofluorescein diacetate
DCF	2',7'-dichlorofluorescein
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
DMEM	Dulbecco's Modified Eagle's Medium
ELS	Electrophoretic Light Scattering
EE	Entrapment Efficiency
EGCG	Epigallocatechin gallate
EDTA	Ethylenediamine tetraacetic acid
FBS	Fetal Bovine Serum
HaCaT	Immortalized Human Cells
HBSS	Hanks' Balanced Salt Solution
HPH	High-Pressure Homogenization
HSH	High-Shear Homogenization
LC	Loading Capacity
LN	Lipid Nanoparticle
MMP	matrix metalloproteinase
MW	Molecular Weight
M	Myricetin
NC	Natural Compound
NLC	Nanostructured Lipid Carriers
NP	Nanoparticle
NR	Neutral red
NADPH	Nicotinamide adenine dinucleotide phosphate
PdI	Polydispersity Index
PBS	Phosphate-Buffered Saline

R	Resveratrol
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
REZ	Resazurin
S	Sonication
SC	Stratum Corneum
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SLN	Solid Lipid Nanoparticles
TEM	Transmission Electron Microscopy
t-BHP	Tert-Butyl Hydroperoxide
UT	Ultra-Turrax T25
UV	Ultraviolet
US	Ultrasonication
UV-VIS	Ultraviolet-Visible
ZP	Zeta Potential

List of symbols

°C	Celsius degree
Da	Dalton
g	Gram
h	Hour
J	Joule
K	Kelvin
L	Liter
μL	Microliters
μm	Micrometers
μM	Micromolar
mL	Mililiter
mg	Miligram
min	Minute
M	Molar
nm	Nanometers
rpm	Rotations Per Minute
s	Second

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Chapter 1: Introduction

1.1 Motivation

Nowadays, the world's population is growing older, and the consequences of aging are gaining special attention. It is believed that in 2050 the world's population over 60 years will almost double from 12% to 22% [1]. People want to improve their quality of life until later years in life and skin aging is one of the central aspects, not only because the appearance of aged skin is obvious, but also because it reflects a person's health [2]. So, with the rising awareness about aging, the pharmaceutical and cosmetic industries are investing even more in the production of anti-aging products [3]. The global cosmeceutical market reached over 37 billions of dollars in 2018 and it is expected to reach over 73 billions of dollars in 2026, exhibiting a compound annual growth rate (CAGR) of 8,9% [4].

The skin provides protection against the external environment. So, an aged skin becomes more fragile and loses some physiological functions, that can lead to an increased risk in developing skin disorders [5]. The aging process can occur due to intrinsic factors, which are genetically determined, or extrinsic factors, which are related to human behavior such as excessive sun exposure or unhealthy habits like smoking [6]. In both aging processes, free radicals are formed, and their uncontrolled production potentiates oxidative stress and can lead to cellular damage and cell apoptosis [7]. In recent years, natural compounds are being investigated by the cosmeceutical industry due to their ability to scavenge free radicals and, therefore, to control the cellular redox imbalance [8]. Furthermore, people are demanding for more ecological and natural products, so the industry is creating more formulations with natural compounds [9]. Polyphenols have not only antioxidant activity, but they are also known for their anti-inflammatory and anti-carcinogenic properties. However, these natural ingredients present a very low bioavailability, hampering their ability to cross the skin barrier and penetrate into deeper skin layers [10].

The skin provides the largest interface for pharmaceutical administration, but also acts as barrier. The skin barrier is known as *stratum corneum* (SC) and constitutes a physical and chemical obstacle for external compounds [11]. The passage of active compounds through this lipidic barrier depends on the drug properties, its physicochemical characteristics such as size, surface charge, lipophilicity, but also depends on the skin type and its health conditions [12]. In the last years, nanotechnology made an important contribution to enhance the permeability of different types of drugs throughout the skin layers. Nanocarriers have the ability to increase the drug stability and protect the compound

against aggressive agents, they enable a controlled drug release, increase the drug bioavailability and are considered non-toxic and biocompatible [13]. There are several types of nanoparticles such as polymeric nanoparticles, magnetic nanoparticles, lipidic nanoparticles, among others. The last ones are particularly interesting in the cosmeceutical point of view.

The lipidic nanoparticles present important properties for topical applications: they are biocompatible with the SC since its constitution is lipidic, exhibit low toxicity and cytotoxicity, they can encapsulate different types of active compounds, having a long-term stability, easy scaled-up and low-cost production. They have a wide range of particle sizes, good encapsulation efficiencies, are suitable for different types of formulations and have a broad application spectrum. Moreover, the use of lipid nanoparticles promotes skin hydration as it can form an occlusive film in the SC, preventing the loss of transepidermal water and forming a reinforcement to the lipid barrier against external agents [14, 15].

Lipid nanoparticles are divided in two types: solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC). The first type is only constituted by solid lipids, where the drug is usually dissolved, surrounded by a layer of emulsifier that stabilizes the emulsion and decreases surface tensions [16]. The latter type, instead of having only solid lipids in the core, also have liquid lipids in the lipidic matrix [17].

In the present research work, SLN were developed to encapsulate three different natural compounds (NC): epigallocatechin gallate, resveratrol and myricetin. These bioactive compounds are present in the vine cane extract, which is a very abundant by-product of winemaking in Portugal. They are known for their high antioxidant, anti-inflammatory and anti-carcinogenic activity, capable of scavenging free radicals, reduce oxidative stress and, therefore, prevent skin aging. In the future, the aim is to encapsulate the total extract in lipid nanoparticles to give this by-product a more profitable finality.

1.2 Main Objectives

The main objective of this dissertation was to produce a cosmetic formulation containing lipidic nanoparticles (SLN) as nanocarriers to transport through the skin layers three well-known natural compounds: epigallocatechin gallate, resveratrol and myricetin. The goal of this formulation is to prevent free radical activity and oxidative stress, that can lead to premature aging of the skin.

First, an optimization process was developed to assess most suitable excipients to formulate the nanoparticles. The final formulation was characterized in terms of size, surface charge and polydispersity index over 30 days. Furthermore, the encapsulation

efficiency and particle morphology were also evaluated, and accelerated stability studies and *in vitro* release profiles were conducted. Additionally, the natural compound's antioxidant activity, alone and in mixtures, was studied to evaluate the occurrence of synergic activities. The natural compounds and the nanoparticles were also tested *in vitro* in immortalized human keratinocytes to evaluate the cytotoxicity of the formulations and their antioxidant activity. This last assay was performed to assess the ability of the formulations to protect the cells against induced oxidative stress.

1.3 Thesis Organization

In the present chapter, an introduction to the topic is presented, which includes a brief description of the project, the main objectives of the developed work, as well as the thesis structure.

The chapter 2 is the state of the art section, where theoretical knowledge essential to the understanding of the project is presented: the skin structure and aging, the natural compounds studied (epigallocatechin gallate, resveratrol and myricetin) as a potential cosmetic treatment, the skin barrier, and the role of nanoparticles to overcome the poor bioavailability of the natural compounds throughout the skin layers. Furthermore, relevant studies were also analyzed to understand the current developments in the nanotechnology area.

The materials and methods used in this work are introduced in chapter 3, where the experimental procedures are detailed. In chapter 4, the results are presented and discussed. Chapter 5 contains the conclusions and future perspectives that should be carried out to improve the present research.

1.4 Thesis outputs

The project developed in this Master Dissertation was presented at IJUP'21- 14th Meeting of Young Researchers of University of Porto: Pereira, Adriana J.; Gonçalves, Catarina; Loureiro, Joana; Silva, Ana C.; Walgode, Ana; Ramalho, Maria J.; Pereira, Maria do Carmo; 2021. Using Nanotechnology to Prevent Skin Aging. Conference: at IJUP'21- 14th Meeting of Young Researchers of University of Porto, 2021, online, Porto, Portugal (presentation).

Furthermore, a research article based on the work developed in this Master Thesis was submitted in the scientific journal *Pharmaceutics* under the title: "Solid lipid nanoparticles comprising vine cane compounds to prevent skin aging".

Chapter 2: State of the art

2.1 Skin and Skin Aging

Skin is known for being the largest organ of the human body in both weight and extension. This organ is characterized for being the external sensor of the human body and has several functions. Many support systems of the body are incorporated into the skin, as this organ supports blood vessels, nerve extremities, muscles, among others [18]. The skin's main function is to protect the body by acting as a physical barrier against many physical, chemical, and microbial agents. Additionally, the skin also protects against UV radiation through the production of melanin and has sensory receptors which detect touch stimulus, pressure, vibration, pain and temperature variations [19]. It has also a thermoregulation function, since several features of the skin are involved in maintaining the body temperature and keeping it in homeostasis, such as sweat glands, hair and adipose tissue [20]. Furthermore, the skin receives numerous endocrine functions, being a target and source of hormones and peptides that were thought to belong only to the central nervous system [21, 22].

The skin of an adult has an average area of 2 m², with a thickness between 0,5 mm and 4 mm, depending on the body region, and weighs about 5 kg. It is divided into three types of tissue: epidermis, dermis and hypodermis [20].

Epidermis is the first layer in contact with the external environment and has a thickness that can vary between 75 and 150 µm throughout the body, except on the palms of the feet and hands, which can reach 600 µm in thickness. This non-vascularized layer is constituted of five strata composed mainly of keratin-producing cells called keratinocytes, melanocytes that are responsible for the production of melanin that gives color to the skin, Langerhans cells which have an immunologic role and Merkel cells that serve a sensory function [22, 23]. In the epidermis, cells are continuously proliferating in the bottom layer (*stratum basale*) and moving up through the different strata. Each strata contains one of the differentiation stages of the keratinocytes. This movement occurs due to the skin vulnerability to external damaging effects, and it takes around 2-4 weeks to be completed, enabling the damaged cells to be replaced by new ones. The epidermis layers are the *stratum basale*, the deepest portion, *stratum spinosum*, *stratum granulosum*, *stratum lucidum* and the outermost layer is called *stratum corneum* (SC) [20]. This last layer is constituted by non-viable keratinocytes (corneocytes) that expel defensive proteins, becoming the first immune response from the body to the external environment, and

therefore preventing the entry of external compounds that can compromise the skin's viability [23]. Additionally, the SC also act as a barrier, preventing the loss of water from the body by the epidermis. The corneocytes are embedded in a lipid matrix consisting of three types of lipids: cholesterol, free fatty acids and ceramides [24].

Dermis is the second more internal layer and is where the main centers of skin activity are located. It has about 2 mm thick, but can reach 4 mm. Although SC is the layer that is in contact with the outside, it is the dermis that really enables the function of pathogen protection that the body needs. The dermis is vascularized and contains fibroblasts, that produce collagen and elastin that support and give structure to the skin. It is in this layer that the secretory glands, nerves and hair follicles are located.

Finally, the hypodermis consists of adipose tissue and sweat glands that connects the dermis to the structural components of the human body [22]. The connective fatty tissue is responsible for vitamin D and triglycerides production [20]. Figure 1 is an illustrative image of the different skin layers.

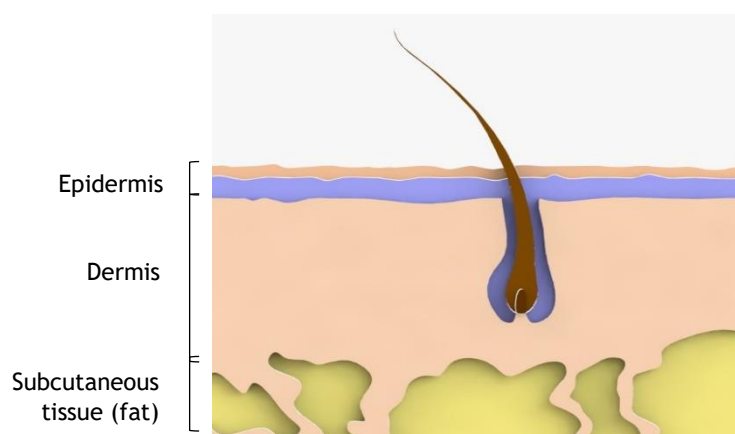


Figure 1. Representative image of the different skin layers.

Skin aging can occur due to intrinsic and extrinsic factors. The first process is inevitable, varies from individual to individual and is genetically determined. There are many theories trying to explain the intrinsic aging process, but the most studied is related to DNA damage and the repairing process [25]. For example, the decrease in telomere length and the presence of telomerase, the reverse transcriptase responsible for telomeres elongation, are DNA-related factors that influence intrinsic cell aging [26]. Telomeres are structures found at the end of eukaryotic chromosomes and have been studied due to their relationship with cell aging. They are repetitive and non-codifying DNA sequences that stabilize and protect the chromosomes from degrading or fusion, preserving genetic information throughout cell replication processes [27]. In each cell division cycle, telomeres erode and lose parts of their DNA sequence as a consequence of incomplete DNA synthesis

and oxidative damage, leading to loss of the cell's ability to replicate and, consequently, loss of proper cell function and, therefore, cell senescence or cell apoptosis. So, telomeres are biological clocks that reflect the number of cell divisions, informing the cells if they are young or old [28]. This shortening process can be accelerated or delayed depending on the existence of certain proteins or the presence of telomere-damaging radicals. There are several proteins associated to these structures that are responsible for their size regulation and protection from DNA repairing systems. For example, telomerase has the function of adding the telomeric sequences at the end of the telomeres, stabilizing and elongating them [29]. This enzyme is expressed in 90% of all human tumors, but it is not present in many somatic tissues, leading to associate its absence or deficiency with telomeres length decrease, cell death and cell aging [30]. Epidermis is one of the few tissues to express telomerase, but this enzyme is almost undetectable in the dermis [31].

Telomeres in skin cells, are more susceptible to accelerated shortening due to both replication processes and DNA-damaging agents such as free radicals. A free radical is a molecular species capable of living independently that contains an unpaired electron in an atomic orbital. They are known for being unstable and highly reactive, since they can behave as oxidants, if they donate electrons, or as reductants if they accept electrons from other molecules [32]. It is widely believed that intrinsic aging is primarily caused by oxidative damage induced by free radicals and reactive oxygen species (ROS), which are reactive molecules with an oxygen atom [25]. These species are capable of reacting with DNA components, proteins and lipids, attacking nitrogenous bases and, therefore, leading to DNA mutations that can compromise the cell viability. They also can induce protein aggregation and lipid peroxidation, among other harmful processes that can lead to cell damage, homeostatic disruption and, finally, premature cell aging and cell apoptosis [33]. Despite this, free radicals are also a vital part of the cell metabolism, since humans are aerobes who need oxygen due to its role in the electron transport chains and other enzyme systems. Therefore, the cells need to create a homeostasis environment in which they find a balance between ROS production and antioxidative defense [25].

Over the years, ROS production levels increase in skin cells and the ability to repair damaged DNA decreases. So, it is crucial to control the ROS levels in order to prevent a premature aged skin, by providing antioxidant molecules to cells so they suffer oxidation instead of the cell components. Skin aged by intrinsic factors is smooth, without blemishes and with pronounced expression lines. Internally, there is a flattening of the epidermal layer, a decrease in the thickness of the dermis becoming less vascularized, a production of fragmented elastic fibers, among other events [26]. In Figure 2, there are presented some internal skin modifications due to intrinsic aging.

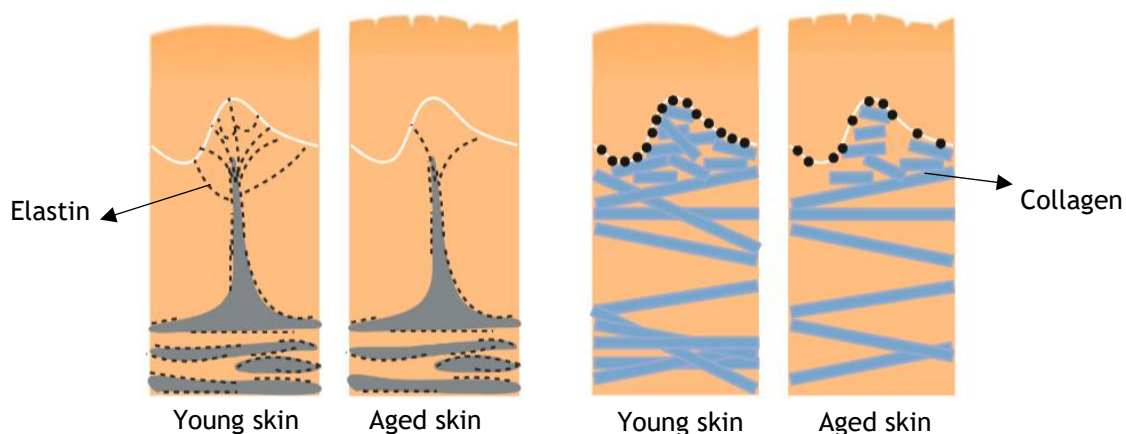


Figure 2. Representation of some internal modifications of the skin due to intrinsic aging.

As for the extrinsic aging process, it is caused by factors controlled by humans, such as smoking, excessive consumption of alcoholic beverages, poor nutrition and chronic sun exposure. This last factor is what causes the most skin deterioration over time, with 80% of facial skin aging being originated due to prolonged sun exposure [26] [34]. In this case, the telomeres shortening is not directly related to extrinsic aging, since, in a study presented by Sugimoto *et al.* (2006), there was almost no evident differences in the length of telomeres in cells before and after being exposed to sunlight [35]. As with intrinsic aging, the direct production of reactive oxygen species or free radicals due to sun exposure or other factors, contributes to the aging process. These free radicals have the power to degrade collagen, increase the thickness of the elastin and its folding, and induce proteins that degrade the cell matrix [18]. In addition, the oxidative stress caused by these free radicals acts on the mitochondrial DNA and eliminates a specific 4977 base pair of that DNA. This process, called common deletion, happens 10 times more in skin damaged by sun exposure in comparison with sun-protected skin, leading to associate this process as a molecular marker for photoaging [36].

A skin aged by extrinsic factors is characterized by having spots of hyperpigmentation or depigmentation, low elasticity and wrinkles. Internally, there is an atrophy of elastin fibers and a decrease in collagen fibers [26]. In Figure 3, there are presented some internal skin modifications due to extrinsic aging.

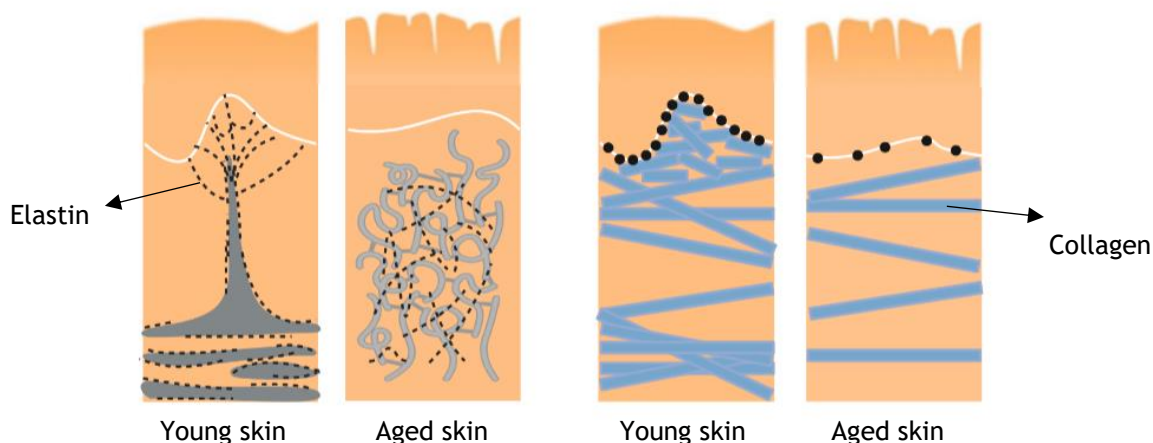


Figure 3. Representation of some internal modifications of the skin due to extrinsic aging.

2.2 Natural Compounds

A natural compound is a molecule that can be found in plants, fungi, microorganisms and other living bodies. They can be classified as secondary metabolites, since they are not essential for the living organism, in opposition to the primary metabolites that play a major role in the organism metabolism such as lipids, amino acids, among others [37]. Natural compounds are considered a successful source of drugs for the treatment of several diseases and illnesses, such as neurodegenerative diseases. The use of these compounds dates ancient times when medicinal plants were already used for the treatment of many human diseases. Due to their unique antioxidant and anti-inflammatory properties, natural compounds have been gaining interest in the scientific community and the pharmaceutical industry [38]. The development of new technologies related to chemistry and biology, is allowing scientists to understand more easily the biological effects of natural compounds on the human body, leading to the development of new therapies for diseases such as cancer and dementia [39]. Nowadays, people are trying to pursue a more natural lifestyle and, therefore, they are seeking for more natural treatments, so it is expected an increase in the use of natural compounds.

Regarding its uses in the cosmeceutical industry, natural compounds are being incorporated in creams, serums and hair products for their ability to scavenge free radicals and, therefore, protect the cell components from being oxidized [40]. For example, in an inflammatory reaction in the skin, the *stratum corneum*'s function will be compromised and, therefore, there will be an increase in the penetration of external molecules into the skin. This inflammatory reaction will lead to the occurrence of oxidative reactions induced by free radicals released by immune cells such as mast cells. Furthermore, deeper in the dermis, proteolytic enzymes will eliminate not only bacteria but also do a significant damage in proteins of the extracellular matrix such as collagen and elastin [41]. As mentioned

before, oxidative stress plays a major role in the intrinsic and extrinsic skin aging process, in the inflammatory response of the body and in the development of several skin diseases such as skin cancer, so the cosmeceutical industry is currently implementing natural compounds in their products due to their antioxidant properties that can prevent many undesirable health conditions.

In this project, three natural compounds were used: epigallocatechin gallate (EGCG), resveratrol and myricetin. These compounds were chosen due to its presence in the vine cane extract. Normally, after the grapes are harvested, the canes are burned or used as a natural fertilizer. To make vine cane more profitable, new applications are currently being studied, such as its use as a source of bioactive compounds [42]. This by-product of winemaking is very abundant in Portugal, which is one of the countries with the largest wine production in the world [43]. In the future, the goal will be to encapsulate the complete extract.

2.2.1 Portuguese vine cane extract

The main phenolic compounds present in the vine cane extract are divided in six groups: flavanols (catechin and epicatechin), phenolic acids (gallic, ellagic, vanillic acids, among others), stilbenes (resveratrol); flavanones (naringin, naringenin and pinocembrin), flavonols (rutin, myricetin, kaempferol and quercetin) and condensed tannins [43]. The natural compounds chosen for this project are usually the most abundant compounds in the vine cane extract [42, 44]. If these compounds are able to be encapsulated in the same nanoparticles, then the encapsulation of the total extract will be successfully accomplished in the future.

2.2.1.1 Epigallocatechin gallate (EGCG)

Epigallocatechin gallate (EGCG) is a polyphenol present in green tea (*Camelia sinensis*) and grapes, acting as a secondary metabolite. This compound is the most abundant in this type of tea and has a high antioxidant, anti-inflammatory and anti-carcinogenic activity [45, 46]. EGCG is water-soluble and is sparingly soluble in hydrophobic mediums. Due to its hydrophilic nature, EGCG is known for being poorly absorbed by cells [47]. Extract of green tea were found to have high scavenger activity when in contact with hydrogen peroxide, and a significantly inhibition of DNA damage induced by UV (ultraviolet) radiation, which is particularly relevant to anti-aging effects and a lower risk of carcinogenesis [48]. Polyphenols are known for their diverse applications in the treatment of numerous diseases such as cancer, obesity, diabetes, cardiovascular and neurodegenerative diseases, among others [49]. EGCG is one of the most studied polyphenols and is known for helping to control oxidation and inflammatory processes at different cellular levels such as free radical

scavenging and inhibition of cyclooxygenase, lipoxygenase and nitric oxide synthase [50, 51]. Moreover, this compound has been shown to prevent the production of free radicals in skin cells where topical EGCG solutions were applied before sun exposure, showing its potential as an anti-aging agent [46, 52]. Furthermore, EGCG increases the antioxidant activity of other enzymes, acting as a great tool to prevent free radicals formation [53]. Its chemical structure is shown in Figure 4.

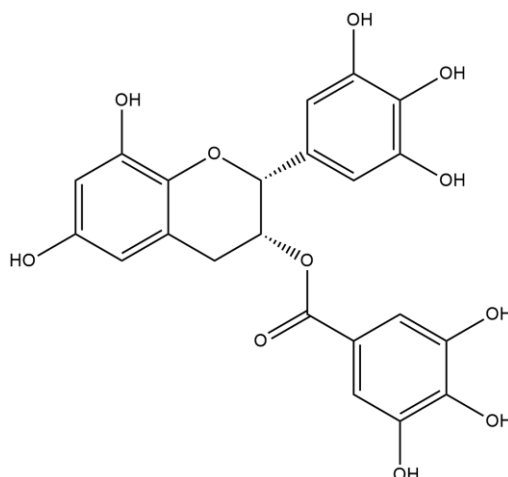


Figure 4. EGCG chemical structure.

2.2.1.2 Resveratrol

Resveratrol is a stilbene that is present in more than 70 species of plants, in blackberries, peanuts, grapes, rhubarb, among others. This natural compound is known for its antioxidant potential, since it has a dual antioxidant capacity, it eliminates free radicals directly and increases the cellular expression of other antioxidant enzymes [54]. Resveratrol is present in nature in two isomers, with trans-resveratrol being more stable and biologically active than the cis-resveratrol isomer. However, resveratrol presents a low bioavailability in *in vivo* applications due to its poor aqueous solubility and limited stability under certain conditions [55]. This compound is also characterized by having anti-carcinogenic, anti-inflammatory and anti-obesity properties [56]. Resveratrol has been shown in several studies to be an inhibitor of lipid peroxidation, ultraviolet light-induced lipid peroxidation and NADPH⁺ molecules, as well as an efficient peroxide radical removal agent [57]. For example, the application of resveratrol in mouse skin before UVB exposure resulted in a significant decrease in lipid peroxidation and edema formation, inflammatory enzymes and enzymes associated with tumor promotion [58]. Furthermore, resveratrol has the ability to lower levels of reactive oxygen species in skin cells exposed to UVA radiation [59]. These strong and unique properties are contributing to a more extensive use of resveratrol in cosmetic

products to prevent skin aging. In Figure 5 the molecular structure of resveratrol is represented.

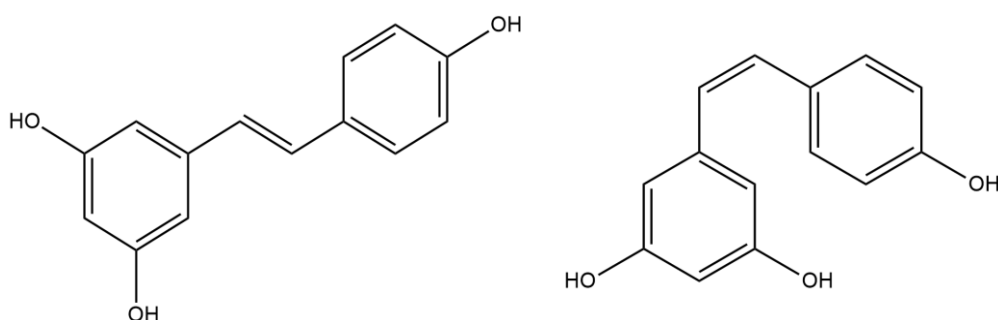


Figure 5. Resveratrol chemical structure. In the left is presented the trans-resveratrol and the right structure is the cis-resveratrol.

2.2.1.3 Myricetin

Myricetin is a flavonoid present in many foods and drinks such as onions, grapes, berries, red wine, among others. This compound is known for its antioxidant, anti-tumor, anti-inflammatory, antibacterial, antiviral and anti-obesity properties [60, 61]. Myricetin is a lipophilic molecule with low water solubility and is susceptible to degradation if exposed to high temperatures and to solutions with high pH values [62]. Flavonoids such as myricetin, are known for protecting the cell against oxidative stress in different paths, for example, in a direct way by acting as antioxidants and neutralizing ROS by donating hydrogen ions, and in an indirect way by inducing antioxidant enzymes or modulating cell signaling pathways [63, 64]. Jung *et al.* (2010), suggested that myricetin suppresses some enzymes of the matrix metalloproteinase (MMP) family [61]. This family is induced by UV radiation, and expresses some enzymes that degrade collagen and other proteins in the extracellular matrix, compromising the viability of the dermal layer and contributing to the aged aspect of the skin [65]. In Figure 6, the molecular structure of myricetin is represented.

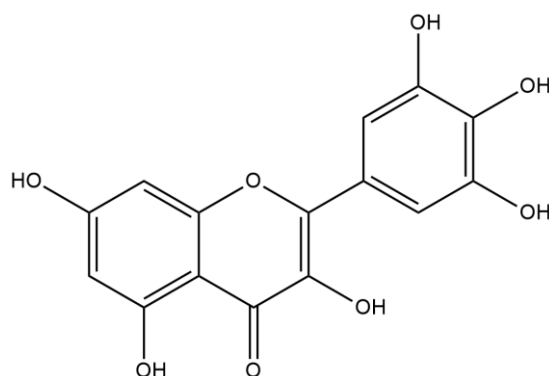


Figure 6. Representation of the molecular structure of myricetin.

2.3 The Skin Barrier

As previously mentioned, the skin's main function is to protect the body from external agents and to prevent water loss through the epidermis. In order to reach the different layers of the skin, the external compounds have to cross the most external protection barrier, the *stratum corneum*. Usually, the SC is composed by 10 to 25 layers of corneocytes rich in keratin and surrounded by intercellular lipids such as ceramides, cholesterol and free fatty acids. These lipids are the most important permeability barrier against dehydration and external agents. The SC structure is compared to a wall of bricks and mortar, where the bricks correspond to the corneocytes and the lipids to the mortar [66]. In Figure 7, it is possible to observe a representative image of the “bricks in mortar” model.

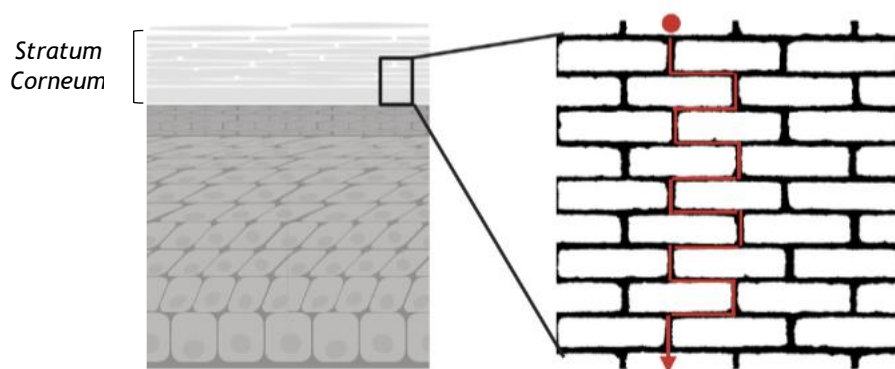


Figure 7. Representative scheme of the "bricks and mortar" model.

Depending on the aim of the product there are three main types of formulations. Firstly, if the aim is to have the active compound only on the skin's surface, for example insect repellents, makeup or disinfection products, the formulation is called epidermal formulation. Secondly, if the objective is to reach deeper regions of the skin such as viable epidermis and dermis, then the formulation is called endodermal or diadermal formulation. In this type, the formulation is not supposed to enter in the systemic circulation, since if it occurs can lead to unwanted side effects. Finally, if the active compound needs to reach the blood circulation, the formulation is called transdermal formulation [12, 14]. Furthermore, the effectiveness of the drug administration is controlled by different factors: the drug properties, for example its lipophilicity and size; the skin type and its condition and the formulation features, for example its rheological properties [12].

Active compounds are able to penetrate the skin through two different routes: the transepidermal route and the pores route. The first one is divided in transcellular via, when the drug crosses the skin through both the lipidic structures of the SC and dead keratinocytes, and intercellular via, when the drug overcomes the SC crossing between the corneocytes [67]. On one hand, the transcellular route is more direct and shorter, but it

offers more resistance because the active compound needs to cross both lipophilic and hydrophilic environments. So, the more common route is the intercellular via because drugs only will need to cross a lipophilic barrier [12]. The pores route, which is divided in transglandular or transfollicular via, has been under studied in recent years. Firstly, it was believed that if the skin appendices (hair follicles and glands) only covered 0,1% of the skin surface, their contribution to the drug penetration was very small. Nowadays, this route is considered a direct and vertical pathway for nano and microparticles to penetrate the skin without having to cross the SC barrier via intercellular penetration [68]. Figure 8 represents all four possible ways for drug penetration into the skin.

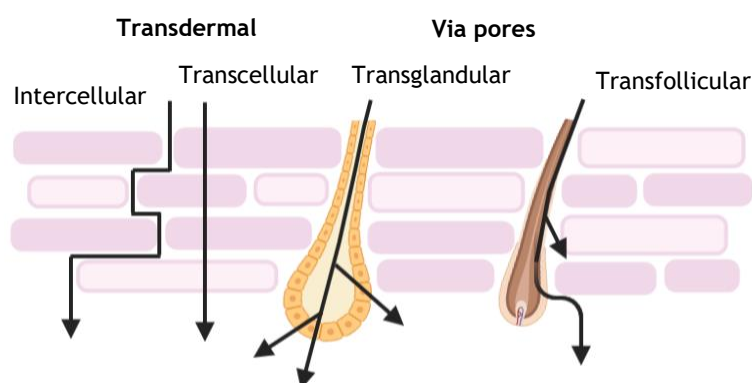


Figure 8. Schematic representation of the four possible ways of drug penetration into the skin.

This work will focus on the optimization of nanoparticles to improve the transport of the chosen natural compounds throughout the SC, with the aim of preventing premature skin aging and cell apoptosis.

2.4 Nanotechnology: lipid nanoparticles

For the formulation to be able to penetrate the lipid layer, it should have something lipophilic in its constitution [14]. Since lipidic nanoparticles are capable of load both lipophilic and hydrophilic drugs, encapsulating the bioactive compounds in lipid structures will help them overcome the SC. The lipid nanoparticles physiological composition interacts with SC making it rearrange the lipid molecules, letting the bioactive compound penetrate the internal layers of the skin [14]. Additionally, the application of these type of nanoparticles promotes skin hydration, because there is a formation of an occlusive film preventing water loss from the SC and a reinforcement of the SC lipidic structure [14]. Furthermore, these lipid nanoparticles facilitate the entry of molecules through hair follicles and allow a controlled release of compounds by creating deposits on the skin. In hair follicles the sebum is the main environment in which lipids are the main constituent, so this represents an important via for the nanoparticles to reach deeper layers of the skin [69]. The size of these particles is also a factor to take into account when talking about

nanoparticle penetration, since the smaller the particles are, the greater their adhesion and contact area, promoting their flow through the skin layers [14]. In Figure 9, the mechanism of penetration of lipid nanoparticles (shown in red) is exemplified.

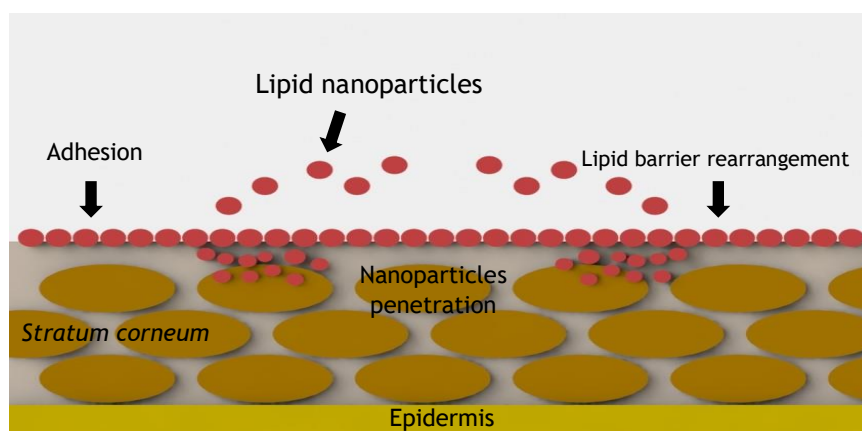


Figure 9. Penetration mechanism of lipid nanoparticles.

2.4.1 Solid lipid nanoparticles (SLN)

In the beginning of the 90s, the need to find an alternative to carrier systems such as nanoemulsions, liposomes and polymeric nanoparticles was evident [15]. Thus, by replacing the liquid lipid (oil) of an o/w (oil in water) emulsion for a solid lipid or a mixture of solid lipids, gave origin to Solid Lipid Nanoparticles (SLN). SLNs are composed of a solid lipid dispersed in an aqueous medium, stabilized by an emulsifier that reduce surface tension and avoid the aggregation between the nanoparticles. The most used lipids for the production of these nanoparticles are triglycerides, fatty acids, waxes and partial glycerides [70]. The size of these nanoparticles is usually between 150-300 nm, but larger or smaller sizes can be achieved depending on the final aim of the project. Nanoparticles with a size smaller than 100 nm can achieve systemic circulation [14]. So, in cosmetic products, nanoparticles size usually is between 200 and 300 nm [17]. These lipid nanoparticles have several advantages compared to other colloidal systems, such as their high biocompatibility and no biotoxicity since they are composed of physiological lipids or lipid molecules, good physical stability, wide range of particles size (nano to sub-micron scale), the possibility to control the release of drugs or active compounds, easy large-scale production, the use of cheap and accessible raw materials and a broad application spectrum such as dermal, oral, pulmonary applications, among others [70, 71]. In Figure 10 is presented a representative image of the composition of a SLN.

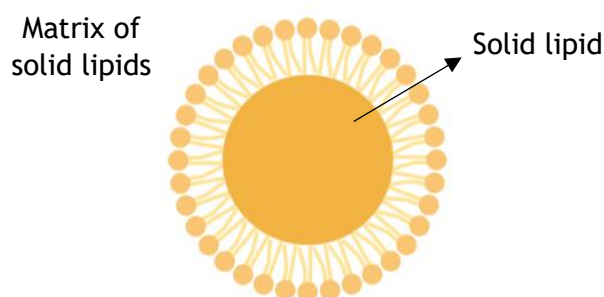


Figure 10. Representative image of SLN composition.

2.4.2 Nanostructured lipid carriers (NLC)

Another type of lipid nanoparticle are the Nanostructured Lipid Carriers (NLC) and were developed a few years later. In these particles, the matrix is composed of a mixture of a solid lipid and a liquid lipid, instead of only presenting solid lipids as in SLN [17]. NLCs are produced by mixing a solid lipid and a liquid lipid in different proportions such as 70:30 [70]. Figure 11 represents the composition of NLC.

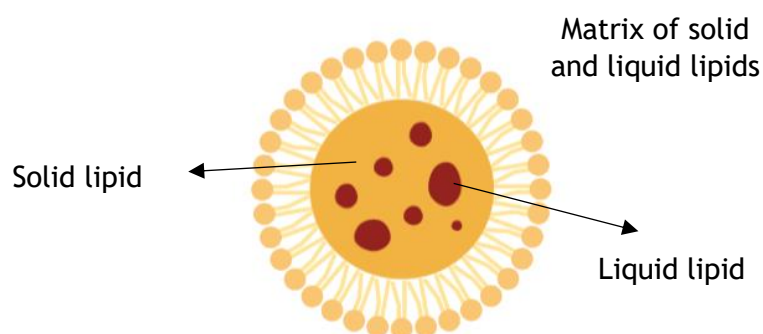


Figure 11. Representative image of NLC composition.

NLC's main advantage is the ability to retain active compounds. The risk of release of the active drug/compound is lower than in SLN. Since SLN are made only of solid lipids, they can go under modifications over time to achieve a stable crystalline structure. This change can lead to the release of the active compound [17]. On the other hand, the addition of a liquid lipid to a solid lipid prevents the recrystallization of the solid lipid and, therefore, no structural changes in the NLCs occur, so there is no release of the active compound over a long period of time. Thus, NLCs have a more stable configuration than SLNs [17]. In Figure 12, it is possible to compare the drug loading capacity and the structural difference between the two types of lipid nanoparticles [17].

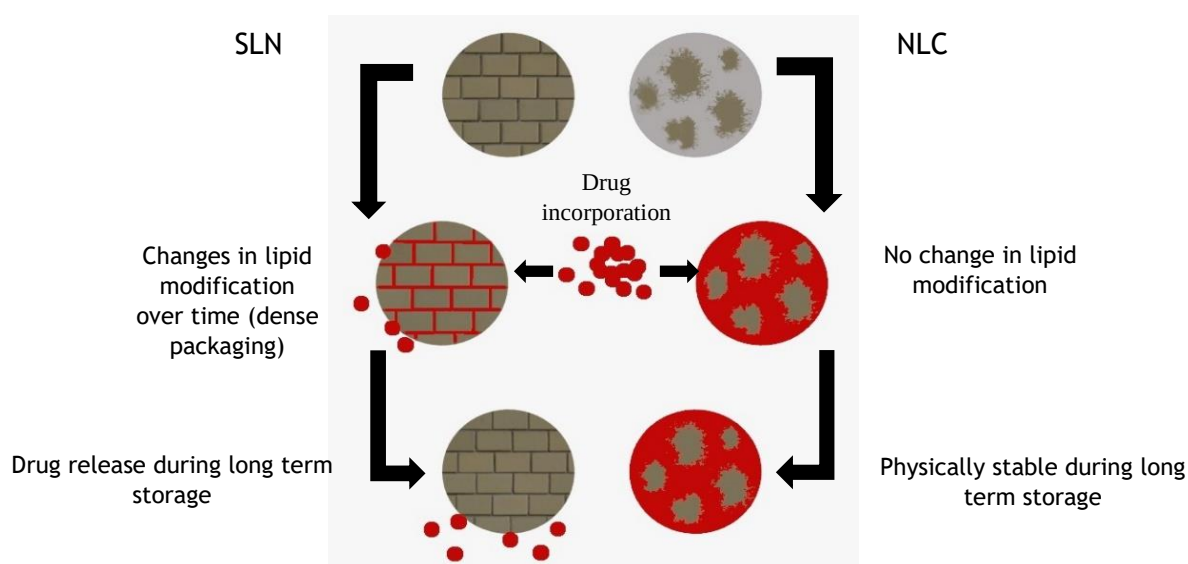


Figure 12. Representative image showing the drug delivery capacity and the structural difference between the two types of lipid nanoparticles.

2.4.3 Drug incorporation models

For drug incorporation into the nanoparticles, both SLN and NLC present different morphologies or models. For SLN, the homogeneous matrix model (i) is achieved when the active compound is homogeneously dissolved in the lipid matrix. This model can occur when the drug is highly lipophilic or when the formulation is rapidly cooled after production and there is no separation between the lipidic phase and the drug. This type of drug loading is known for long drug release periods [72]. The drug-enriched shell model (ii) occurs when a drug shell surrounds a lipid core. This can be formed, for example, when the drug is more soluble in the emulsifier solution than in the lipid phase. This type of morphology leads to a very fast drug release [73]. Finally, the third SLN type of morphology is the drug-enriched core model (iii) and it happens when the drug solubilization in the lipid is close to the saturation point so, during the cooling process, the active compound has a tendency to recrystallize before the lipid that forms a membrane around the active compound. This model is known for a prolonged drug release over time [16, 72]. In Figure 13 are represented the three types of SLN morphology.

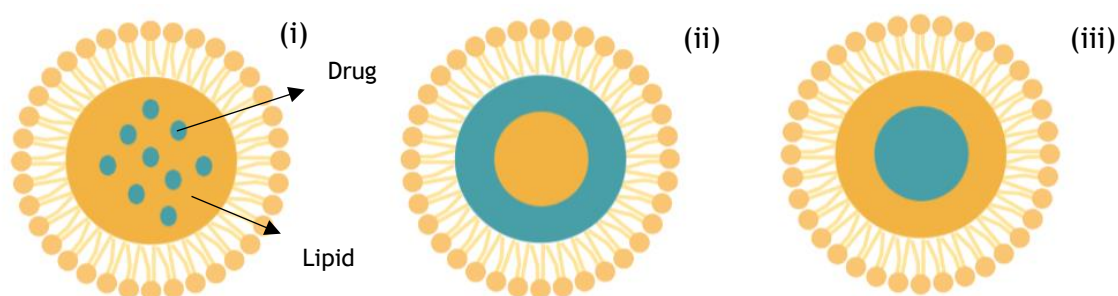


Figure 13. Drug incorporation models of SLN. (i) Homogenous matrix model, (ii) Drug-enriched shell model, (iii) Drug-enriched core model.

Regarding the NLC, the “Imperfect crystal” type (i) NLCs are obtained when a solid lipid is mixed with smaller amounts of a liquid lipid, resulting in imperfections that can easily accommodate drug molecules [16]. The second type is the “Amorphous” type (ii) and these NLCs are obtained by mixing special lipids to the solid lipid such as isopropyl myristate or hydroxyoctacosanyl hydroxy stearate, among others. These lipids create solid particles of amorphous structure which avoid the lipid recrystallization after cooling, increasing the shelf life of this type of nanoparticles [74]. Finally, the “Multiple” type (iii) is derived from multiple w/o/w (water in oil in water) emulsions. In this case, the concentration of liquid lipid exceeds its solubility in the solid lipid leading to the formation of small oil compartments. Usually, the active compound will exhibit greater solubility in the liquid lipid, getting entrapped in the compartments. This indicates that the drug release will be slow and controlled [72]. In Figure 14 are represented the three types of NLC morphology.

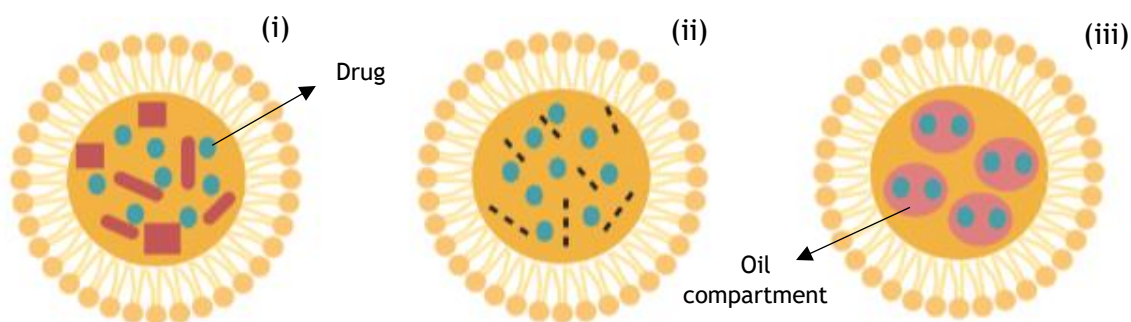


Figure 14. Drug incorporation models of NLC. (i) Imperfect crystal type, (ii) Amorphous type, (iii) Multiple type.

In order to achieve the desired SLN and NLC formulation, it is important to consider several parameters, such as the drug solubility in the lipid, the melting point of the lipid, the compatibility between the selected solid and liquid lipids in NLC formulations, the choice of emulsifier and its Hydrophilic-Lipophilic Balance (HLB) and the producing method [16].

2.4.4 Lipidic nanoparticle production

There are several methods for producing these nanoparticles, but generally all techniques require the formation of an o/w nanoemulsion followed by the solidification of the dispersed lipid phase. This last stage is a critical phase when it is necessary to produce nanoparticles with very small sizes and low polydispersity. Thus, to achieve the desired characteristics, equipment that produces high mechanical forces such as UltraTurrax or/and ultrasonicators are usually used. High pressure homogenization (HPH) is the most studied method for the production of nanoparticles and is subdivided into hot and cold techniques [70, 75]. This production method is easily scaled-up for industrial productions, does not use organic solvents that need to be removed until the end of the process, produces particles

that are in the submicron scale and the necessary equipment is not expensive [76]. However, it is not suitable for active compounds that are extremely heat sensitive, since the high-energy intensity of this technique, produces high temperatures that can cause the degradation of the compound. In this method, the active compound is dissolved in the melted solid lipid, in the SLN, or in a mixture of liquid and solid lipids in the NLC. In the hot technique, the process is conducted at temperatures above the melting point of the solid lipid. The hot surfactant solution is dispersed in a hot lipid solution containing the active compound. The sample is then subjected to a high-pressure homogenizer that creates a high shear stress and cavitation forces that reduces the particles to the submicron size. In the cold technique the lipids containing the active compound are cooled and after solidification, they are crushed and dispersed in a cold surfactant solution. Finally, the sample is subjected to a high-pressure homogenizer [15]. This production process reduces the sample's exposure time to the high temperatures used in the hot technique, so it is more appropriated for extremely heat sensitive compounds [16]. There are more production techniques such as the solvent injection technique or the emulsification-solvent evaporation technique that use solvents that need to be removed at the end of the process, which is why they are not so widely used [70].

2.5 Literature Review

The application of lipid nanoparticles in products for cutaneous use has been widely investigated due to the numerous advantages of these nanocarriers, such as the chemical protection of the incorporated molecules, the improvement of the bioavailability of the compounds and their controlled release, leading to a better penetration and retention in the skin [14]. In this section, several articles are going to be analyzed to understand the current achievements of this field.

Shrotriya *et al.* (2017) developed a semi-solid formulation as a novel topical treatment for irritant contact dermatitis [77]. In this study, resveratrol-loaded solid lipid nanoparticles using precirol ATO 5 as solid lipid and tween 20 and span 80 as emulsifiers, were made through the ultrasonication production method. The obtained nanoparticles presented a mean size below 100 nm and an encapsulation efficiency between 68-89%. Furthermore, the formulation was tested in both human cadaver skin and rabbits, showing a drug release and deposition up to 24 h and no skin irritation effects. The Resveratrol-SLN gel demonstrated an effective suppression of ear swelling and a reduction in skin water content when compared to a commercial gel, showing the potential of the nanoparticles in reducing the skin inflammation.

Chen *et al.* (2017) studied different NLC formulations and their ability to carry, protect, control the release and deliver resveratrol, EGCG and vitamin E to the skin [78]. The nanoparticles were produced by high pressure homogenization technique and the final formulations were characterized in terms of size, zeta potential, encapsulation efficiency and UV induced degradation. For resveratrol nanoparticles, they also studied its release profile and penetration through the *stratum corneum*. Compritol 888, cetyl palmitate and phospholipon 80 were used as solid lipids, organic sesame oil as liquid lipid and tween 80 as emulsifier. The nanoparticles presented a range size between 100-300 nm, a zeta potential between -53 mV and -25 mV and an encapsulation efficiency above 94%, showing that lipid nanoparticles are a potential method to deliver both hydrophobic and hydrophilic actives in one formulation. Resveratrol and vitamin E nanoparticles demonstrated effective protection of the active compound against UV induced degradation, but for EGCG that was not the case, since the % of active compound remaining in the samples after 168 h of UV exposure was less than the control (free EGCG). Finally, the release study of resveratrol showed a 70% release after 24 h and the nanoparticles successfully demonstrated an improvement in the resveratrol skin penetration, suggesting that lipid nanoparticles are a promising method to encapsulate and deliver antioxidants to the skin.

Dzulhi *et al.* (2018) produced SLN loaded with green tea leaves extract in order to characterize and evaluate the skin penetration of the active compounds [79]. The lipid nanoparticles were prepared by emulsification-solvent evaporation technique where glyceryl monostearate (solid lipid) and lecithin (emulsifier) were dissolved in dichloromethane. In terms of size, nanoparticles had between 155-280 nm, a zeta potential between -42 mV and -30 mV and an encapsulation efficiency above 81%. For the penetration study, green tea nanoparticles and the control (free green tea extract) were made into a cream dosage form and applied to female rats. After 12 h, the SLN cream showed a higher EGCG flux through the skin than the control cream, demonstrating the ability of SLN to increase the skin penetration of the active compounds.

Ban *et al.* (2020) studied the effect of lipid nanoparticles loaded with myricetin as a novel carrier to increase the penetration of the active compound into the sudomotor nerves located underneath sweat glands to reduce the focal sweating [80]. The NLC nanoparticles were produced combining high shear homogenization and ultrasonication techniques. The lipids used were tristearin and tricaprylin and the emulsifiers were Pluronic P-123 and Brij S 100. The produced nanoparticles had sizes between 170-184 nm, the surface charges were between -4,6 mV and -2,5 mV and the entrapment efficiencies were approximately 99%. The biocompatibility of the myricetin-loaded nanoparticles was evaluated, and the results suggested that this type of nanoparticles can be safely applied for treating topical

hyperhydrosis. Furthermore, the transport of myricetin throughout the skin was simulated with Nile Red (lipophilic fluorescent dye) applied on pig skin. Two types of formulation were applied: NR free and NR encapsulated. The results showed that NR free was not detected in any pig skin slice, but the NR encapsulated in the lipidic nanocarrier was detected in the *stratum corneum*, epidermis and even in the dermis, suggesting that lipid nanoparticles are a great deliver vehicle to carry myricetin throughout the skin layers and treat this disease.

Rigon *et al.* (2016) developed a formulation with resveratrol-loaded SLN to evaluate its possible use in therapies against hyperpigmentation and skin aging [81]. The nanoparticles were obtained by a ultrasonication technique, where stearic acid was the solid lipid and poloxamer 407 with methylparaben and propylparaben was the emulsifier and stabilizer solution used. In terms of size, the loaded nanoparticles had between 155-166 nm and the zeta potential was around -2,60 mV. The developed SLN presented a good tyrosinase inhibition activity (enzyme present in melanin production, consequently related to hyperpigmentation of the skin) in comparison to a control solution, which means that the nanocarrier increases the drug inhibition activity. To study the *in vitro* skin permeation of resveratrol, SLN solutions were applied in pig skin and after 24 h, the cumulative amount of resveratrol permeated was 45%. Finally, cytotoxicity assays were carried out and the formulations showed no toxic effects in keratinocytes for concentration below 250 µg/mL. In conclusion, resveratrol-loaded nanoparticles could be used in potential treatments for hyperpigmentation and skin aging disorders.

Abbas *et al.* (2018) produced SLN loaded with resveratrol to evaluate its antioxidant, anti-inflammatory and anti-aging capacity *in vivo* [82]. The nanoparticles were prepared by high pressure homogenization technique, where compritol 888 was used as a solid lipid and different mixtures of poloxamer 188, poloxamer 407 and tween 80 were used as emulsifier. Six formulations were prepared with different combinations of emulsifiers, so the particle size ranged between 230-841 nm and the zeta potential varied between -28 mV and -6 mV, showing that different emulsifiers will lead to very different particle sizes. Additionally, the six types of formulation had encapsulation efficiencies between 55-65% and release efficiencies above 54% in the first day. In the occlusive properties test, the smaller nanoparticles had a better packing and fitting, so they were able to create a continuous film of nanoparticles on the skin surface, combating moisture loss and, therefore, aging and loss of skin elasticity. The formulation with 248 nm was chosen to perform the antioxidant, anti-inflammatory and anti-aging essays. In this tests, dorsal rat's skin with treatment (resveratrol-SLN and free resveratrol) were exposure to UVB radiation and compared with skin that was not treated and skin that was not exposed. In the three tests, several enzymes related to UVB exposure were quantified and the skin treated with the nanocarriers had a

superior dermal photo-protection compared to the free resveratrol treated skin, demonstrating the potential of lipid nanoparticles in the transport of active compounds through the skin layers.

Eiras *et al.* (2017) developed NLC containing vitamin E to incorporate in a hydrogel and evaluate its suitability for cutaneous applications [83]. The nanoparticles were obtained by high shear homogenization combined with sonication technique. The excipients used were precirol ATO 5 (solid lipid), vitamin E (oil), tween 80 (emulsifier) and cetrimide. To evaluate the particle stability, the particle size was measured after 7 months of storage: 90% of the particles were lower than 328 nm and 50% lower than 105 nm. There was a slight decrease in size since the time they were produced but it is irrelevant for cutaneous applications. Several *in vitro* tests were performed to the hydrogel during storage: organoleptic analysis, accelerated stability, pH analysis, rheological measurements and texture analysis. After these tests, the formulation was considered stable. The last two tests were related to its biocompatibility (using human keratinocytes) and irritant potential (using fertilized chicken eggs). The developed formulation demonstrated to be biocompatible and non-irritant, stable and suitable for experiments in human volunteers.

Clares *et al.* (2014) produced three different lipid-based nanocarriers for retinyl palmitate encapsulation in order to evaluate their skin delivery, photostability and biocompatibility [84]. The SLN were prepared by high shear homogenization combined with ultrasonication technique, where compritol 888 was the solid lipid used and span 80 was the emulsifier. The lipid nanoparticles had approximately 271 nm and a zeta potential of -55 mV. In terms of encapsulation efficiency, SLN were the second better lipidic nanocarrier with almost 95% of encapsulation. In the photo-stability studies, the solid lipid nanoparticles offered significantly better protection than liposomes and nanoemulsions, showing SLN's potential in physical UV-blocking protection. In the permeation and retention studies, the nanoemulsions showed a higher permeation flux than the other two nanocarriers, probably due to its smaller size (approximately 15 nm) and greater interfacial surface area. So, depending on the aim of the formulation, the three types were proven to be suitable for cosmetic or dermatologic use, with SLN acting in the superficial layers and the other two formulations reaching deeper skin layers.

Tichota *et al.* (2014) developed NLC loaded with argan oil to improve skin hydration [85]. The NLC were obtained through the combination of high shear homogenization and ultrasonication techniques. Two formulations were produced where the solid lipid was precirol ATO 5 or softisan 142, the argan oil was the liquid lipid and tween 80 was the emulsifier. Both NLC formulations were monodispersed, but the one made with precirol had smaller and more homogeneous sizes, so it was chosen to continue the study. The size and

zeta potential were measured in the production day and after 90 days at different storage temperatures. Moreover, no significant variation was observed, indicating good stability throughout storage. The formulation was incorporated into a hydrogel and characterized in terms of pH, texture, viscosity and color for 30 days. The results showed no major alteration in the formulation, indicating absence of lipid degradation. The *in vivo* studies were carried out in healthy human volunteers and after 30 days of application, the skin hydration increased, suggesting the existence of a synergic effect between the NLC and argan oil hydrating properties.

In Table 1 are presented the main features of the studies analyzed. Overall, they demonstrated that lipid nanoparticles are efficient nanocarriers for natural compounds, increasing their penetration and bioavailability throughout the skin layers. Furthermore, some of the studies incorporated their nanoparticles into hydrogels or creams, for easy application into the skin, and the results were very promising, showing that the formulations were suitable for cosmetic or therapeutic use.

Table 1. Research studies previously analyzed.

Types of nanoparticles	Lipids/emulsifiers	Production technique	Active compound	Purpose	Reference
SLN	Precirol ATO 5 / Tween 20, Span 80	Ultrasonication	Resveratrol	Topical treatment for irritant contact dermatitis	[77]
NLC	Compritol 888, Cetyl palmitate, Phospholipon 80, Organic sesame oil / Tween 80	High pressure homogenization	Resveratrol, EGCG, Vitamin E	Carry, protect, control the release, and deliver natural compounds	[78]
SLN	Glyceryl monostearate / Lecithin	Emulsification-solvent evaporation	Green tea extract	Characterize and evaluate the skin penetration of active compounds	[79]
NLC	Tristearin, Tricaprylin / Pluronic P-123 and Brij S 100	High shear homogenization and ultrasonication	Myricetin	Increase the penetration of the active compound for treating topical hyperhydrosis	[80]
SLN	Stearic acid / poloxamer 407 with methylparaben and propylparaben	Ultrasonication	Resveratrol	Control of hyperpigmentation and skin aging	[81]
SLN	Compritol 888 / poloxamer 188, poloxamer 407 and Tween 80	High pressure homogenization	Resveratrol	Evaluation of the antioxidant, anti-inflammatory and anti-aging capacity of resveratrol	[82]
NLC	Precirol ATO 5, vitamin E / Tween 80	High shear homogenization and ultrasonication	Vitamin E	Skin treatment	[83]
SLN	Compritol 888 / Span 80	High shear homogenization and ultrasonication	Retinyl palmitate	Improve retinyl palmitate's skin delivery, photostability and biocompatibility.	[84]
NLC	Precirol ATO 5 or softisan 142, Argan oil / Tween 80	High shear homogenization and ultrasonication	Argan oil	Improve skin hydration	[85]

Chapter 3: Materials and Methods

3.1 Materials

3.1.1 Natural Compounds

Epigallocatechin Gallate (EGCG, (-)-epigallocatechin-3-gallate, $\geq 94\%$ purity, molecular weight 458 g/mol) was purchased from Kosher, Myricetin (3,3',4',5,5',7-Hexahydroxyflavone, $\geq 97\%$ purity, molecular weight 318,24 g/mol) and Resveratrol (*trans*-1,2-(3,4',5-Trihydroxydiphenyl)ethylene, $\geq 99\%$ purity, molecular weight 228,25 g/mol) were both purchase from TCI Chemicals.

3.1.2 Formulation studies

3.1.2.1 Lipid screening

For the nanoparticle optimization process, several solid lipids were studied to evaluate their compatibility with the natural compounds. In Table 2 are listed the lipids used and the respective supplier.

Table 2. List of solid lipids used in the nanoparticle optimization process and their supplier.

Solid lipids	Supplier
Precirol® 5 ATO (glyceryl distearate), Gelucire® 39/01, Cetyl palmitate, Suppocire DM Pellets, Compritol® HD5 ATO, Gelucire® 50/13 (Stearoyl polyoxyl-32 glycerides), Suppocire NA15 Pellets, Gelucire® 43/01 (hard fat compounds), Apifil® (PEG-8 beeswax)	Gattefossé (Nanterre, France)
Dynasan® 114 (glyceryl tristearate), Softisan® 100 (hydrogenated coco-glycerides), Softisan® 154 (hydrogenated palm oil)	IOI Oleo GmbH (Hamburg, Germany)
Witepsol® E76 (hard fat compounds)	Oxi-Med (Barcelona, Spain)
Glyceryl monostearate, Stearic acid	Acopharma S.A. (Terrassa, Spain)

3.1.2.2 Emulsifier compatibility evaluation

The emulsifiers used were pluronic F-127 (molecular weight 12600 g/mol) and tween 80, both acquired from Sigma-Aldrich Química S.A.

3.1.3 *In vitro* cytotoxicity assay and evaluation of the protection of the formulation against oxidative stress

Dulbecco's modified Eagle's medium (DMEM) with 4,5 g/L glucose and GlutaMAX™, fetal bovine serum (FBS), 0,25% trypsin/1 mM EDTA and Hanks' balanced salt solution (HBSS) without calcium and magnesium [HBSS (-/-)] were obtained from Gibco™ (Thermo Fisher Scientific). Neutral red (NR) solution, resazurin (REZ) sodium salt, tert-butyl hydroperoxide (t-BHP) solution, 2',7'-Dichlorofluorescein diacetate (DCFH-DA) and Triton™ X-100 detergent solution were obtained from Sigma-Aldrich Química S.A. Antibiotic (10000 U/mL penicillin, 10000 µg/mL streptomycin) was obtained from Biochrom. Dimethyl sulfoxide (DMSO) was obtained from Merck. All sterile plastic material was obtained from Corning Costar. All the reagents used were of analytical grade or of the highest grade available.

3.2 Methods

3.2.1 Formulation studies

3.2.1.1 Lipid screening

Firstly, each solid lipid was tested to establish their ability to dissolve the three NC of interest.

The experimental procedure was based in a process described by Tichota *et al.* (2014) [85]. Firstly, 100 mg of each solid lipid was mixed with 1 mg of the NC. If the lipid did not dissolve the compound in this proportion, another tentative with 1:200 (w/w) was carried out. The mixture of lipid/natural compound was warmed up to 80 °C (above the lipid phase melting point) for 1 hour, with magnetic agitation. The solubility of the natural compounds was visually determined, at 15-minute intervals, observing the presence or absence of crystals of the compounds in the lipid.

3.2.1.2 Emulsifier compatibility evaluation

In order to perform an emulsifier optimization, the nanoparticles were prepared without the natural compounds according to the experimental procedure detailed in the following section. Two different emulsifiers were tested and analyzed: pluronic-F127 and tween 80. Furthermore, different Ultra-Turrax T25 (0,5; 1 and 2 min) and sonication (5, 10, 15 and 30 min) times were tested to select the best production conditions. The resultant

nanoparticles were studied throughout time in terms of size, Pdl and zeta potential to evaluate if they were stable and had the required characteristics to be chosen for final formulation.

3.2.2 Nanoparticle preparation

Nanoparticles were produced by two methods combined: the high shear homogenization followed by ultrasonication. The experimental preparation was based in a procedure described by Vaz *et al.* (2019) [86]. First, a mixture of 1 g of the solid lipid and 20 mg of one natural compound was heated at 80 °C (5 °C to 10 °C above the melting point of the lipid) to melt completely. At the same time, 8,7 mL of an aqueous solution (10% w/v) of emulsifier was heated separately. When the lipid solution was completely melted and both solutions were at the same temperature, the aqueous phase was added to the lipidic phase.

For homogenization, the resultant mixture was stirred in the Ultra-Turrax T25 (Janke and Kunkel IKA-Labortechnik, Staufen, Germany) at 13500 rpm, followed by an 80% intensity sonication using a Vibra-Cell™ CV15 (Sonics and Materials, Newtown, CT, USA). The samples were cooled down to room temperature with magnetic agitation and were stored protected from the light. The nanoparticles were made in triplicate and un-loaded nanoparticles were also prepared as control samples. In Figure 15, is presented a graphic illustration of the experimental procedure.

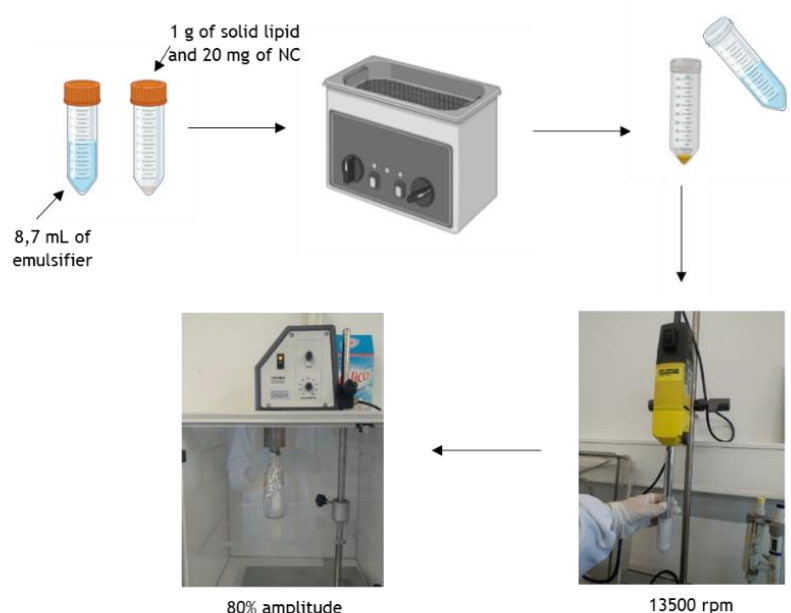


Figure 15. Graphic illustration of the experimental procedure.

3.2.3 Nanoparticle characterization

Nanoparticles were characterized in terms of size, zeta potential and morphology. The particle size, polydispersity index (Pdl) and zeta potential (ZP) were measured by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS (Malvern Instrument, UK) at day 1, 7, 14 and 30, to evaluate the nanoparticle stability. Before the measurement, the formulations were diluted in ultrapure water (1:100 v/v) and every measurement was run in triplicate.

The morphology was measured by transmission electron microscopy (TEM) using a JEM-1400 electron microscope (JEOL, Tokyo, Japan) with an accelerating voltage of 80 kV. The experimental procedure was based in a procedure described by Loureiro *et al.* (2017) [56]. Briefly, 5 μ L of each sample were deposit on a 400 mesh carbon-formvar copper grids (Agar Scientific, Essex UK) and let absorb for 5 min. Then, the samples were stained with 10 μ L of 2% (w/v) uranyl acetate ($\geq 98\%$ purity, molecular weight 424,15 g/mol was purchase from Sigma-Aldrich Química S.A.) solution and the excess was removed. After this, the samples were once again stained with 10 μ L of 2% (w/v) uranyl acetate solution for 45 s, the excess was removed, and the grid was ready for the transmission electron microscope.

3.2.3.1 Dynamic Light Scattering (DLS)

Dynamic Light Scattering (DLS) technique makes it possible to analyze the average particle size as well as the size distribution of the particles (Pdl), which indicates the heterogeneity of sizes between the nanoparticles. In samples with Pdl < 0,3 is acceptable to consider that the solution is monodisperse for lipid nanoparticles (the nanoparticles have approximately the same size) [87]. DLS can analyze sizes on the scale of nanometers and micrometers. This technique is based on the Brownian movement of dispersed particles. These particles move randomly in all directions and collide with solvent molecules. The collisions cause a certain amount of energy to be transferred, which induces particle movement. The release of energy has an effect on the speed movement of the particles, with smaller particles moving faster than larger particles [88]. From the Stokes-Einstein equation (eq. 1) is possible to understand the relationship between the speed of the particles and the particle size.

$$D = \frac{k_B T}{6 \pi \eta R_H} \quad (1)$$

Where D is the translational diffusion coefficient (m^2/s), k_B is the Boltzman constant ($\text{m}^2 \cdot \text{kg} / \text{K} \cdot \text{s}^2$), T is the temperature (K), η is the viscosity (Pa.s) and R_H the hydrodynamic radius (m) [88]. In a DLS instrument, a laser is directed to a sample in a cuvette. If there are any particles, the laser light gets scattered in all direction. The light is detected at a certain

angle over time, and this is used to calculate the translation diffusion coefficient and the particle size [88]. Figure 16 shows a schematic representation of DLS measurement system.

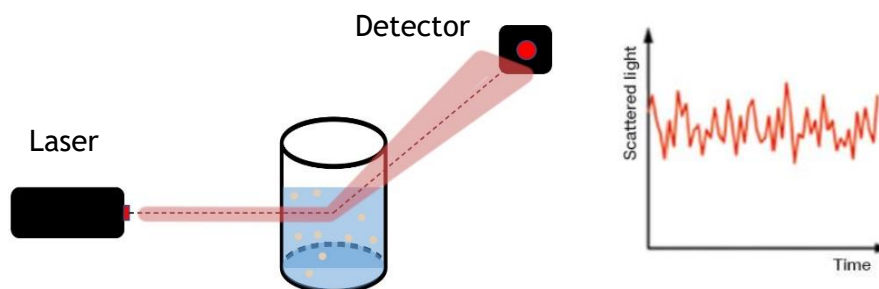


Figure 16. DLS measurement system.

The zeta potential is the electric charge of the particles that are dispersed in the liquid. It is a good parameter to check the physical stability of the nanoparticles since particles with high zeta potential (negative or positive) are stable while particles with low zeta potentials tend to coagulate or flocculate [89]. To measure the zeta potential, the technique used was Electrophoretic Light Scattering (ELS), integrated in the same equipment as the DLS technique [90]. The ELS technique is used to measure the electrophoretic mobility of particles in a solution. The solution is introduced into a cuvette with two electrodes, an electrical field is applied, and the velocity of the nanoparticles is measured. The particles that have a net charge will travel to the opposite charged electrode with a certain velocity [91]. The zeta potential is related to the electrophoretic mobility by the Henry's equation (eq. 2) presented below.

$$U_E = \frac{2 \varepsilon \zeta F(ka)}{3 \eta} \quad (2)$$

Where U_E is the electrophoretic mobility, which is the particle's velocity per unit of electric field strength ($\mu\text{m/s}/(\text{V/cm})$), ε is the dielectric constant, ζ is the zeta potential, $F(ka)$ is the Henry's function (1,5 for aqueous media) and η is the dispersion viscosity [92], [93].

3.2.3.2 Transmission electron microscopy (TEM)

The transmission electron microscopy (TEM) is an important tool to analyze nanoparticles, since it has a high resolution that can reach about 0,07 nm, depending on the sample thickness and on the accelerating voltage, and gives detailed images of the nanoparticles. It is also possible to check if they are aggregating and if the size is similar to the one from the DLS tests [94].

The microscope operation shares some basic principles with light microscopes but uses electrons instead of light. A beam of electrons hits the sample and parts of it are

transmitted and reach a phosphor screen where the image is being formed. The darker parts of the image are areas where fewer electrons were transmitted, and the lighter parts are those areas of the sample where more electrons were transmitted [95].

Since the electrons wavelength is much smaller than the light wavelength, the optimal resolution is many orders of magnitude better. This microscope is able to reveal the smaller details in the internal structure of a variety of materials [95].

3.2.4 Compounds characterization

In order to assess the maximum absorption wavelength for each compound, the natural compound's spectrum (200-600 nm) was obtained using a BioTek Synergy 2 Multi-Mode Reader (Winooski, Vermont, USA). To perform this experiment, EGCG, myricetin and resveratrol were dissolved in a 70% (v/v) aqueous ethanol (pure anhydrous ethanol, molecular weight 46,07 g/mol, from Sigma-Aldrich Química S.A.) solution due to the hydrophobicity of the natural compounds. The spectrums were made in triplicate.

For quantification purposes, the linear calibration curve for each compound was made. A stock solution was prepared in triplicate where EGCG, myricetin and resveratrol were dissolved in a 70% (v/v) aqueous ethanol solution (final concentration: 0,0133 mg/mL). To obtain samples with different concentrations, the stock solution was diluted. The absorbance of every sample was measured at 274 nm for EGCG, 305 nm for resveratrol and 377 nm for myricetin, which are the characteristic obtained peak for each compound.

3.2.4.1 Spectrophotometry

Spectrophotometry is an analytical technique very commonly used to quantify and analyze a certain substance. This technique can easily measure the amount of light absorbed by or transmitted through the sample [96]. There are several advantages in using UV-Vis spectrophotometry for example, this technique is non-destructive and samples can be reused, it is a quick and easy process, does not require extensive processing and is generally inexpensive to operate [96].

A UV-Vis spectrophotometer is the instrument used to perform this technique and is composed by a light source, a wavelength selector such as a monochromator (transforms polychromatic light in a selected beam of monochromatic light), a detector and a computer for signal processing [96]. The sample is subjected to a beam of light and will absorb, reflect and transmit a certain amount of light that will be detected by the detector.

The results from UV-Vis spectroscopy are usually presented as a graph of absorbance of radiation as a function of wavelength. This graph is known as the UV spectrum and provides information about the sample, for example, what is the maximum absorbance

wavelength [96, 97]. The absorbance (A) is the amount of light absorbed by the sample and is expressed by the following equation (eq. 3).

$$A = \log_{10} \left(\frac{I_0}{I} \right) \quad (3)$$

Where I_0 is the intensity of light before passing through the sample and I is the intensity of light after passing through the sample. To obtain the concentration of the sample, the Beer-Lambert law is usually applied (eq. 4) [96].

$$A = \varepsilon c L \quad (4)$$

Where A is the absorbance, ε is the molar absorptivity (L/mol), c is the concentration (mol/L·cm) and L is the optical path length (cm).

3.2.5 Determination of encapsulation efficiency and loading capacity

To obtain the encapsulation efficiency (EE) and the loading capacity (LC), the loaded nanoparticles were diluted in ultrapure water (1:50) and 500 μ L of the dilution were filtered using centrifugal filters (Amicon Ultra Centrifugal Filters Ultracell- 30 kD, Merck Millipore Ltd., Tullagreen, Carrigtwohill; from Sigma-Aldrich Química S.A.), at 14500 g for 3 min. After the centrifugation, 60 μ L of the supernatant were collected and mixed with 140 μ L of ethanol 100% (v/v). This experiment was run in triplicate for each type of nanoparticle. The amount of free natural compound was quantified measuring the absorbance with the UV-Vis spectrophotometer at the characteristic wavelength of each compound. Through the calibration curve, the concentration of free compound was calculated, and the encapsulation efficiency was determined as described in the following equation (eq. 5).

$$EE (\%) = \frac{\text{Total amount of compound in formulation} - \text{amount of free compound}}{\text{Total amount of compound in formulation}} \times 100 \quad (5)$$

For the loading capacity, it was possible to calculate the amount of compound encapsulated through the amount of free compound obtained. Then, with the total mass of SLN, the loading capacity was determined following the equation bellow (eq. 6).

$$LC (\%) = \frac{\text{Total mass of natural compound encapsulated}}{\text{Total mass of SLN} + \text{Total mass of natural compound encapsulated}} \times 100 \quad (6)$$

3.2.6 In vitro release

To assess the natural compounds release, loaded nanoparticles were dissolved in HEPES buffer (1:50 proportion, with a final volume of 5 mL). HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, $\geq 99,5\%$ purity, molecular weight 249,30 g/mol) was purchase from Sigma Aldrich Química S.A. is known for being one of the best

biological buffers with a pH between 6,8 and 8,2 [98]. The mixtures were incubated at 37 °C with magnetic agitation at 200 rpm. At specific timepoints, 200 µL of the solution were filtered using centrifugal filters (Amicon Ultra Centrifugal Filters Ultracell- 30 kD, Merck Millipore Ltd., Tullagreen, Carrigtwohill; from Sigma-Aldrich), at 14500 g for 3 min. After the centrifugation, 60 µL of the supernatant were collected and mixed with 140 µL of ethanol 100% (v/v). The absorbance was read at the characteristic wavelength of the compound and through the calibration curve it was possible to obtain the amount of free compound and calculate the % compound released at a certain moment (eq. 7).

$$\text{Natural compound released at time } t \text{ (\%)} = \frac{\text{Total compound at time } t}{\text{Total amount of compound in formulation}} \times 100 \quad (7)$$

3.2.7 Antioxidant assay

To evaluate the antioxidant potential of the natural compounds, the DPPH technique was used, not only in the natural compounds alone, but also in mixtures of two and three compounds to assay possible synergies. DPPH (2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl, molecular weight 394,23 g/mol from Sigma-Aldrich Química S.A.) is a stable free radical with a deep violet color that has a strong absorption band at 517 nm. When in contact with an antioxidant substance, DPPH's color vanishes to a yellow pale as the electron pairs off [99].

The experimental preparation was based in a procedure described by Duan *et al.* (2006) [100]. Firstly, stock solutions were prepared dissolving the natural compounds in methanol (≥99,8% purity, molecular weight 32,04 g/mol purchased from Sigma-Aldrich Química S.A.). Each stock solution was added to 175 µL of the DPPH methanolic solution (0,16 mM) to obtain a final concentration of natural compound or mixture of 4×10^{-6} M and a final volume of 200 µL. The studied mixtures were: EGCG + myricetin; EGCG + resveratrol, resveratrol + myricetin and resveratrol + myricetin + EGCG. The solutions were vortexed for 1 min and then were kept in the dark for 30 min. Finally, the absorbance was read at 517 nm and the scavenging potential was calculated with the following equation (eq. 8).

$$\text{Scavenging effect (\%)} = \left(1 - \frac{A_{\text{sample}} - A_{\text{sample blank}}}{A_{\text{control}}} \right) \times 100 \quad (8)$$

Where A_{control} is the absorbance of the control (DPPH solution without the sample), $A_{\text{sample blank}}$ is the absorbance of the sample without DPPH and A_{sample} is the absorbance of the test sample (DPPH with the mixtures or natural compound).

3.2.8 Accelerated stability

To study possible changes that may occur during storage, an accelerated stability assay was performed. The experimental preparation was based in a procedure described by

Eiras *et al.* (2017) [83]. Firstly, 20 μL of the loaded nanoparticles were diluted in 1980 μL of ultrapure water. Then the samples were centrifuge twice (MiniSpin eppendorf centrifuge) at 3000 g for 30 min. After each cycle the samples were visually examined to verify phase separation, creaming or flocculation and analyzed in the DLS in terms of particle size, Pdl and zeta potential.

3.2.9 Statistical analysis

The results were expressed as the average $\pm$ standard deviation. The statistical analysis of the results was made using the student's t-test for independent samples or paired samples in Excel. Differences were considered to be significant for p values lower than 0,05 (confidence level $\geq 95\%$).

3.2.10 *In vitro* cytotoxicity assay and antioxidant assay in immortalized human cells (HaCaT)

The tests presented bellow were carried out in collaboration with the Faculty of Pharmacy of University of Porto.

3.2.10.1 Cultured of immortalized human cells (HaCaT)

HaCaT cells were used as an *in vitro* model, as it is a widely used human keratinocyte model for biological and medical research [101]. These cells were cultured in 75 cm^2 flasks using DMEM with 4,5 g/L glucose and GlutaMAX™, supplemented with 10% FBS, 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin. The cells were kept in a 5% CO_2 - 95% air atmosphere, at 37 °C, and the cell culture medium was changed every 2 days. When at 80-90% of confluence, cultures were passaged by trypsinization (0,25% trypsin/1 mM EDTA). For the experiments, the cells were always seeded in 96 well plates at a density of 20000 cells/well. The cells used in all experiments were taken between the 42nd and 50th passages.

3.2.10.2 Cytotoxicity of the formulations

The cytotoxicity of the tested formulations was evaluated in immortalized human keratinocytes, the HaCaT cells. The procedure was based in a procedure previously described by Vaz *et al.* (2019) [86]. Briefly, 24 hours after seeding the cells were exposed to the formulations (0 - 1000 $\mu\text{g}/\text{mL}$) prepared in fresh cell culture medium. Twenty-four hours after exposure the formulations cytotoxicity was evaluated by the neutral red (NR) uptake and resazurin (REZ) reduction assays. Triton™ X-100 (1%) was used as positive control.

3.2.10.2.1 Neutral red (NR) uptake assay

The neutral red uptake assay is a viable assay based on the ability of living cells to incorporate the cationic dye. This dye can penetrate the viable cells and is accumulated

intracellularly in lysosomes. If a toxic substance is present, the cell's uptake of the neutral red decreases. So, this cytotoxicity assay allows to assess membrane permeability and lysosomal activity, and to conclude if the cell is viable, damaged, or dead. Cytotoxicity is presented as a concentration-dependent reduction uptake of NR [102].

Twenty-four hours after exposure to the tested formulations, the cell culture medium was aspirated, followed by the addition of fresh cell culture medium containing NR (50 µg/mL). The cells were then incubated, at 37 °C, in a humidified 5% CO₂ - 95% air atmosphere, for 90 min. After incubation, the cell culture medium was removed, and the NR dye retained only by viable cells was extracted with lysis buffer (absolute ethyl alcohol/distilled water (1:1) with 5% acetic acid). The absorbance was measured, at 540 nm, in a multiwell plate reader (PowerWaveX BioTek Instruments, Vermont, USA). The percentage of NR uptake relatively to that of the control cells (0% formulation present) was used as the cytotoxicity measure. Four independent experiments were performed, in triplicate. The cytotoxicity of the free drugs (0,81 µg/mL) was also evaluated by the NR uptake assay, 24 h after exposure, as performed for the evaluation of the formulation's cytotoxicity.

3.2.10.2.2 Resazurin (REZ) reduction assay

Resazurin is a cell permeable redox indicator that viable cells with active metabolism are able to reduce it into resorufin, which is fluorescent. The quantity of resorufin produced is proportional to the number of viable cells, and can be quantified through a fluorometer equipped with a 560/590 nm emission filter set [103].

Twenty-four hours after exposure to the tested formulations, the cell culture medium was aspirated, followed by the addition of fresh cell culture medium containing REZ at a concentration of 10 µg/mL. Then, incubation of the cells occurred at 37 °C, in a humidified 5% CO₂ - 95% air atmosphere, for 90 minutes. After that, resorufin fluorescence was measured at excitation/emission wavelengths of 560/590 nm, in a multiwell plate reader (PowerWaveX BioTek Instruments, USA). The percentage of REZ reduction relatively to the control cells (0%) was used as the cytotoxicity measure. Four independent experiments were performed, in triplicate.

3.2.10.3 Evaluation of the protection of the formulations against oxidative stress: effects on t-BHP induced increase in ROS levels

The expected antioxidant effect of the tested formulations was evaluated *in vitro*, in HaCaT cells, through the evaluation of their ability to protect against t-BHP-induced increase in the intracellular levels of ROS and reactive nitrogen species (RNS). For that

purpose, the DCFH-DA probe was used, which is hydrolyzed once in the cytoplasm, leading to the formation of 2',7'-dichlorodihydrofluorescein (DCFH). In the presence of ROS/RNS, DCFH is easily oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF), which can be easily quantified and which fluorescence intensity is proportional to the levels of ROS/RNS [104]. Briefly, HaCaT cells were seeded in 96-well plates (20000 cells/well) and, 24 h after seeding, the cells were pre-incubated with 20 μ M DCFH-DA, protected from light, at 37 °C, in a 5% CO₂ - 95% air atmosphere. After 60 min of pre-incubation, DCFH-DA was removed and the cells exposed to tert-butyl hydroperoxide (0 - 500 μ M) in the presence or absence of the tested formulations (50 μ g/mL) or free drugs (alone or in mixtures of 0,81 μ g/mL, the concentration present in the 50 μ g/mL concentration of the developed formulations), at 37 °C, in a 5% CO₂ - 95% air atmosphere. After 24h of exposure, the fluorescence was measured at 485 nm excitation and 530 nm emission wavelengths in a multi-well plate reader (PowerWaveX BioTek Instruments, United States of America). Four independent experiments were performed, in triplicate.

3.2.10.4 Statistical analysis

All statistical calculations were performed using the GraphPad Prism 8 for Windows (GraphPad Software, San Diego, CA, USA). The normality of the data distribution was assessed using the KS, D'Agostino & Pearson omnibus and Shapiro-Wilk normality tests. For data with a parametric distribution One-way ANOVA was used to perform the statistical comparisons, followed by the Dunnett's multiple comparisons test. For data with a non-parametric distribution, the Kruskal-Wallis test was used to perform the statistical comparisons, followed by the Dunn's multiple comparisons test. In the evaluation of the antioxidant effect of the formulations, the comparisons were performed using Two-way ANOVA followed by the Tukey's multiple comparisons test. Details of the performed statistical analysis are described in the figure legend. Differences were considered to be significant for *p* values lower than 0,05.

Chapter 4: Results and Discussion

4.1 Formulation studies

4.1.1 Lipid screening

The lipid screening consists in analyzing the dissolution capacity of the natural compounds in the available lipids. These lipids were selected due to their biocompatibility with the skin since the aim of this work is to develop a skin formulation. EGCG was the first natural compound tested. The results are presented in the following table, where the negative sign means that there were no crystals of compound formed after each 15-minute interval and the positive sign means that there were visible crystals of the compound.

Table 3. Dissolution capacity of EGCG in the solid lipids available.

	Solid lipids essay (1:100)			
	15 min	30 min	45 min	60 min
Gelucire® 50/13	-	-	-	-
Compritol® HD5 ATO	-	-	-	-
Softisan® 100	-	-	-	-
Precirol® 5 ATO	+	+	+	+
Stearic acid	+	+	+	+
Softisan® 154	+	+	+	+
Dynasan® 118	+	+	+	+
Apifil®	+	+	+	+
Glyceryl monostearate	+	+	+	+
Gelucire® 43/01	+	+	+	+
Witepsol® E76	+	+	+	+
Cetyl palmitate	+	+	+	+
Gelucire 39/01	+	+	+	+
Suppocire DM Pellets	+	+	+	+
Suppocire NA15 pellets	+	+	+	+

Table 3 shows that only three lipids were able to 100% dissolve EGCG: gelucire® 50/13, compritol® HD5 ATO and softisan® 100. In Annex A- Lipid screening, images of EGCG samples are presented, where it is possible to easily see the dissolution and non-dissolution

of the natural compound in two of the studied lipids. Then, for resveratrol, only the three lipids that dissolved the EGCG were tested. In Table 4, are presented the dissolution results.

Table 4. Dissolution capacity of resveratrol in the solid lipids selected.

Solid lipids essay (1:100)				
	15 min	30 min	45 min	60 min
Gelucire® 50/13	+	-	-	-
Compritol® HD5 ATO	+	+	+	+
Softisan® 100	+	+	+	+

As it can be seen from the table above, only gelucire® 50/13 was able to dissolve resveratrol in a 1:100 ratio (natural compound: lipid). Both compritol® HD5 ATO and softisan® 100 had a very small number of undissolved particles so it was necessary to try again, but in a 1:200 ratio. In Annex A- Lipid screening, images of resveratrol samples are presented, where it is possible to easily see the dissolution and non-dissolution of the natural compound in two of the studied lipids. In Table 5, it is possible to verify that only compritol® HD5 ATO presented good results.

Table 5. Dissolution capacity of resveratrol in the solid lipids selected (1:200).

Solid lipids essay (1:200)				
	15 min	30 min	45 min	60 min
Compritol® HD5 ATO	+	+	+	-
Softisan® 100	+	+	+	+

Finally, for myricetin only gelucire® 50/13 and compritol® HD5 ATO were tested. Annex A- Lipid screening presents images of the myricetin samples, where it is possible to easily see the dissolution of the natural compound in the studied lipids. Table 6 presents the results obtained for the two lipids.

Table 6. Dissolution capacity of myricetin in the solid lipids selected.

Solid lipids essay (1:100)				
	15 min	30 min	45 min	60 min
Gelucire® 50/13	-	-	-	-
Compritol® HD5 ATO	-	-	-	-

After these experiments, the solid lipids selected were gelucire® 50/13 and compritol® HD5 ATO. So, two possible nanoparticle combinations were obtained: SLN with gelucire® 50/13 or SLN with compritol® HD5 ATO.

4.1.2 Emulsifier compatibility evaluation

To select the best emulsifier (tween 80 or pluronic-F127) and to optimize the production times, the nanoparticles were first produced without the natural compounds. Different combinations of Ultra-Turrax (UT) (0,5; 1; and 2 min) and Sonication (S) (5, 10, 15 and 30 min) times were tested and the samples were visually analyzed and characterized in the DLS.

The first combination to be produced was SLN with gelucire® 50/13 and pluronic-F127. The combinations between ultra-turrax and ultrasonication times tested for this first type of nanoparticles are presented in Table 7, as well as the solution characteristics.

Table 7. Ultra-Turrax (UT) and sonication (S) times tested for gelucire 50/13 and pluronic-F127, as well as the solution characteristics.

SLN: Gelucire® 50/13 + Pluronic® F-127				
S (min)	UT (min)	0,5	1	2
5		Consistency and colour not acceptable	-	-
10		Consistency and colour not acceptable	-	-
15		Consistency and colour not acceptable	-	-
30		Consistency and colour not acceptable	-	-

As can be seen, four combinations were produced maintaining the ultra-turrax time constant and varying the sonication time. The obtained emulsions presented a colour and consistency not acceptable, not having the usual white and creamy consistency. Since the visual appearance was not satisfactory, the nanoparticles were not analyzed in the DLS and the rest of the combinations were not produced. So, this combination of SLN with gelucire® 50/13 and pluronic-F127 was discarded.

Then, another SLN with gelucire® 50/13 were made, but this time with tween 80. The tested combinations and their characteristics are presented in Table 8.

Table 8. Ultra-Turrax (UT) and sonication times (S) tested for gelucire 50/13 and tween 80, as well as the solution characteristics.

SLN: Gelucire® 50/13 + Tween 80				
S (min)	UT (min)	0,5	1	2
5		Consistency and colour not acceptable	-	-
10		-	-	-
15		-	-	-
30		Consistency and colour not acceptable	-	Consistency and colour not acceptable

In this attempt, three combinations were performed: the extreme conditions (lowest UT and S times and the longest UT and S times) and one more with UT=0,5 min and S=30 min. Once again, the samples had a color and consistency not acceptable so, it was thought that this could be happening due to the lipid, since it is the main excipient in the formulation, and this occurred with the two emulsifiers tested.

The third combination to be performed was SLN with compritol® HD5 ATO and tween 80. In the following table are presented the samples produced for this type of nanoparticle.

Table 9. Ultra-Turrax (UT) and sonication (S) times tested for compritol HD5 ATO and tween 80, as well as the solution characteristics (size, zeta potential (ZP) and Pdl).

SLN: Compritol® HD5 ATO + Tween 80				
S (min)	UT (min)	0,5	1	2
5		Size: 81,5%- 1844 nm and 18,5%- 90 nm Pdl: 0,631 ZP: 37,1%- (-13,1) mV and 62,9%-14,8 mV	-	-
10		-	-	-
15		-	-	Solidified
30		Size: 92,2%- 2025 nm and 7,8%- 63 nm Pdl: 0,560 ZP: -6,39 mV	-	Solidified

In this case, four combinations were performed: the extreme UT and S times and two more combinations. From Table 9, it is possible to observe that two combinations of ultra-turrax and sonication times led to the solidification of the emulsions, that were excluded in the day after the preparation. The remaining combinations were analyzed in the DLS but the nanoparticles presented more than one peak and a Pdl above 0,3, which indicated that the emulsions were not monodispersed, having the highest % of the nanoparticles with more than

1000 nm. As mentioned before, the aim of the formulation is to deliver the active compounds to the upper skin layers, acting as a cosmetic formulation. In this type of formulations, the nanoparticles should have between 200 nm and 300 nm, so this type of nanoparticles were not suitable to be used [17].

The next combination was SLN with compritol® HD5 ATO and pluronic-F127. The nanoparticles obtained from the different combinations of UT and S times are presented and characterized in Table 10.

Table 10. Ultra-Turrax (UT) and sonication (S) times tested for compritol HD5 ATO and pluronic F-127, as well as the solution characteristics (size, zeta potential (ZP) and Pdl).

SLN: Compritol® HD5 ATO + Pluronic-F127				
S (min)	UT (min)	0,5	1	2
		Size: 83%- 716 nm and 16%- 138 nm Pdl: 0,409 ZP: -0,6 mV		Size: 350 ± 17 nm Pdl: 0,353 ± 0,007 ZP: -0,4 ± 0,3 mV
	5		-	
	10	-	-	-
	15	-	-	Size: 206 ± 5 nm Pdl: 0,21 ± 0,01 ZP: -1,9 ± 0,4 mV
	30	Size: 315 ± 10 nm Pdl: 0,434 ± 0,003 ZP: -0,5 ± 0,1 mV	-	Size: 96 ± 33 nm Pdl: 0,307 ± 0,004 ZP: -1,7 ± 0,7 mV

This time, five combinations of ultra-turrax and ultrasonication times were developed and analyzed in the DLS, in the day after. In this case, the nanoparticle's size varied according to the different combinations of ultra-turrax and sonication times: less time in the ultra-turrax and sonicator gave origin to larger particles and more exposition time to these equipments led to smaller particles. The combination where the exposition time was the longest (UT= 2 min, S=30 min) led to the smallest particles with 96 ± 33 nm. As already mentioned, this formulation is for topical cosmetic application and nanoparticles with less than 100 nm can reach the systemic circulation, which is not supposed to happen in this case [14]. This way, this batch was discarded.

In the remaining four combinations, three of them presented sizes higher than 300 nm and Pdl's above 0,3, which was an indicative of polydispersity in the formulation [17], [87]. So, these combinations were discarded. One combination achieved the desired characteristics: 2 minutes in the ultra-turrax and 15 min in the ultrasonicator. These nanoparticles were analyzed in the DLS and their size was 206 ± 5 nm in the day after the production. Furthermore, the Pdl was below 0,3, which was an indicative that the

formulation was monodispersed. So, this combination was suitable for the final aim of the formulation.

The chosen combination was studied to assess its stability over time. The nanoparticles were analyzed in the DLS (size, Pdl and zeta potential) at day 1, 7, 14 and 138. In Table 11, are presented the results of the stability studies.

Table 11. DLS characterization (size, Pdl and zeta potential) for the chosen combination at day 1, 7, 14 and 138.

Compritol® HD5 ATO + Pluronic-F127			
	Size (nm)	Pdl	Zeta Potential (mV)
Day 1	206 ± 5	0,21 ± 0,01	-1,9 ± 0,4
Day 7	228 ± 4	0,27 ± 0,03	-3,1 ± 0,7
Day 14	233 ± 6	0,26 ± 0,02	-3 ± 2
Day 138	237 ± 5	0,236 ± 0,002	-2,5 ± 0,4

Analyzing Table 11, it is possible to observe that between day 1 and day 7 the size increased 22 nm ($p < 0,05$), but after that day the size remained constant ($p > 0,05$). This small increase occurred probably because the particles were not fully stabilized in the day after their production. The Pdl was below 0,3 (monodispersed formulation) and the zeta potential was stable over time ($p > 0,05$). So, this combination was selected to encapsulate the natural compounds.

The lipid screening and emulsifier optimization are crucial experimental parts to develop nanoparticles with the desired characteristics and will influence the stability of the formulation, the success of the drug entrapment, its release, among other factors. The chosen lipid, compritol® HD5 ATO, is mixture of glyceryl and polyethylene glycol behenate. This fatty acid is solid at room and body temperature with a melting point that ranges from 60 °C to 67 °C [105].

The selected emulsifier, pluronic-F127, is a tri-block copolymer of poly(ethylene oxide), poly(propylene oxide) and another poly(ethylene oxide). This synthetic polymer has a hydrophilic part (ethylene oxide) and a hydrophobic part (propylene oxide) resulting in an amphiphilic structure, meaning that pluronic-F127 acts as an emulsifier, forming micelles in aqueous solutions [106]. Emulsifiers have an important role in the nanoparticle's formation, since they are used to stabilize the lipids after cooling, reducing the surface tension and avoiding nanoparticle's aggregation. In Figure 17, the chemical structure of the emulsifier is presented.

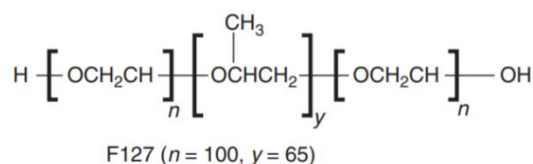


Figure 17. Chemical structure of pluronic-F127.

4.2 Nanoparticle characterization and stability studies

4.2.1 DLS characterization

The nanoparticles encapsulating the natural compounds were then produced. In Annex B- Nanoparticle production, are presented pictures of the three types of nanoparticle batches. A stability study was performed throughout 30 days, where the size, zeta potential and Pdl were analyzed. In Table 12, 13 and 14 are presented the mean and standard deviation (SD) of the size, zeta potential and Pdl for each type of nanoparticle (EGCG, resveratrol and myricetin). In Annex C- Nanoparticle characterization and stability studies, are presented DLS outputs comparing the size of the nanoparticles at day 1 and day 30.

Table 12. Size, Pdl and zeta potential of the nanoparticles with EGCG (mean $\pm$ SD, $n=3$).

Compritol® HD5 ATO + Pluronic-F127 + EGCG			
	Size (nm)	Pdl	Zeta Potential (mV)
Day 1	215 $\pm$ 7	0,224 $\pm$ 0,006	-2 $\pm$ 2
Day 7	234 $\pm$ 15	0,256 $\pm$ 0,004	-4,7 $\pm$ 0,7
Day 14	248 $\pm$ 8	0,268 $\pm$ 0,006	-6,8 $\pm$ 0,9
Day 30	242 $\pm$ 9	0,266 $\pm$ 0,007	-5,7 $\pm$ 0,4

Table 13. Size, Pdl and zeta potential of the nanoparticles with resveratrol (mean $\pm$ SD, $n=3$).

Compritol® HD5 ATO + Pluronic-F127 + Resveratrol			
	Size (nm)	Pdl	Zeta Potential (mV)
Day 1	218 $\pm$ 9	0,23 $\pm$ 0,02	-1,4 $\pm$ 0,6
Day 7	230 $\pm$ 17	0,25 $\pm$ 0,03	-2,7 $\pm$ 0,5
Day 14	240 $\pm$ 6	0,26 $\pm$ 0,02	-1,4 $\pm$ 0,5
Day 30	237 $\pm$ 16	0,26 $\pm$ 0,03	-1 $\pm$ 1

Table 14. Size, Pdl and zeta potential of the nanoparticles with myricetin (mean $\pm$ SD, n=3).

Compritol® HD5 ATO + Pluronic-F127 + Myricetin			
	Size (nm)	Pdl	Zeta Potential (mV)
Day 1	187 $\pm$ 5	0,196 $\pm$ 0,005	-3,7 $\pm$ 0,3
Day 7	211 $\pm$ 2	0,25 $\pm$ 0,02	-2,7 $\pm$ 0,4
Day 14	215 $\pm$ 10	0,27 $\pm$ 0,01	-5,8 $\pm$ 0,8
Day 30	211 $\pm$ 4	0,265 $\pm$ 0,004	-4,7 $\pm$ 0,4

Regarding the size, EGCG nanoparticles demonstrated to be similar between day 1 and day 7 ($p > 0,05$), but when comparing day 1 with day 30, significant changes occurred ($p < 0,05$). Overall, for resveratrol nanoparticles, no significant changes in size occurred ($p > 0,05$). At last, for the myricetin nanoparticles it was observed a slight increase in size between day 1 and day 7 ($p < 0,05$). After day 7, the size of all nanoparticles did not change significantly until the end of the stability study (day 30) ($p > 0,05$). Furthermore, it was possible to conclude that at the end of the study, all nanoparticles maintained the ideal size and proved to be stable over time, not being observed any signs of nanoparticle aggregation.

In terms of Pdl, in all measurements, all types of nanoparticles presented a Pdl below 0,3, which is the threshold value for this type of nanoparticles [87]. Thus, it is possible to conclude that all samples are monodisperse, which means that in the same formulation there were no significant size variations.

Finally, in terms of zeta potential, all samples obtained a negative and very close to zero value. Usually, particles with zeta potentials less than -30 mV or greater than 30 mV are typically considered stable, with no aggregation occurring [107]. Rigon *et al.* (2016), obtained similar zeta potential values for resveratrol loaded lipidic nanoparticles with Pluronic-F127 as an emulsifier [81]. This emulsifier stabilizes steric and electrostatic repulsions, maintaining the solution's stability but decreasing the zeta potential values of the nanoparticles. Thus, these zeta values do not mean instability or aggregation. Overall, the zeta potential of each type of nanoparticle did not change significantly ($p > 0,05$).

Comparing these results with the ones obtained for the unloaded nanoparticles (Table 11), it is possible to conclude that both resveratrol and EGCG-loaded nanoparticles presented a similar size and zeta potential to the unloaded nanoparticles in all measurements ($p > 0,05$). For the myricetin-loaded nanoparticles, this was not observed since a decrease in size occurred, in day 1 and day 7 ($p < 0,05$). These results suggest that encapsulating myricetin can lead to nanoparticles with smaller sizes. In terms of zeta potential, there were no significant changes detected in most measurements ($p > 0,05$).

4.2.2 TEM characterization

The nanoparticles were also characterized in the transmission electron microscope. In Figure 18, are presented two images of the EGCG-loaded nanoparticles obtained with TEM.

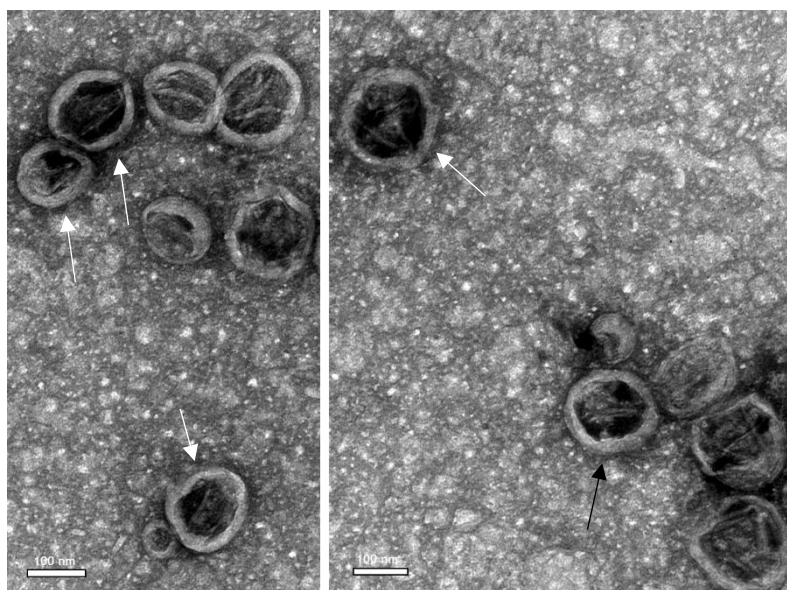


Figure 18. TEM images of the EGCG-loaded nanoparticles.

From the figure shown above, it is possible to see that the EGCG nanoparticles have a round appearance as expected. Furthermore, the particles have a size slightly smaller than the values obtained in the DLS, which may be due to the nanoparticle impregnation technique for analysis in TEM. Some authors also reported mean sizes determined by TEM slightly smaller than the ones obtained by DLS, due to the interference of the dispersant in the hydrodynamic diameter [94]. In Figure 19, are presented two images of the resveratrol-loaded nanoparticles obtained.

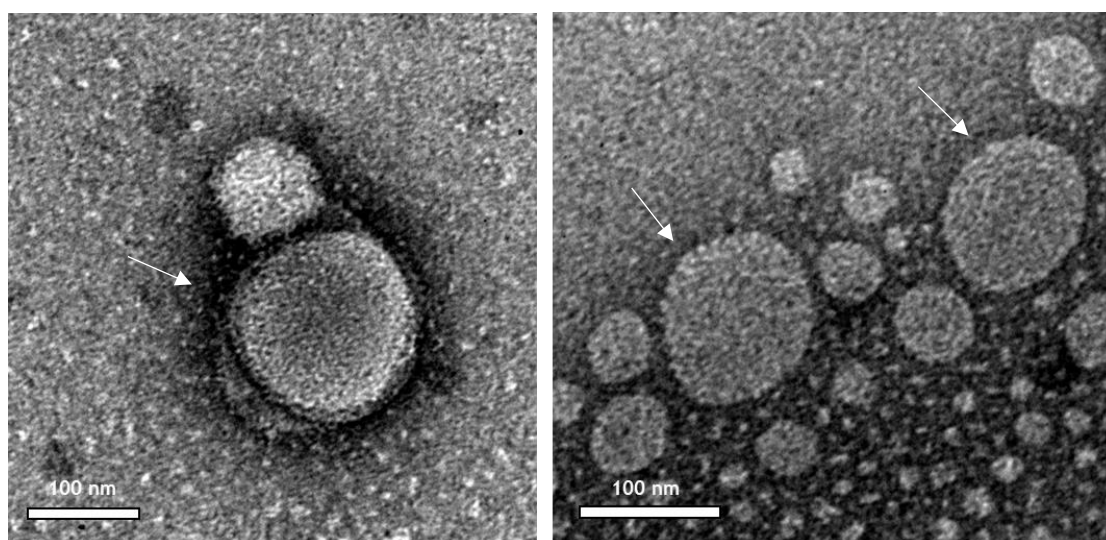


Figure 19. TEM images of the resveratrol-loaded nanoparticles.

In this case, the nanoparticles also present a round shape but, in terms of size, they vary having an average size of about 200 nm. This is expected, since there is always a polydispersity index associated to the formulation. In Figure 20, are presented two images of the myricetin-loaded nanoparticles obtained.

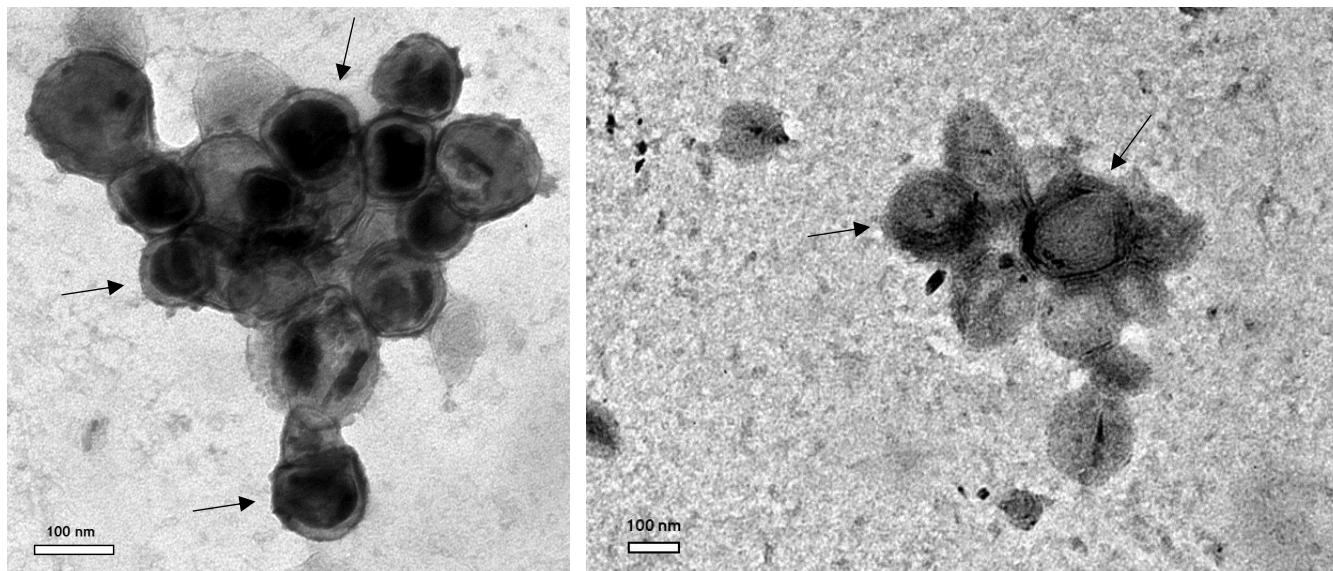


Figure 20. TEM images of the myricetin-loaded nanoparticles.

Analyzing the figure, it is possible to conclude that the particles have a round aspect and their size, once again, varies between 100 nm and around 150 nm. Once again, these smaller sizes compared with the DLS values can occur due to the impregnation technique applied.

4.3 Compound characterization

Firstly, the spectrums (200-600 nm) of the three natural compounds were determined to assess the maximum absorption wavelength of each compound through UV-Vis spectroscopy. This technique is commonly used to characterize phenolic compounds since they present a phenolic ring capable of absorbing UV light [108].

EGCG's UV-Vis spectrum obtained, had one characteristic peak at 274 nm, which is similar to values found in the literature: 272 nm in water and 276 nm in ethanol [109, 110]. The fact that the solvent is 70% (v/v) aqueous ethanol solution can be the cause for the small variance between 274 nm and the other two values.

For resveratrol, the maximum absorption wavelength obtained was at 305 nm which is in accordance with the literature [111, 112]. For myricetin, the complete spectrum had two very distinct peaks, but the one chosen to quantify the natural compound was at 377 nm, which is similar to values found in the literature [113, 114]. The three complete spectrums are presented in Annex D- Compounds characterization.

To quantify each compound, calibration curves were produced, where 70% (v/v) aqueous ethanol solution with known concentrations of each natural compound were prepared in triplicate. The absorbance of the samples was measured at the maximum wavelength of each compound. The calibration curves are displayed in Annex D- Compounds characterization.

Every calibration curve exhibited a high coefficient of determination (R^2), higher than 0,995, which represents a valid linear relationship between concentration and absorbance.

4.4 Encapsulation efficiency and loading capacity

Through the calibration curve previously obtained, it was possible to quantify the amount of natural compound present inside the nanoparticles. Through the encapsulation efficiency and loading capacity it is possible to verify if the lipid chosen for the nanoparticles production was the correct one and if the production process was successfully executed. In Table 15 and 16, are presented the mean and standard deviation (SD) of the encapsulation efficiency and loading capacity values for each type of nanoparticle. In Annex E- Encapsulation efficiency and loading capacity, there is an example of the calculation made to determine the presented values.

Table 15. Encapsulation efficiency for each type of nanoparticle (mean $\pm$ SD, n=3).

Encapsulation efficiency (%)		
EGCG	Resveratrol	Myricetin
(90 $\pm$ 5)%	(95,6 $\pm$ 0,9)%	(98 $\pm$ 2)%

Table 16. Loading capacity for each type of nanoparticle (mean $\pm$ SD, n=3).

Loading capacity (%)		
EGCG	Resveratrol	Myricetin
(0,96 $\pm$ 0,05)%	(1,01 $\pm$ 0,01)%	(1,03 $\pm$ 0,02)%

From Table 15 it is possible to conclude that the encapsulation efficiencies were high for all natural compounds. These values were expected since both resveratrol and myricetin are lipophilic compounds and, therefore, would have affinity to the lipid part, hence their high encapsulation efficiency [55, 62]. Since lipid nanoparticles are capable of load both lipophilic and hydrophilic drugs, the high value obtained for EGCG encapsulation was also expected [14].

Regarding the loading capacity, the solubility of the active compound in the selected lipid is essential to obtain a high drug loading [75, 115]. The proper selection of lipid and

emulsifier through the lipid screening and emulsifier optimization process, positively affected the loading capacity, hence the obtained values are so close to the theoretical loading capacity (1,058). These encapsulation and drug loading results demonstrate that lipid nanoparticles can be effective nanocarriers for the encapsulation and transport of natural compounds that have a low bioavailability. Besides this, these values prove that the lipid screening phase, the emulsifier optimization and the production of nanoparticles were carried out with success.

These results are in accordance with other similar formulations that were previously described. The encapsulation of these natural compounds usually vary from 60% to 100% depending on the quantity of compound in solution, the production conditions, the solubility of the drug in the lipids used, the compatibility between the lipids and emulsifiers selected, the storage conditions, among others. For example, Radhakrishnan *et al.* (2016), obtained encapsulation efficiencies between 67,2% and 89,5% for EGCG-SLN loaded nanoparticles of glycerol monostearate with soya lecithin and pluronic-F68, depending on the concentration of EGCG in the formulations [116]. Shtay *et al.* (2019) obtained an optimum value of 68,5%, also in SLN encapsulating EGCG, constituted by cocoa butter with mono- and diglycerides of fatty acids and sodium stearyl-2-lactylate [117].

For resveratrol, Yadav *et al.* (2018) obtained 91,25% of encapsulation efficiency in SLN constituted by stearic acid and lecithin [118]. Pandita *et al.* (2014) obtained 88,9% in resveratrol-loaded solid lipid nanoparticles of stearic acid and pluronic-F68, values similar to the ones obtained in this study [119]. For myricetin, Gaber *et al.* (2017) obtained 90,93% and 84,5% for SLN produced with different lipids (gelucire 39/01 or gelucire 50/13 and compritol 888 ATO) with pluronic-F127 [120].

4.5 *In vitro* release

Natural compounds as EGCG, resveratrol and myricetin are known for their low bioavailability, limited stability and degradation if exposed to certain external conditions [47, 55, 62]. To achieve a prolonged release of sensitive active compounds with a scarce skin penetration, their encapsulation in nanocarriers is essential and it is one of the main advantageous of this transport systems [70]. Thus, *in vitro* drug release is an important assay which allows a deeper understanding of the nanoparticles as delivery carriers. The release profile was evaluated for each natural compound, where the three types of nanoparticles were subjected to conditions which simulated physiological conditions (buffer at physiological pH, temperature, and agitation). The EGCG, resveratrol and myricetin release profiles are presented in Figure 21, 22 and 23. In Annex F- *In vitro* release studies, a calculation example of the drug release profile values is presented.

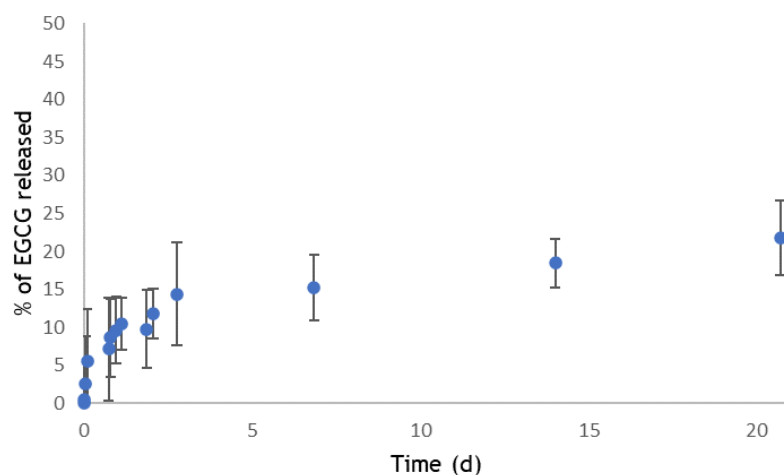


Figure 21. *In vitro* release profile of EGCG nanoparticles in HEPES buffer (mean $\pm$ SD, n=3).

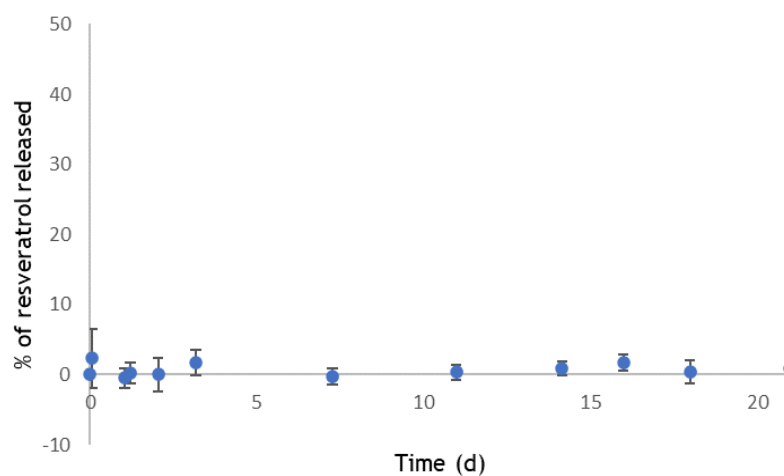


Figure 22. *In vitro* release profile of resveratrol nanoparticles in HEPES buffer (mean $\pm$ SD, n=3).

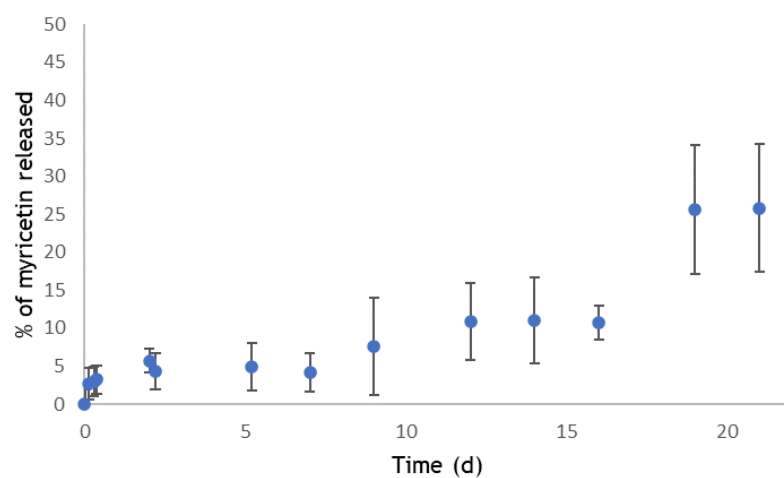


Figure 23. *In vitro* release profile of myricetin nanoparticles in HEPES buffer (mean $\pm$ SD, n=3).

For all release profiles, the assay was carried out for approximately 21 days because the release of the natural compound was very slow. Regarding EGCG, approximately 21 days after the release started, $(22 \pm 5)\%$ of the natural compound was released and for myricetin $(26 \pm 8)\%$. Concerning resveratrol, its release profile proved to be very slow and practically null, $(1 \pm 1)\%$.

The results obtained in this assay can, once again, be related to the hydrophobicity of the compounds in question. As already mentioned, the compounds have affinity with the lipidic part and, in particular with the chosen lipid since a lipid screening was carried out to study the best lipid to dissolve the three natural compounds. So, the compounds will have affinity to stay in the lipidic part rather than transiting to the release medium (aqueous medium), leading to a very slow release. Furthermore, these nanoparticles may present a drug-enriched core, which means that the active compound is surrounded by a lipid layer and, therefore, the release will be delayed [72, 121].

The release profile of lipid nanoparticles is deeply influenced by several factors such as the lipid type, the concentration of emulsifier, the solubility and concentration of the natural compound, the production method, the release medium, among other factors [70], [121]. For example, for Chen *et al.* (2017) the NLC (solid lipids used: cetyl palmitate, compritol 888 ATO or phospholipon 80; sesame oil as liquid lipid and tween 80 as emulsifier) released 70% of the encapsulated resveratrol in the first 24 h, demonstrating a quick and effective release [78]. Although NLC are lipid nanoparticles, the internal structure is different from SLN, and this will influence the release profile. On the other hand, some authors experienced lower release profiles such as Miao *et al.* (2013), where the lipophilic drug paclitaxel (PTX) was loaded in SLN (made up by glyceryl monostearate and poloxamer 188) and had a release of 41,6% after 24 h [122]. To improve the % of release, several attempts were made where the type of medium was changed. The experimental process remained the same as described above.

Before HEPES, PBS was the first medium to be tested as it is the most used medium to simulate physiological conditions. However, when this buffer was mixed with pure ethanol to read the absorbance, the salts of the PBS precipitated and influenced the results. Then, the HEPES buffer was used and gave origin to the present approach which led to values lower than expected. So, the third attempt consisted in adding 1% of tween 80 to the medium, because the presence of an emulsifier in the external environment could enhance the solubilization of the active compound [123]. The release proved to be very slow once again. Another procedure described by Wang *et al.* (2012) was carried out, where 10% of tween 80 was added to the HEPES medium instead of 1%, since it increased the % of release of curcumin (hydrophobic drug) [124]. This time, this increase in the emulsifier led to particle

aggregation and filter breakage. Thus, the particles and the emulsifier began to pass through the filter giving rise to problems in the absorbance reading, as this should not occur. Afterwards, a different medium, described by Hu *et al.* (2005), was tested. A sodium dodecyl sulfate solution (SDS) (1% wt) in HEPES was used, but once again, this approach led to ultrafilter rupture. Finally, one last experiment was performed with the SDS (1% wt) in HEPES medium but instead of using an ultrafilter, a dialysis membrane (Molecular weight cutoff of 12 kDa) was tested. This membrane was equilibrated in ultrapure water for 30 min and in the release medium for another 30 min, before the experiment begun. Although the membrane was not damaged as the ultrafilter, the release profile was slow as well.

Despite these results, this does not mean that the particles cannot release the compound in the *stratum corneum*. As already mentioned, the aim is for the compounds to diffuse through the superficial layers of the skin, which are mainly composed of lipids and corneocytes, so the formulation will be more compatible with this medium than with the buffer (aqueous medium). Furthermore, a prolonged release is more advantageous for cosmetic formulations, since the compound will be released slowly, not requiring multiple applications throughout the day.

4.6 Antioxidant assay

An *in vitro* antioxidant assay was performed to assess the antioxidant activity of each compound and conjugations between the three compounds. The DPPH assay was tested using methanol as the solvent, in which was easy to dissolve the compounds. DPPH is a stable organic radical widely used in the quantification of radical-scavenging capacity (RSC). This technique has many advantages such as credible sensitivity, simplicity and feasibility [125]. In Table 17 are presented the mean and standard deviation (SD) of the antioxidant activity percentages obtained for the natural compounds.

Table 17. Antioxidant activities of EGCG, resveratrol (R), myricetin (M) and mixtures of EGCG and resveratrol, EGCG and myricetin, resveratrol and myricetin and the three of them together (mean $\pm$ SD, n=3).

Scavenging activity (%)	EGCG	R	M	EGCG + R	EGCG + M	R + M	EGCG + R + M
Obtained	26 $\pm$ 1,1	6 $\pm$ 2	14 $\pm$ 2	20 $\pm$ 3	22 $\pm$ 3	12 $\pm$ 3	18 $\pm$ 1
Theoretical	-	-	-	16,2	20,1	9,9	15,4

As it can be seen, all three natural compounds presented an antioxidant activity at the studied concentration. All three were tested in the same conditions in order to be compared and, in this experiment EGCG presented the highest antioxidant activity and resveratrol the lowest one ($p < 0,05$). This type of assay is very susceptible to the conditions

under which it is performed: it depends on the concentrations of the compounds, the concentration of the DPPH solution, the pH, the solvent used, the presence of oxygen, the presence of light, among other factors [126].

After analyzing the three compounds by themselves, the mixtures were made to evaluate possible synergic effects. When different bioactive compounds are mixed their antioxidant properties can behave in three different ways: if the interaction is the sum of the individual effects, which is the most simplistic case, it is called “null interaction”; if the antioxidant activity is greater than the sum it is called synergic effect; and if the interaction leads to lesser values than the sum it is called antagonism [127]. Since the future aim is to encapsulate in lipid nanoparticles the total phenolic extract, it is important to analyze the interactions between the three main components in order to estimate possible advantages in using the complete extract.

Once again, the mixtures were made to have a total concentration of 4 μM to become possible to compare the results with a theoretical antioxidant activity value that the mixture was expected to have, also presented in the Table 17. This value is the average between each natural compound’s antioxidant activity.

From Table 17, it is also possible to see that the mixture of EGCG + resveratrol and the mixture of the three compounds presented a synergic effect, in which the experimental value was higher than the average value of the mixtures (theoretical value) indicating two promising interactions. These combinations were chosen to be evaluated in immortalized human cells. The other two mixtures (EGCG + myricetin and resveratrol + myricetin) presented an approximated null interaction since the experimental value obtained was very close to the theoretical one, taking into account the standard deviation.

4.7 Accelerated Stability

The accelerated stability assay was performed to predict possible changes that can occur during storage. These changes can be seen by visual examination, for example the presence/absence of phase separation, creaming or flocculation, and through DLS measurements, where the size, zeta potential and Pdl are analyzed. In Annex G- Accelerated stability, are presented pictures of the samples taken after each centrifugation. In Tables 18, 19 and 20 are displayed the mean and standard deviation (SD) of the size, Pdl and zeta potential values for each sample after the two centrifugations.

Table 18. Size, Pdl and zeta potential of the nanoparticles loaded with EGCG after the accelerated stability assay (mean $\pm$ SD, n=3).

EGCG nanoparticles			
	Size (nm)	Pdl	Zeta Potential (mV)
30 min centrifugation	221 $\pm$ 29	0,27 $\pm$ 0,01	-5 $\pm$ 1
1 h centrifugation	218 $\pm$ 8	0,26 $\pm$ 0,01	-6 $\pm$ 1

Table 19. Size, Pdl and zeta potential of the nanoparticles loaded with resveratrol after the accelerated stability assay (mean $\pm$ SD, n=3).

Resveratrol nanoparticles			
	Size (nm)	Pdl	Zeta Potential (mV)
30 min centrifugation	216 $\pm$ 7	0,26 $\pm$ 0,02	-1,6 $\pm$ 0,2
1 h centrifugation	216 $\pm$ 11	0,28 $\pm$ 0,02	-2 $\pm$ 1

Table 20. Size, Pdl and zeta potential of the nanoparticles loaded with myricetin after the accelerated stability assay (mean $\pm$ SD, n=3).

Myricetin nanoparticles			
	Size (nm)	Pdl	Zeta Potential (mV)
30 min centrifugation	186 $\pm$ 8	0,257 $\pm$ 0,005	-4 $\pm$ 1
1 h centrifugation	190 $\pm$ 8	0,258 $\pm$ 0,005	-3 $\pm$ 1

From the tables above, it is possible to conclude that no significant alterations occurred in terms of size, Pdl and zeta potentials between the two centrifugations ($p > 0,05$). These results demonstrate that the 30 min and 1 h centrifugations did not significantly affect the samples quality. Visually, the samples appeared to be stable and homogeneous after each centrifugation. No signs of creaming, flocculation or phase separation were detected, indicating a good long-term stability of the tested formulations, as can be seen from the pictures presented in annex. These results suggest that these nanoparticles are adequate for incorporation in cosmetic formulations.

4.8 *In vitro* cytotoxicity assay and antioxidant assay in immortalized human cells (HaCaT)

4.8.1 Cytotoxicity of the formulations

Through the neutral red uptake and resazurin reduction assays, the cytotoxicity of the developed formulations (0 - 1000 $\mu\text{g/mL}$) was evaluated, 24 h after exposure. For that purpose, HaCaT cells were used as an *in vitro* model. The cytotoxicity assays were performed

to select non-cytotoxic concentrations to be used in the evaluation of the presumed effects of the formulations against t-BHP-induced oxidative stress.

In Figure 24, a concentration-dependent reduction in NR uptake was observed for all the formulations, including the unloaded formulation. In accordance with the obtained data, no significant cytotoxicity was detected 24 hours after exposure to the 50 µg/mL concentration of the unloaded, EGCG, Resveratrol and Myricetin formulations, while a significant reduction in NR uptake was observed for concentrations equal or higher than 100 µg/mL. For the EGCG + Resveratrol and the EGCG + Resveratrol + Myricetin formulations, a significant reduction in the NR uptake was detected for all the tested concentrations. In fact, NR uptake slightly decreased to 91,8 and 91,7 % 24 h after exposure to 50 µg/mL EGCG + Resveratrol and 50 µg/mL EGCG + Resveratrol + Myricetin, respectively.

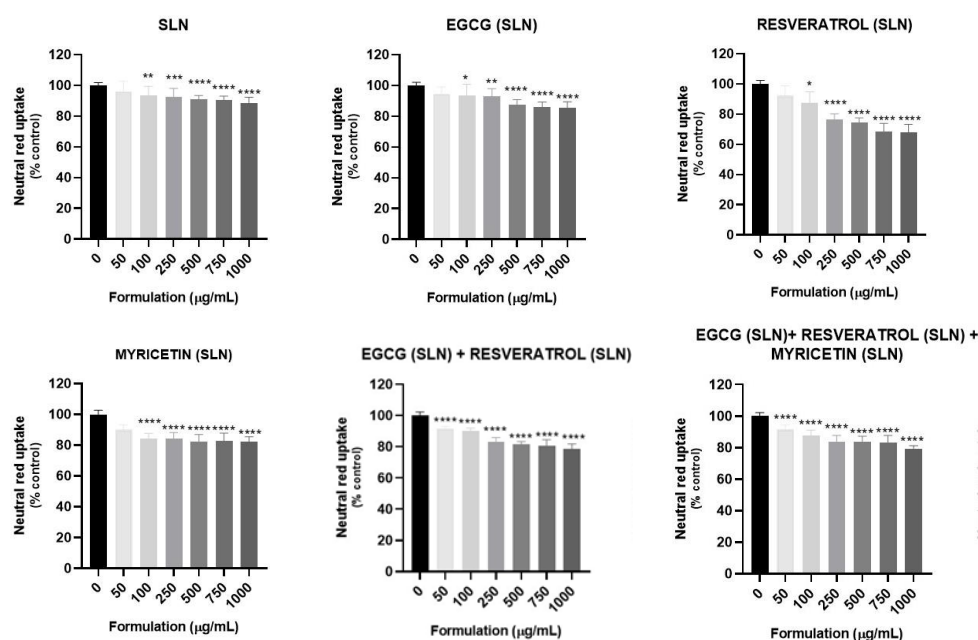


Figure 24. Cytotoxicity of the developed formulations evaluated in HaCaT cells by the neutral red uptake assay 24 hours after exposure. Results are expressed as mean $\pm$ SD from 4 independent experiences, performed in triplicate. Statistical comparisons were made using One-way ANOVA followed by the Tukey's multiple comparisons test (for data with a parametric distribution) or using the Kruskal-Wallis test followed by the Dunn's multiple comparisons test (for data with a non-parametric distribution) (* $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$; **** $p < 0,0001$ for each formulation vs. 0 µg/mL). In all cases, p values $< 0,05$ were considered significant.

The resazurin reduction assay was also performed, and the cytotoxicity was evaluated 24 hours after exposure. Nevertheless, as can be observed in Figure 25, this cytotoxicity assay proved to be less sensitive than the NR uptake assay in the evaluation of the cytotoxicity of the developed formulations. In fact, no significant effects on REZ reduction were observed 24 hours after exposure to unloaded, EGCG and Myricetin formulations, at all the tested concentrations (0 - 1000 µg/mL). For the Resveratrol formulation, a significant decrease in REZ reduction was detected for concentrations equal or higher than 500 µg/mL

(REZ reduction significantly decreased to 93,9, 90,9 and 76,4% 24 hours after exposure to the 500, 750 and 1000 $\mu\text{g/mL}$ concentrations). Similar results were detected for the mixture EGCG + Resveratrol formulation. For the other formulation also containing Resveratrol, namely the mixture of the three natural compounds, a significant decreased in REZ reduction was observed for the two highest tested concentrations (REZ reductions significantly decreased to 90,5 and 88,8%, 24 h after exposure to the 750 and 1000 $\mu\text{g/mL}$ concentrations).

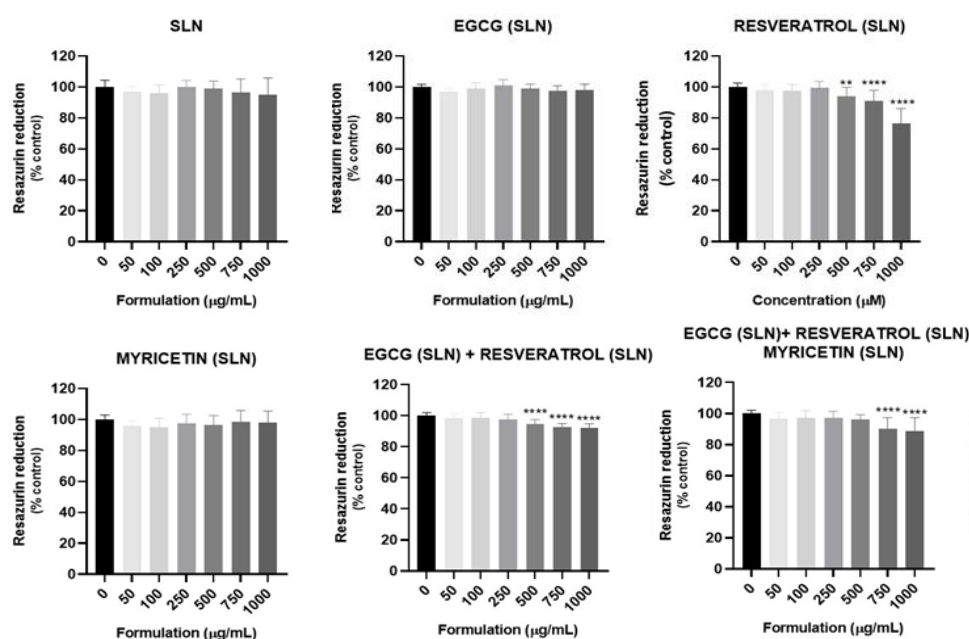


Figure 25. Cytotoxicity of the developed formulations evaluated in HaCaT cells by the resazurin reduction assay 24 hours after exposure. Results are expressed as mean $\pm$ SD from 4 independent experiences, performed in triplicate. Statistical comparisons were made using One-way ANOVA followed by the Tukey's multiple comparisons test (** $p < 0,01$; *** $p < 0,001$; **** $p < 0,0001$ for each formulation vs. 0 $\mu\text{g/mL}$). In all cases, p values $< 0,05$ were considered significant.

The 50 $\mu\text{g/mL}$ concentration was selected for the antioxidant assay, since there was no significant cytotoxicity at this concentration, 24 h after exposure. The cytotoxicity evaluation was only performed by the most sensitive assay, the NR uptake assay (according to the results), to assure that this was a non-cytotoxic concentration. In fact, although significantly decreasing for some formulations, NR uptake was always higher than 90% of the control cells (0 $\mu\text{g/mL}$).

The cytotoxicity of the free compounds (alone or in the same mixtures presented in the developed formulations) was also evaluated but only at the 0,81 $\mu\text{g/mL}$ concentration, the final concentration present in the 50 $\mu\text{g/mL}$ concentration of each formulation. As observed in Figure 26, no significant effects on NR uptake were detected 24 hours after exposure to all the tested active compounds, both alone and in combination suggesting that this concentration is non-cytotoxic towards HaCaT cells.

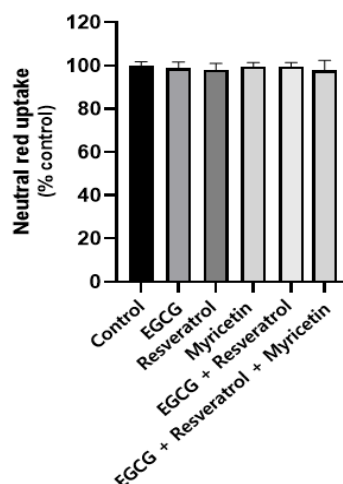


Figure 26. Cytotoxicity of the free drugs (alone or in combination) evaluated in HaCaT cells by the neutral red uptake assay 24 hours after exposure. The compounds were only evaluated at the 0,81 $\mu\text{g/mL}$ concentration, the concentration present in the 50 $\mu\text{g/mL}$ concentration of each formulation. Results are expressed as mean $\pm$ SD from 4 independent experiences, performed in triplicate. Statistical comparisons were made using One-way ANOVA followed by the Tukey's multiple comparisons test. In all cases, p values $< 0,05$ were considered significant.

4.8.2 Evaluation of the protection of the formulations against oxidative stress: effects on t-BHP induced increase in ROS levels

The predicted protective effects of the developed formulations against t-BHP - induced oxidative stress were further studied. For that purpose, HaCaT cells were incubated with t-BHP (0 - 500 μM) in the presence or absence of the formulations (50 $\mu\text{g/mL}$), and ROS/RNS intracellular levels were then quantified 24 hours after exposure. As observed in Figure 27, t-BHP induced a significant and concentration-dependent increase in ROS/RNS intracellular levels. When compared to control cells (0 μM), ROS/RNS levels significantly increased to 119,2, 171,7 and 254,1%, 24 h after exposure to 100, 250 and 500 μM t-BHP, respectively.

Furthermore, the simultaneous exposure to t-BHP and loaded SLN formulations resulted in a significant decreased in ROS/RNS production when compared to t-BHP alone, suggesting a protective antioxidant effect of these SLN formulations against t-BHP-induced oxidative stress. At the concentration of 100 μM t-BHP, only a slight tendency for a protective antioxidant effect was observed. In the following concentrations (250 and 500 μM t-BHP) it was possible to verify a more accentuated effect. From the SLN formulations loaded with just one natural compound, the EGCG-loaded SLN formulation was considered the most effective in decreasing ROS/RNS production (ROS/RNS levels significantly decreased to 138,5 and 172,5% 24 hours after exposure to 250 and 500 μM t-BHP, respectively, in the presence of EGCG SLN, when compared to 171,7 and 254,1% 24 hours after exposure to 250 and 500 μM t-BHP alone). This result was expected since in the DPPH antioxidant test, EGCG was also the compound with the higher antioxidant activity. In addition, from all the SLN-loaded

formulations, the EGCG + Resveratrol combination was the responsible for the highest protection against t-BHP-induced oxidative stress, decreasing ROS/RNS intracellular levels to 123,6 and 160,5% 24 h after exposure to 250 and 500 μM t-BHP, respectively. Once again, this is in accordance with the DPPH antioxidant assay. As expected, the unloaded SLN formulation was not able to decrease the t-BHP-induced ROS/RNS production when compared to t-BHP alone, further suggesting that the observed protective effects observed for all the formulations seem to be a result of the antioxidant effects of the loaded natural compounds.

Besides, although not being the most efficient formulation, the mixture of the three loaded-SLNs formulations still proved to significantly improve the protective effects against oxidative stress. The obtained results suggest that the developed loaded SLNs are adequate for the development of novel cosmetic formulations with antioxidant activity.

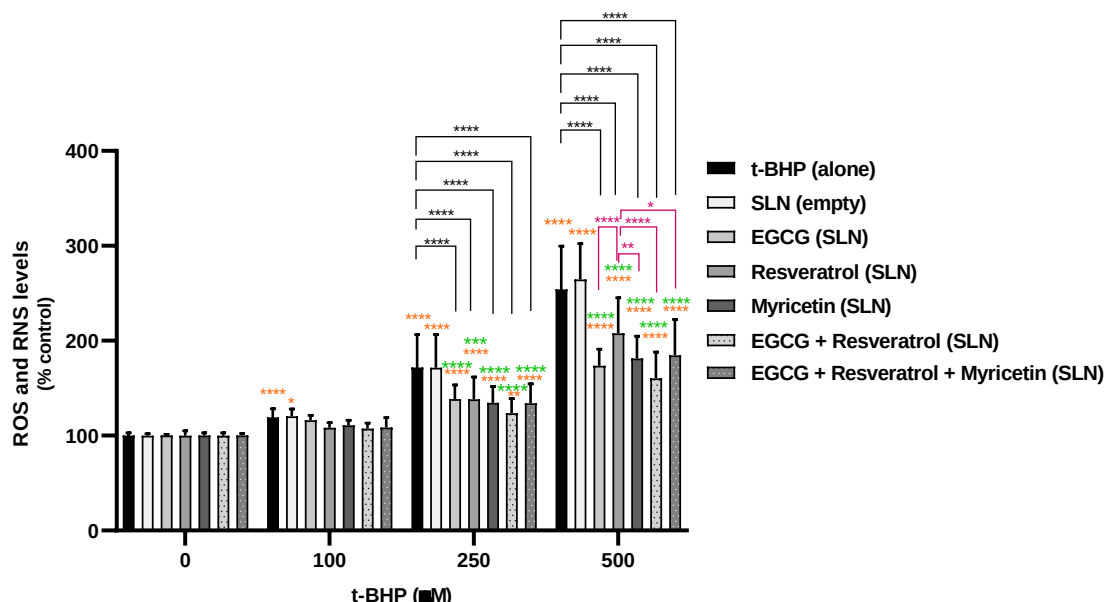


Figure 27. Intracellular levels of ROS/RNS 24 hours after exposure to t-BHP (0 - 500 μM) in the presence or absence of the developed SLN formulations. Results are expressed as mean $\pm$ SD from 6 independent experiences, performed in triplicate. Statistical comparisons were made using Two-way ANOVA followed by the Tukey's multiple comparisons test (* $p < 0,05$; **** $p < 0,0001$; in orange are represented the statistical analysis for each condition versus 0 μM t-BHP; in green are represented the statistical analysis at each t-BHP concentration for the comparison between drug-loaded SLN formulations and empty SLN formulation). In all cases, p values $< 0,05$ were considered significant.

Chapter 5: Conclusions and Future Perspectives

Skin aging is a natural process, but due to external agents or even genetic factors this process is more evident in some people. One way to slow the premature aging of the skin is to decrease the concentration of free radicals in the skin's cells. The use of natural compounds with great antioxidant activity has been gaining the interest of the scientific community and of the pharmaceutical and cosmetic industry due to their ability to scavenge free radicals and, therefore, protect the cell components from being oxidized. However, these natural compounds present low bioavailability and difficulties in crossing the superficial barrier of the skin. One way to overcome these problems is to encapsulate them in lipid nanoparticles that are compatible with the most external layer of the skin, the *stratum corneum*. These nanoparticles are being increasingly used in cosmetic products and have the ability to protect and regulate the compounds penetration in the skin layers. The three natural compounds chosen in this project (EGCG, resveratrol and myricetin) are part of the vine cane extract, which is a by-product of the vineyards that is often burned and exists in large quantities in Portugal. They are known not only for their antioxidant activity, but also for their anti-carcinogenic, anti-inflammatory, and anti-obesity properties.

The aim of this project was to develop a formulation that would incorporate the three natural compounds encapsulated in lipid nanoparticles. To complete this aim, firstly a lipid screening was carried out to evaluate which lipid was the most compatible with the three compounds. After this, an emulsifier compatibility study was assayed, in which the nanoparticles were produced without the natural compounds to determine the most suitable emulsifier and the optimum production parameters. In the end, it was possible to select one SLN formulation that presented the desired characteristics for the aim of the project. The unloaded nanoparticles presented a size of approximately 200 nm in the day after the production and a slight increase of around 20 nm was observed on the 7th day onwards. They maintained these characteristics throughout 138 days, proving their stability over time.

Then, the loaded nanoparticles were produced and proved to be stable over a 30-day stability study, in which their sizes also increased about 20 nm between day 1 and day 7, but after that they maintained this value constant. In terms of Pdl and zeta potential there were no significant changes throughout the stability study. They were also characterized through TEM, in which their round shape and sizes were confirmed. Furthermore, high encapsulation efficiencies, above $(90 \pm 5)\%$, and loading capacity values close to the theoretical one were obtained, revealing that the lipid screening and nanoparticle

production were essential to achieve these great values. Besides this, it was possible to infer that lipid nanoparticles are suitable nanocarriers for the transportation of these three natural compounds. Additionally, the *in vitro* release profile presented a prolonged release, also beneficial for the aim of the formulation, since the compound will be released slowly, not requiring multiple formulation applications throughout the day. One of the possible causes for obtaining these release profiles could rely on the fact that the natural compounds have affinity to stay in the lipid part instead of transiting to the aqueous release medium, since a lipid screening was developed to enhance their compatibility with the lipid.

To assess the antioxidant activity of each compound and possible synergic effects in certain compound combinations, an antioxidant test was carried out. Firstly, the three natural compounds presented antioxidant activity as expected. Secondly, The EGCG and resveratrol mixture and the mixture of the three natural compounds presented a synergetic effect, which revealed a promising effect in the *in vitro* antioxidant tests performed in HaCaT. Furthermore, an accelerated stability assay was performed to predict possible stability problems in the formulation over time. The test demonstrated that the nanoparticles have a long-term stability, not presenting any signal of aggregation, size alteration, flocculation, phase separation or creaming.

Finally, the cytotoxicity of the SLN formulations and free natural compounds was assessed *in vitro* in immortalized human keratinocytes. For that, two assays were performed: the neutral red uptake assay and the resazurin reduction assay. The limiting concentration of exposition to the formulations was 50 µg/mL, since there was no significant cytotoxicity detected at this concentration. Regarding the free natural compounds, there was no cytotoxicity associated at this concentration. Afterwards, the ability of the formulations to protect the skin cells against induced oxidative stress was tested. From the SLN formulations loaded with just one natural compound, the EGCG-loaded formulation was considered the most effective in decreasing ROS/RNS production, as expected from the DPPH assay. From the mixtures, the one that presented the better ability to reduce the oxidative stress was EGCG + resveratrol, also as expected from the DPPH assay.

To conclude, the solid lipid nanoparticles demonstrated to be suitable deliver carriers for these three natural compounds, since a successful encapsulation was obtained. These nanoparticles constitute a viable strategy for the transport of natural compounds since they will protect the NC against external agents and enhance their bioavailability in the skin cells. Therefore, these loaded-SLNs could be used to develop new cosmetic products with health-promoting properties for skin aging prevention. Furthermore, this study highlights the application of the circular economy through the possibility of repurposing the vine cane waste into cosmetic formulations, generating more sustainable products.

For future work, more experiments could be made. For example, the encapsulation of the natural compounds in a NLC formulation could be tested since a preliminary study has already been conducted (lipid screening and emulsifier optimization) and is presented in Annex G- Preliminary studies in NLC formulations. Furthermore, the resultant nanoparticles could be incorporated in a hydrogel to perform *in vivo* tests in human volunteers to evaluate the possibilities of creating a new cosmetic formulation. As oxidative stress is also related with other disorders, such as neurodegenerative diseases, this formulation could also be tested as a preventive treatment, to combat several problems associated with free radicals. However, different *in vivo* and *in vitro* tests would be necessary to implement these nanoparticles in a pharmaceutical formulation.

References

1. Organization, W.H. *Ageing and health*. 2021 26/07/2021]; Available from: <https://www.who.int/news-room/fact-sheets/detail/ageing-and-health>.
2. Makrantonaki, E., V. Bekou, and C.C. Zouboulis, *Genetics and skin aging*. *Dermato-Endocrinology*, 2012. 4(3): p. 280-284.
3. Intelligence, M. *Cosmeceuticals Market- Growth, trends, covid-19 impact, and forecasts (2021-2026)*. 26/07/2021]; Available from: <https://www.mordorintelligence.com/industry-reports/global-cosmeceuticals-market-industry>.
4. Insights, F.B. *Cosmeceuticals Market size, share and Industry analysis, by product (skin care, hair care, injectables and others), by distribution channel (Pharmacy/Drug stores, online stores, and others), and regional forecast, 2019-2026*. 26/07/2021]; Available from: <https://www.fortunebusinessinsights.com/cosmeceuticals-market-102521>.
5. Fang, J.Y., et al., *Skin aging caused by intrinsic or extrinsic processes characterized with functional proteomics*. *Proteomics*, 2016. 16(20): p. 2718-2731.
6. Kammeyer, A. and R.M. Luiten, *Oxidation events and skin aging*. *Ageing Research Reviews*, 2015. 21: p. 16-29.
7. Birch-Machin, M. and A. Bowman, *Oxidative stress and ageing*. *British Journal of Dermatology*, 2016. 175: p. 26-29.
8. Tundis, R., et al., *Potential role of natural compounds against skin aging*. *Current medicinal chemistry*, 2015. 22(12): p. 1515-1538.
9. Carvalho, I.T., B.N. Estevinho, and L. Santos, *Application of microencapsulated essential oils in cosmetic and personal healthcare products-a review*. *International journal of cosmetic science*, 2016. 38(2): p. 109-119.
10. Maleki, S.J., J.F. Crespo, and B. Cabanillas, *Anti-inflammatory effects of flavonoids*. *Food Chemistry*, 2019. 299: p. 125124.
11. Liu, X. and G.K. German, *The effects of barrier disruption and moisturization on the dynamic drying mechanics of human stratum corneum*. *Journal of the Mechanical Behavior of Biomedical Materials*, 2015. 49: p. 80-89.
12. Trommer, H. and R. Neubert, *Overcoming the stratum corneum: the modulation of skin penetration*. *Skin pharmacology and physiology*, 2006. 19(2): p. 106-121.
13. Banerjee, R., *Overcoming the stratum corneum barrier: a nano approach*. *Drug delivery and translational research*, 2013. 3(3): p. 205-208.
14. Garcês, A., et al., *Formulations based on solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) for cutaneous use: A review*. *European Journal of Pharmaceutical Sciences*, 2018. 112: p. 159-167.
15. Pardeike, J., A. Hommoss, and R.H. Müller, *Lipid nanoparticles (SLN, NLC) in cosmetic and pharmaceutical dermal products*. *International journal of pharmaceutics*, 2009. 366(1-2): p. 170-184.
16. Souto, E.B., et al., *SLN and NLC for topical, dermal, and transdermal drug delivery*. *Expert opinion on drug delivery*, 2020. 17(3): p. 357-377.
17. H Muller, R., R. Shegokar, and C. M Keck, *20 years of lipid nanoparticles (SLN & NLC): present state of development & industrial applications*. *Current drug discovery technologies*, 2011. 8(3): p. 207-227.
18. Tobin, D.J., *Introduction to skin aging*. *Journal of tissue viability*, 2017. 26(1): p. 37-46.
19. Archer, C., *Functions of the skin*. *Rook's textbook of dermatology*, 2004. 1: p. 4.1.
20. Leeds, U.o. *The Histology Guide*. 13/06/2021]; Available from: https://www.histology.leeds.ac.uk/skin/skin_layers.php.
21. Chuong, C.-M., et al., *What is the true function of skin?* *Experimental dermatology*, 2002. 11(2): p. 159-187.
22. Tobin, D.J., *Biochemistry of human skin—our brain on the outside*. *Chemical Society Reviews*, 2006. 35(1): p. 52-67.
23. Yousef, H., M. Alhajj, and S. Sharma, *Anatomy, skin (integument), epidermis*. 2017.
24. van Smeden, J. and J.A. Bouwstra, *Stratum corneum lipids: their role for the skin barrier function in healthy subjects and atopic dermatitis patients*, in *Skin barrier function*. 2016, Karger Publishers. p. 8-26.
25. Poljšak, B., R.G. Dahmane, and A. Godić, *Intrinsic skin aging: the role of oxidative stress*. *Acta Dermatovenereol Alp Pannonica Adriat*, 2012. 21(2): p. 33-36.

26. Baumann, L., *Skin ageing and its treatment*. The Journal of Pathology: A Journal of the Pathological Society of Great Britain and Ireland, 2007. 211(2): p. 241-251.
27. Kosmadaki, M. and B. Gilchrest, *The role of telomeres in skin aging/photoaging*. Micron, 2004. 35(3): p. 155-159.
28. Azevedo, C. and C. E. Sunkel, *Biologia Celular e Molecular*. Vol. 5ª Edição. 2012: LIDEL-Edições Técnicas, Lda.
29. Geserick, C. and M.A. Blasco, *Novel roles for telomerase in aging*. Mechanisms of ageing and development, 2006. 127(6): p. 579-583.
30. Boukamp, P., *Ageing mechanisms: the role of telomere loss*. Clinical and experimental dermatology, 2001. 26(7): p. 562-565.
31. Buckingham, E. and A. Klingelutz, *The role of telomeres in the ageing of human skin*. Experimental dermatology, 2011. 20: p. 297-302.
32. Lobo, V., et al., *Free radicals, antioxidants and functional foods: Impact on human health*. Pharmacognosy reviews, 2010. 4(8): p. 118-126.
33. Nita, M. and A. Grzybowski, *The Role of the Reactive Oxygen Species and Oxidative Stress in the Pathomechanism of the Age-Related Ocular Diseases and Other Pathologies of the Anterior and Posterior Eye Segments in Adults*. Oxidative medicine and cellular longevity, 2016. 2016: p. 3164734-3164734.
34. Uitto, J., *Understanding premature skin aging*. 1997, Mass Medical Soc.
35. Sugimoto, M., R. Yamashita, and M. Ueda, *Telomere length of the skin in association with chronological aging and photoaging*. Journal of dermatological science, 2006. 43(1): p. 43-47.
36. Berneburg, M., et al., *Induction of the Photoaging-Associated Mitochondrial Common Deletion In Vivo in Normal Human Skin*. Journal of Investigative Dermatology, 2004. 122(5): p. 1277-1283.
37. Springob, K. and T.M. Kutchan, *Introduction to the different classes of natural products*, in *Plant-derived natural products*. 2009, Springer. p. 3-50.
38. Lacerda-Neto, L.J., et al., *The complex pharmacology of natural products*. Future medicinal chemistry, 2019. 11(08): p. 797-799.
39. Ji, H.-F., X.-J. Li, and H.-Y. Zhang, *Natural products and drug discovery. Can thousands of years of ancient medical knowledge lead us to new and powerful drug combinations in the fight against cancer and dementia?* EMBO reports, 2009. 10(3): p. 194-200.
40. Mukherjee, P.K., et al., *Bioactive compounds from natural resources against skin aging*. Phytomedicine, 2011. 19(1): p. 64-73.
41. Angerhofer, C.K., D. Maes, and P.U. Giacomoni, *The use of natural compounds and botanicals in the development of anti-aging skin care products*, in *Skin aging handbook*. 2009, Elsevier. p. 205-263.
42. Moreira, M.M., et al., *Potential of Portuguese vine shoot wastes as natural resources of bioactive compounds*. Science of The Total Environment, 2018. 634: p. 831-842.
43. Dorosh, O., et al., *Vine-Canes Valorisation: Ultrasound-Assisted Extraction from Lab to Pilot Scale*. Molecules, 2020. 25(7): p. 1739.
44. Moreira, M.M., et al., *Vine-Canes as a Source of Value-Added Compounds for Cosmetic Formulations*. Molecules, 2020. 25(13): p. 2969.
45. Kim, H.-S., M.J. Quon, and J.-a. Kim, *New insights into the mechanisms of polyphenols beyond antioxidant properties; lessons from the green tea polyphenol, epigallocatechin 3-gallate*. Redox biology, 2014. 2: p. 187-195.
46. Katiyar, S.K., *Skin photoprotection by green tea: antioxidant and immunomodulatory effects*. Current Drug Targets-Immune, Endocrine & Metabolic Disorders, 2003. 3(3): p. 234-242.
47. Zhong, Y., C.-M. Ma, and F. Shahidi, *Antioxidant and antiviral activities of lipophilic epigallocatechin gallate (EGCG) derivatives*. Journal of Functional Foods, 2012. 4(1): p. 87-93.
48. Wei, H., et al., *Scavenging of hydrogen peroxide and inhibition of ultraviolet light-induced oxidative DNA damage by aqueous extracts from green and black teas*. Free Radical Biology and Medicine, 1999. 26(11-12): p. 1427-1435.
49. Krupkova, O., S.J. Ferguson, and K. Wuertz-Kozak, *Stability of (-)-epigallocatechin gallate and its activity in liquid formulations and delivery systems*. The Journal of nutritional biochemistry, 2016. 37: p. 1-12.
50. Ahmed, S., et al., *Green tea polyphenol epigallocatechin-3-gallate inhibits the IL-18-induced activity and expression of cyclooxygenase-2 and nitric oxide synthase-2 in human chondrocytes*. Free Radical Biology and Medicine, 2002. 33(8): p. 1097-1105.

51. Choi, J.-H., et al., *Effects of green tea catechin on polymorphonuclear leukocyte 5'-lipoxygenase activity, leukotriene B₄ synthesis, and renal damage in diabetic rats*. *Annals of nutrition and metabolism*, 2004. **48**(3): p. 151-155.
52. Katiyar, S.K., et al., *Green tea polyphenol (-)-epigallocatechin-3-gallate treatment of human skin inhibits ultraviolet radiation-induced oxidative stress*. *Carcinogenesis*, 2001. **22**(2): p. 287-294.
53. Chakrawarti, L., et al., *Therapeutic effects of EGCG: a patent review*. *Expert Opinion on Therapeutic Patents*, 2016. **26**(8): p. 907-916.
54. Patricia Farris, M., et al., *Resveratrol: A unique antioxidant offering a multi-mechanistic approach for treating aging skin*. *J Drugs Dermatol*, 2013. **12**(12): p. 1389-1394.
55. Zupančič, Š., Z. Lavrič, and J. Kristl, *Stability and solubility of trans-resveratrol are strongly influenced by pH and temperature*. *European Journal of Pharmaceutics and Biopharmaceutics*, 2015. **93**: p. 196-204.
56. Loureiro, J.A., et al., *Resveratrol and grape extract-loaded solid lipid nanoparticles for the treatment of Alzheimer's disease*. *Molecules*, 2017. **22**(2): p. 277.
57. Baxter, R.A., *Anti-aging properties of resveratrol: review and report of a potent new antioxidant skin care formulation*. *Journal of cosmetic dermatology*, 2008. **7**(1): p. 2-7.
58. Afaq, F., V.M. Adhami, and N. Ahmad, *Prevention of short-term ultraviolet B radiation-mediated damages by resveratrol in SKH-1 hairless mice*. *Toxicology and applied pharmacology*, 2003. **186**(1): p. 28-37.
59. Chen, M.-L., et al., *Protective effect of resveratrol against oxidative damage of UVA irradiated HaCaT cells*. *Zhong nan da xue xue bao. Yi xue ban= Journal of Central South University. Medical sciences*, 2006. **31**(5): p. 635-639.
60. Song, X., et al., *Myricetin: A review of the most recent research*. *Biomedicine & Pharmacotherapy*, 2021. **134**: p. 111017.
61. Jung, S.K., et al., *Myricetin suppresses UVB-induced wrinkle formation and MMP-9 expression by inhibiting Raf*. *Biochemical pharmacology*, 2010. **79**(10): p. 1455-1461.
62. Yao, Y., et al., *Preformulation studies of myricetin: a natural antioxidant flavonoid*. *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, 2014. **69**(1): p. 19-26.
63. Wang, Z.H., et al., *Myricetin suppresses oxidative stress-induced cell damage via both direct and indirect antioxidant action*. *Environmental toxicology and pharmacology*, 2010. **29**(1): p. 12-18.
64. Williams, R.J., J.P.E. Spencer, and C. Rice-Evans, *Flavonoids: antioxidants or signalling molecules?* *Free Radical Biology and Medicine*, 2004. **36**(7): p. 838-849.
65. Quan, T., et al. *Matrix-degrading metalloproteinases in photoaging*. in *Journal of Investigative Dermatology Symposium Proceedings*. 2009. Elsevier.
66. van Smeden, J., et al., *The important role of stratum corneum lipids for the cutaneous barrier function*. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 2014. **1841**(3): p. 295-313.
67. Ita, K.B., *Transdermal drug delivery: progress and challenges*. *Journal of Drug Delivery Science and Technology*, 2014. **24**(3): p. 245-250.
68. Patzelt, A., et al., *Selective follicular targeting by modification of the particle sizes*. *Journal of Controlled Release*, 2011. **150**(1): p. 45-48.
69. Lauterbach, A. and C.C. Müller-Goymann, *Applications and limitations of lipid nanoparticles in dermal and transdermal drug delivery via the follicular route*. *European Journal of Pharmaceutics and Biopharmaceutics*, 2015. **97**: p. 152-163.
70. Puglia, C. and F. Bonina, *Lipid nanoparticles as novel delivery systems for cosmetics and dermal pharmaceuticals*. *Expert Opinion on Drug Delivery*, 2012. **9**(4): p. 429-441.
71. Lingayat, V.J., N.S. Zarekar, and R.S. Shendge, *Solid lipid nanoparticles: a review*. *Nanoscience and Nanotechnology Research*, 2017. **2**: p. 67-72.
72. Shah, R., et al., *Lipid nanoparticles: Production, characterization and stability*. 2015: Springer.
73. Yadav, N., S. Khatak, and U.S. Sara, *Solid lipid nanoparticles-a review*. *Int. J. Appl. Pharm*, 2013. **5**(2): p. 8-18.
74. Souto, E., A. Almeida, and R. Müller, *Lipid nanoparticles (SLN®, NLC®) for cutaneous drug delivery: structure, protection and skin effects*. *Journal of Biomedical Nanotechnology*, 2007. **3**(4): p. 317-331.
75. Naseri, N., H. Valizadeh, and P. Zakeri-Milani, *Solid Lipid Nanoparticles and Nanostructured Lipid Carriers: Structure, Preparation and Application*. *Advanced pharmaceutical bulletin*, 2015. **5**(3): p. 305-313.

76. Belouqui, A., et al., *Nanostructured lipid carriers: promising drug delivery systems for future clinics*. Nanomedicine: Nanotechnology, biology and medicine, 2016. 12(1): p. 143-161.
77. Shrotriya, S., N. Ranpise, and B. Vidhate, *Skin targeting of resveratrol utilizing solid lipid nanoparticle-engrossed gel for chemically induced irritant contact dermatitis*. Drug delivery and translational research, 2017. 7(1): p. 37-52.
78. Chen, J., et al., *Development and evaluation of resveratrol, Vitamin E, and epigallocatechin gallate loaded lipid nanoparticles for skin care applications*. European Journal of Pharmaceutics and Biopharmaceutics, 2017. 117: p. 286-291.
79. Dzulhi, S., E. Anwar, and T. Nurhayati, *Formulation, characterization and in vitro skin penetration of green tea (Camellia sinensis L.) leaves extract-loaded solid lipid nanoparticles*. J. Appl. Pharm. Sci, 2018. 8(08): p. 057-062.
80. Ban, C., et al., *Reduction of focal sweating by lipid nanoparticle-delivered myricetin*. Scientific reports, 2020. 10(1): p. 1-14.
81. Rigon, R.B., et al., *Skin delivery and in vitro biological evaluation of trans-resveratrol-loaded solid lipid nanoparticles for skin disorder therapies*. Molecules, 2016. 21(1): p. 116.
82. Abbas, H., R. Kamel, and N. El-Sayed, *Dermal anti-oxidant, anti-inflammatory and anti-aging effects of Compritol ATO-based Resveratrol colloidal carriers prepared using mixed surfactants*. International Journal of Pharmaceutics, 2018. 541(1): p. 37-47.
83. Eiras, F., et al., *Characterization and biocompatibility evaluation of cutaneous formulations containing lipid nanoparticles*. International Journal of Pharmaceutics, 2017. 519(1-2): p. 373-380.
84. Clares, B., et al., *Nanoemulsions (NEs), liposomes (LPs) and solid lipid nanoparticles (SLNs) for retinyl palmitate: Effect on skin permeation*. International Journal of Pharmaceutics, 2014. 473(1): p. 591-598.
85. Tichota, D.M., et al., *Design, characterization, and clinical evaluation of argan oil nanostructured lipid carriers to improve skin hydration*. International journal of nanomedicine, 2014. 9: p. 3855-3864.
86. Vaz, S., et al., *Evaluation of the biocompatibility and skin hydration potential of vitamin E-loaded lipid nanosystems formulations: In vitro and human in vivo studies*. Colloids and Surfaces B: Biointerfaces, 2019. 179: p. 242-249.
87. Danaei, M., et al., *Impact of particle size and polydispersity index on the clinical applications of lipidic nanocarrier systems*. Pharmaceutics, 2018. 10(2): p. 57.
88. Paar, A. *The principles of dynamic light scattering*. 02/01/2021]; Available from: <https://wiki.anton-paar.com/en/the-principles-of-dynamic-light-scattering/>.
89. Lu, G.W. and P. Gao, *CHAPTER 3 - Emulsions and Microemulsions for Topical and Transdermal Drug Delivery*, in *Handbook of Non-Invasive Drug Delivery Systems*, V.S. Kulkarni, Editor. 2010, William Andrew Publishing: Boston. p. 59-94.
90. Samimi, S., et al., *Chapter 3 - Lipid-Based Nanoparticles for Drug Delivery Systems*, in *Characterization and Biology of Nanomaterials for Drug Delivery*, S.S. Mohapatra, et al., Editors. 2019, Elsevier. p. 47-76.
91. Panalytical, M. *Electrophoretic Light Scattering (ELS)*. 02/01/2021]; Available from: <https://www.malvernpanalytical.com/en/products/technology/light-scattering/electrophoretic-light-scattering>.
92. Bhattacharjee, S., *DLS and zeta potential - What they are and what they are not?* Journal of Controlled Release, 2016. 235: p. 337-351.
93. Swan, J.W. and E.M. Furst, *A simpler expression for Henry's function describing the electrophoretic mobility of spherical colloids*. Journal of Colloid and Interface Science, 2012. 388(1): p. 92-94.
94. Souza, T.G., V.S. Ciminelli, and N.D.S. Mohallem. *A comparison of TEM and DLS methods to characterize size distribution of ceramic nanoparticles*. in *Journal of physics: conference series*. 2016. IOP Publishing.
95. Leadley, D. *Transmission Electron Microscopy (TEM)*. 2010 14/08/2021]; Available from: <https://warwick.ac.uk/fac/sci/physics/current/postgraduate/regs/mpagswarwick/ex5/techniques/structural/tem/>.
96. Tom, J. *UV-Vis Spectroscopy: Principle, Strength and Limitations and Applications*. 2021 12/08/2021]; Available from: <https://www.technologynetworks.com/analysis/articles/uv-vis-spectroscopy-principle-strengths-and-limitations-and-applications-349865>.
97. Evans, L., *UV-VIS Spectrophotometry A Brief Background to Spectrophotometry*. Biochrom-Harvard Bioscience, 2018. 1: p. 1-15.
98. Aldrich, S. *HEPES*. 09/08/2021]; Available from: <https://www.sigmaaldrich.com/PT/en/product/sigma/h3375?context=product>.

99. Kedare, S.B. and R. Singh, *Genesis and development of DPPH method of antioxidant assay*. Journal of food science and technology, 2011. **48**(4): p. 412-422.
100. Duan, X.-J., et al., *Evaluation of antioxidant property of extract and fractions obtained from a red alga, Polysiphonia urceolata*. Food Chemistry, 2006. **95**(1): p. 37-43.
101. Wilson, V.G., *Growth and differentiation of HaCaT keratinocytes, in Epidermal cells*. 2013, Springer. p. 33-41.
102. Ates, G., et al., *Assaying cellular viability using the neutral red uptake assay, in Cell Viability Assays*. 2017, Springer. p. 19-26.
103. Riss, T.L., et al., *Cell viability assays*. Assay Guidance Manual [Internet], 2016.
104. He, Y.-Y. and D.-P. Häder, *Involvement of reactive oxygen species in the UV-B damage to the cyanobacterium Anabaena sp.* Journal of Photochemistry and Photobiology B: Biology, 2002. **66**(1): p. 73-80.
105. Aburahma, M.H. and S.M. Badr-Eldin, *Compritol 888 ATO: a multifunctional lipid excipient in drug delivery systems and nanopharmaceuticals*. Expert Opinion on Drug Delivery, 2014. **11**(12): p. 1865-1883.
106. Shirwaiker, R.A., M.F. Purser, and R.A. Wysk, *6 - Scaffolding hydrogels for rapid prototyping based tissue engineering, in Rapid Prototyping of Biomaterials*, R. Narayan, Editor. 2014, Woodhead Publishing. p. 176-200.
107. Ojha, S., *In vitro and In vivo neuroprotective study of solid lipid nanoparticles loaded with dimethyl fumarate*. Asian Journal of Pharmaceutics (AJP): Free full text articles from Asian J Pharm, 2018. **12**(01).
108. Aleixandre-Tudo, J.L. and W. Du Toit, *The role of UV-visible spectroscopy for phenolic compounds quantification in winemaking*. Frontiers and new trends in the science of fermented food and beverages, 2018: p. 1-21.
109. Atomssa, T. and A. Gholap, *Characterization and determination of catechins in green tea leaves using UV-visible spectrometer*. Journal of Engineering and Technology Research, 2015. **7**(1): p. 22-31.
110. Zheng, X., et al., *Electrochemical studies of (-)-epigallocatechin gallate and its interaction with DNA*. Analytical and Bioanalytical Chemistry, 2006. **386**(6): p. 1913-1919.
111. Zhang, J., X.-F. Dai, and J.-Y. Huang, *Resveratrol Binding to Fibrinogen and its Biological Implication*. Food Biophysics, 2012. **7**(1): p. 35-42.
112. Allan, K.E., C.E. Lenehan, and A.V. Ellis, *UV Light Stability of -Cyclodextrin/Resveratrol HostGuest Complexes and Isomer Stability at Varying pH*. Australian Journal of Chemistry, 2009. **62**(8): p. 921-926.
113. Yang, W., et al., *Hydrophobically Modified Glucan as an Amphiphilic Carbohydrate Polymer for Micellar Delivery of Myricetin*. Molecules, 2019. **24**(20): p. 3747.
114. Stecher, G., et al., *Determination of flavonoids and stilbenes in red wine and related biological products by HPLC and HPLC-ESI-MS-MS*. Fresenius' journal of analytical chemistry, 2001. **371**(1): p. 73-80.
115. Müller, R.H., K. Mäder, and S. Gohla, *Solid lipid nanoparticles (SLN) for controlled drug delivery - a review of the state of the art*. European Journal of Pharmaceutics and Biopharmaceutics, 2000. **50**(1): p. 161-177.
116. Radhakrishnan, R., et al., *Encapsulation of biophenolic phytochemical EGCG within lipid nanoparticles enhances its stability and cytotoxicity against cancer*. Chemistry and Physics of Lipids, 2016. **198**: p. 51-60.
117. Shtay, R., et al., *Encapsulation of (-)-epigallocatechin-3-gallate (EGCG) in solid lipid nanoparticles for food applications*. Journal of Food Engineering, 2019. **244**: p. 91-100.
118. Yadav, A., et al., *Resveratrol loaded solid lipid nanoparticles attenuate mitochondrial oxidative stress in vascular dementia by activating Nrf2/HO-1 pathway*. Neurochemistry International, 2018. **112**: p. 239-254.
119. Pandita, D., et al., *Solid lipid nanoparticles enhance oral bioavailability of resveratrol, a natural polyphenol*. Food Research International, 2014. **62**: p. 1165-1174.
120. Gaber, D.M., N. Nafee, and O.Y. Abdallah, *Myricetin solid lipid nanoparticles: Stability assurance from system preparation to site of action*. European Journal of Pharmaceutical Sciences, 2017. **109**: p. 569-580.
121. Son, G.-H., B.-J. Lee, and C.-W. Cho, *Mechanisms of drug release from advanced drug formulations such as polymeric-based drug-delivery systems and lipid nanoparticles*. Journal of Pharmaceutical Investigation, 2017. **47**(4): p. 287-296.
122. Miao, J., et al., *Drug resistance reversal activity of anticancer drug loaded solid lipid nanoparticles in multi-drug resistant cancer cells*. Colloids and Surfaces B: Biointerfaces, 2013. **110**: p. 74-80.

123. Baek, J.-S. and C.-W. Cho, *Controlled release and reversal of multidrug resistance by co-encapsulation of paclitaxel and verapamil in solid lipid nanoparticles*. International Journal of Pharmaceutics, 2015. **478**(2): p. 617-624.
124. Wang, W., et al., *Enhanced bioavailability and efficiency of curcumin for the treatment of asthma by its formulation in solid lipid nanoparticles*. International journal of nanomedicine, 2012. **7**: p. 3667.
125. Deng, J., W. Cheng, and G. Yang, *A novel antioxidant activity index (AAU) for natural products using the DPPH assay*. Food Chemistry, 2011. **125**(4): p. 1430-1435.
126. Schaich, K.M., X. Tian, and J. Xie, *Hurdles and pitfalls in measuring antioxidant efficacy: A critical evaluation of ABTS, DPPH, and ORAC assays*. Journal of Functional Foods, 2015. **14**: p. 111-125.
127. Prieto, M.A., M.A. Murado, and J.A. Vázquez, *Quantification, characterization and description of synergy and antagonism in the antioxidant response*. Food Research International, 2014. **60**: p. 218-229.

Annex

Annex A- Lipid screening

In this annex are presented three examples (Figure A1, A2 and A3) of the results obtained in the lipid screening for each natural compound.

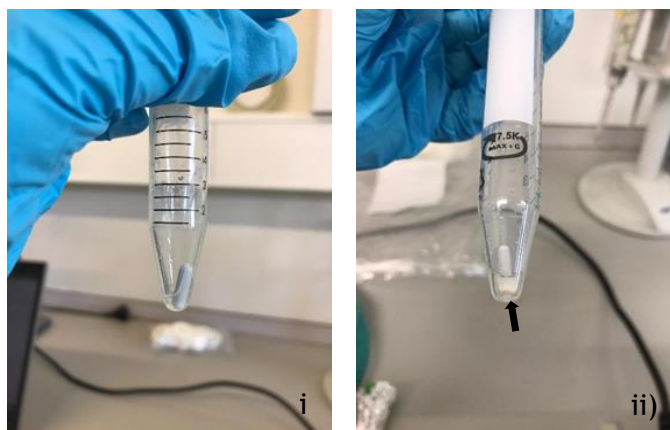


Figure A 1. i) EGCG-compritol HD5 ATO (1:100); ii) EGCG-Glyceryl monostearate (1:100). Glyceryl monostearate did not dissolve EGCG so was excluded from the process.

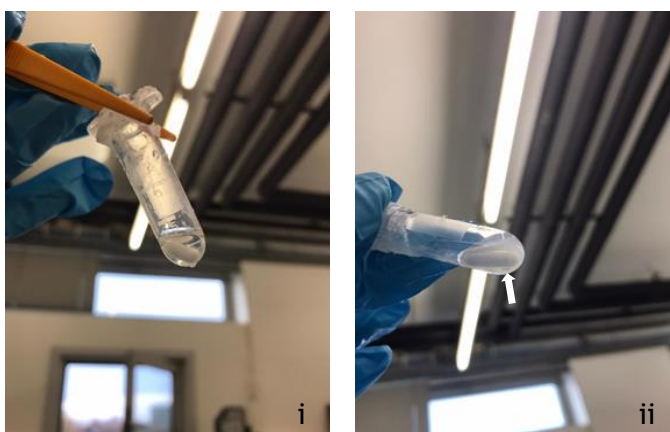


Figure A 2. Resveratrol-compritol HD5 ATO (1:200); ii) Resveratrol-softisan 100 (1:200). The last one did not dissolve resveratrol so was excluded from the process.

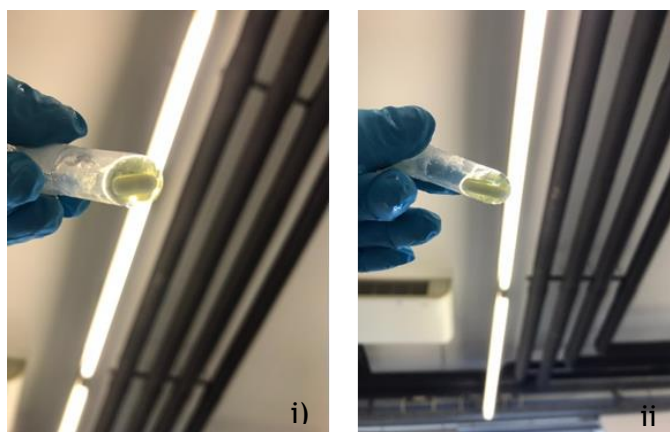


Figure A 3. i) Myricetin- compritol HD5 ATO (1:100); ii) Myricetin-gelucire 50/13 (1:100). Both dissolved myricetin.

Annex B- Nanoparticle production

In Figure B1, B2 and B3 are presented the obtained formulations of EGCG, resveratrol and myricetin, after their production.



Figure B 1. Resveratrol-loaded SLN formulations.



Figure B 2. EGCG-loaded SLN formulations.



Figure B 3. Myricetin-loaded SLN formulations.

Annex C- Nanoparticle characterization and stability studies

The pictures below (Figure C1, C2 and C3) contain the DLS data for the size distribution obtained for the nanoparticles produced with the natural compounds on the day after the production and the final day of the stability studies (day 30).

Temperature (°C): 25,0	Duration Used (s): 50
Count Rate (kcps): 312,4	Measurement Position (mm): 1,05
Cell Description: Disposable sizing cuvette	Attenuator: 5

	Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 177,3	Peak 1: 221,9	100,0	94,92
Pdl: 0,217	Peak 2: 0,000	0,0	0,000
Intercept: 0,936	Peak 3: 0,000	0,0	0,000
Result quality : Good			

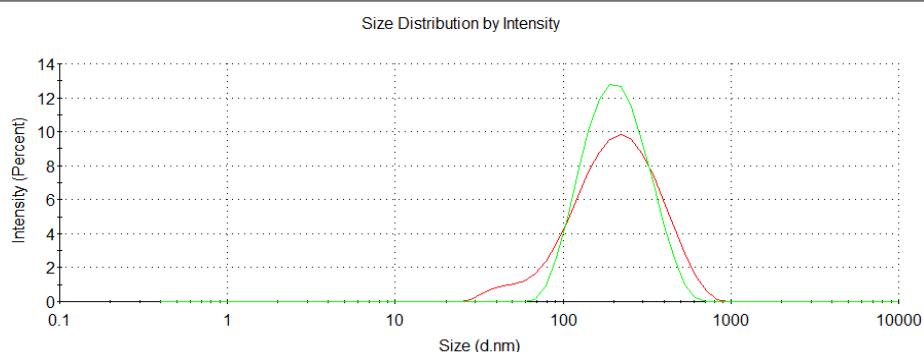


Figure C 1. DLS data for two size measurements of EGCG-loaded nanoparticles: day 1 (green) and day 30 (red).

Temperature (°C): 25,0	Duration Used (s): 50
Count Rate (kcps): 381,8	Measurement Position (mm): 4,65
Cell Description: Disposable sizing cuvette	Attenuator: 6

	Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm): 163,5	Peak 1: 222,3	100,0	118,5
Pdl: 0,258	Peak 2: 0,000	0,0	0,000
Intercept: 0,938	Peak 3: 0,000	0,0	0,000
Result quality : Good			

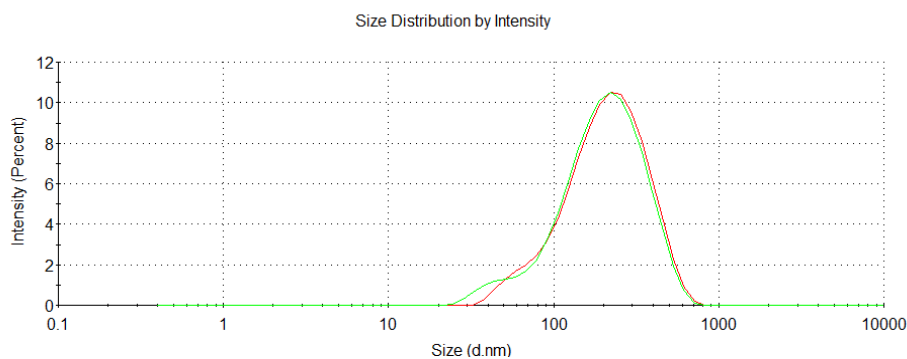


Figure C 2. DLS data for two size measurements of resveratrol-loaded nanoparticles: day 1 (green) and day 30 (red).

Temperature (°C): 25,0		Duration Used (s): 60	
Count Rate (kcps): 349,9		Measurement Position (mm): 1,25	
Cell Description: Disposable sizing cuvette		Attenuator: 5	
		Size (d.nm):	% Intensity:
Z-Average (d.nm): 153,4	Peak 1:	195,2	100,0
Pdl: 0,205	Peak 2:	0,000	0,0
Intercept: 0,931	Peak 3:	0,000	0,0
Result quality : Good		St Dev (d.nm):	95,88

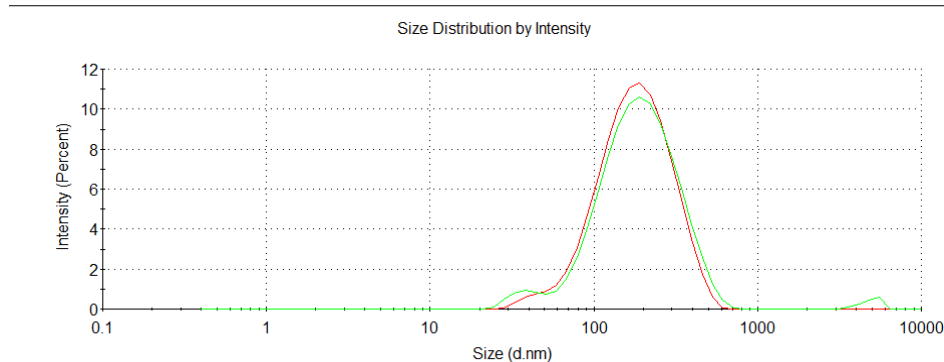


Figure C 3. DLS data for two size measurements of myricetin-loaded nanoparticles: day 1 (green) and day 30 (red).

Annex D- Compounds characterization

To quantify the amount of natural compound that was encapsulated and to perform the release studies, it was necessary to find the maximum absorbance wavelength for each compound, so the UV-Vis spectrum was obtained. In Figures D1, D2 and D3 are presented the three spectrums.

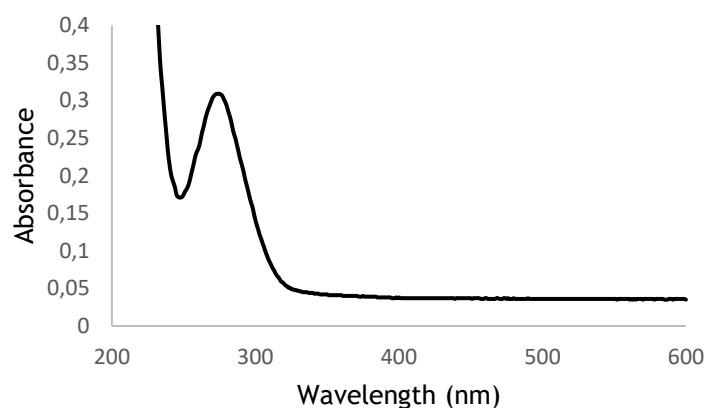


Figure D 1. UV-Vis spectrum obtained for EGCG with the characteristic peak at 274 nm.

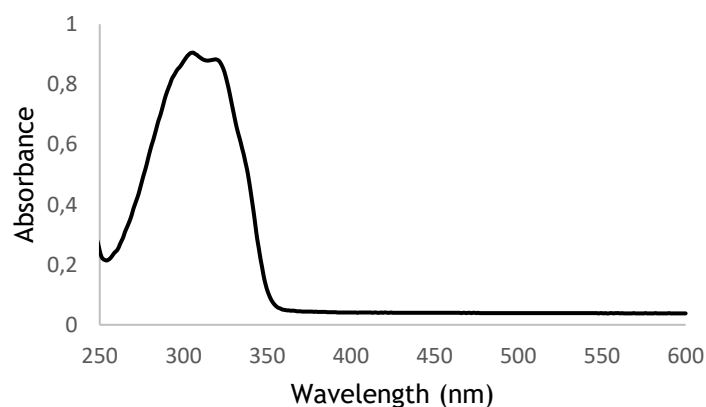


Figure D 2. UV-Vis spectrum obtained for resveratrol with the characteristic peak at 305 nm.

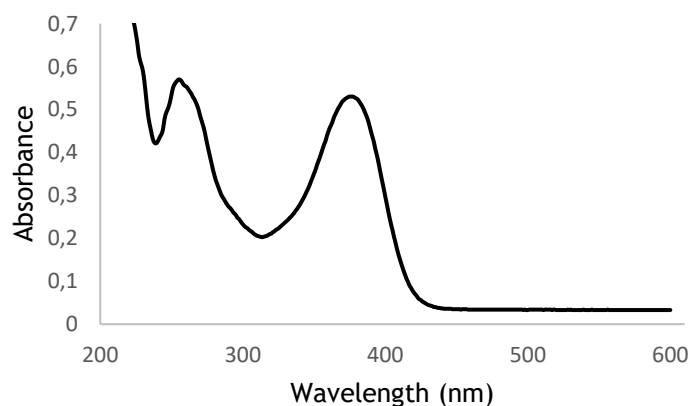


Figure D 3. UV-Vis spectrum obtained for myricetin with the characteristic peaks at 257 nm and 377 nm.

After obtaining the complete spectrum, a calibration curve was developed for each natural compound. In Figure D4, D5 and D6 are displayed the three calibration curves that were made in triplicate, so the standard deviation is also presented.

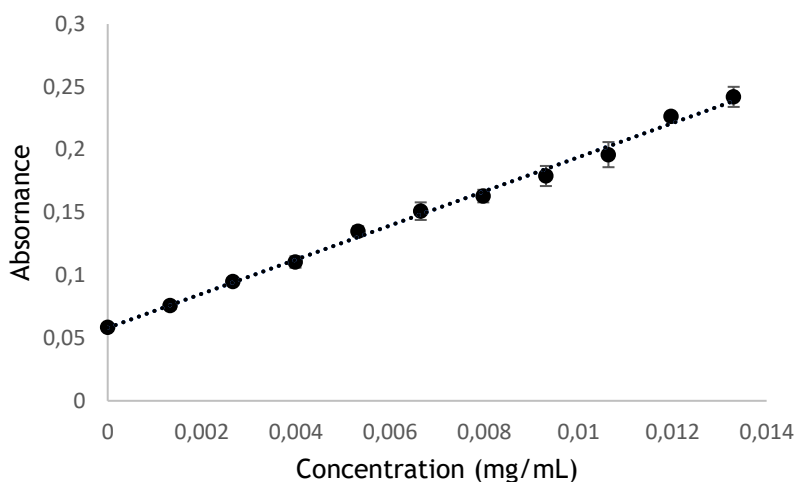


Figure D 4. Calibration curve obtained for EGCG at 274 nm. $Abs = 13,585 \cdot C \text{ (mg/mL)} + 0,0581$; $R^2 = 0,9955$.

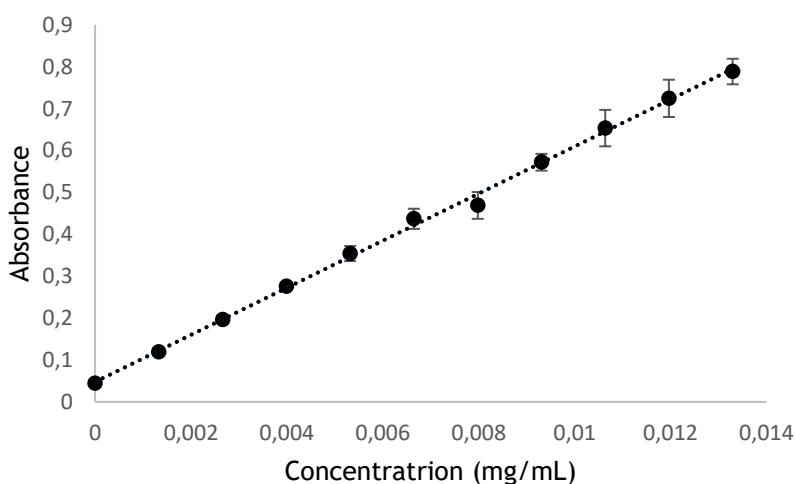


Figure D 5. Calibration curve obtained for resveratrol at 305 nm. $Abs = 56,193 \cdot C \text{ (mg/mL)} + 0,0476$; $R^2 = 0,9981$.

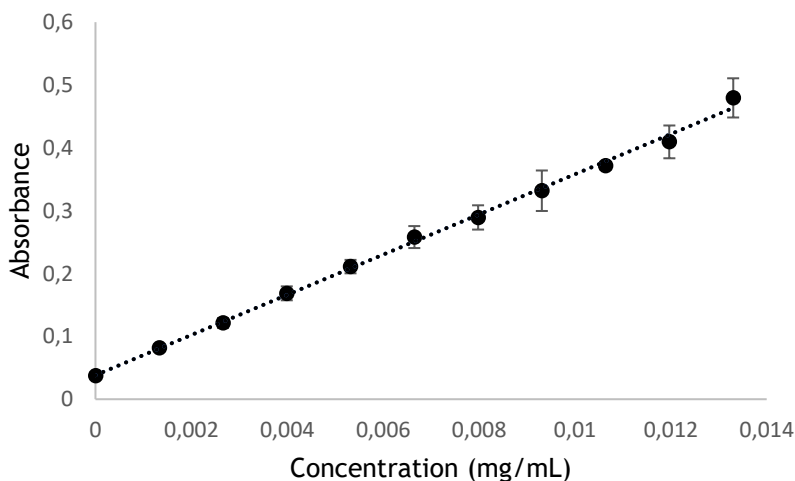


Figure D 6. Calibration curve obtained for myricetin at 377 nm. $Abs = 31,974 \cdot C \text{ (mg/mL)} + 0,0382$; $R^2 = 0,9973$.

Annex E- Encapsulation efficiency and loading capacity

In this section, a calculation example of how the entrapment efficiency was determined will be given. The experiment was made in triplicate, but the calculations are the same for the three measurements and samples.

Firstly, the volume of the solution was measured and knowing the amount of EGCG in the formulation it was possible to determine the concentration of the natural compound through the following equation (eq. i):

$$C = \frac{m}{V} = \frac{20 \text{ mg}}{8,05 \text{ mL}} = 2,48 \text{ mg/mL} \quad \text{i}$$

Secondly, an absorbance was read in the spectrophotometer (0,090) in the end of the procedure described in the 3.2.5 Determination of entrapment efficiency (chapter 3). Using the calibration curve, which correlates absorbances with concentrations, it was possible to obtain the amount of EGCG detected in the spectrophotometer (eq. ii):

$$Abs = 13,585 \times C + 0,0581 \leftrightarrow C = \frac{0,090 - 0,0581}{13,585} \leftrightarrow C = 0,0023 \text{ mg/mL} \quad \text{ii}$$

Throughout the process, the samples were diluted in ethanol two times, firstly, when the ultracentrifugation was performed (1:50) and when they were placed in the microplate to read the absorbance (120 μ L of ethanol mixed with 60 μ L of the sample). So, it was necessary to calculate the real EGCG detected (eq. iii):

$$C = 0,0023 \times 50 \times \frac{10}{3} = 0,3914 \text{ mg/mL} \quad \text{iii}$$

Finally, the entrapment efficiency equation can be applied to calculate the % of entrapped active compound (eq. iv):

$$\begin{aligned} EE(\%) &= \frac{\text{Total amount of EGCG} - \text{amount of free compound}}{\text{Total amount of EGCG}} \times 100 \quad \text{iv} \\ &= \frac{2,48 - 0,3914}{2,48} \times 100 = 84,2\% \end{aligned}$$

For the loading capacity, the mass of encapsulated EGCG was obtained subtracting the mass of free compound to the total amount of EGCG in the formulation (eq. v):

$$m_{\text{encapsulated EGCG}} = 20 \text{ mg} - (C \times V) = 20 - (0,3914 \times 8,05) = 16,850 \text{ mg} \quad \text{v}$$

Then, the total mass of SLN was calculated adding the mass of the excipients: solid lipid (1000 mg) and pluronic F127 (870 mg). Finally, the loading capacity was determined with the following equation (eq. vi):

$$LC (\%) = \frac{\text{Total mass of natural compound encapsulated}}{\text{Total mass of SLN} + \text{Total mass of natural compound encapsulated}} \times 100 \quad \text{vi}$$
$$= \frac{16,850}{(1000 + 870) + 16,850} \times 100 = 0,89\%$$

Annex F- *In vitro* release studies

In this annex, a calculation example of how the release profile points were obtained will be presented. The experiment was made in triplicate for each point of the release, but the calculations are the same for each measurement and sample.

In the timepoint 20,7 days after the starting of the EGCG's release profile, a sample was analyzed in the spectrophotometer and the absorbance of 0,153 was obtained. As described in the entrapment efficiency determination, through the calibration curve it was possible to determine the concentration of free compound (eq. vii):

$$Abs = 13,585 \times C + 0,0581 \leftrightarrow C = \frac{0,133 - 0,0581}{13,585} \leftrightarrow C = 0,0055 \text{ mg/mL} \quad \text{vii}$$

Once again, the sample was diluted two times, when the ultracentrifugation was performed (1:50) and when they were placed in the microplate to read the absorbance (120 μL of ethanol mixed with 60 μL of the sample). So, it was necessary to calculate the real EGCG detected (eq. viii):

$$C = 0,0055 \times 50 \times \frac{10}{3} = 0,920 \text{ mg/mL} \quad \text{viii}$$

Finally, the % of EGCG that was free was (eq. ix):

$$\begin{aligned} \text{Free natural compound}(\%) &= \frac{\text{Amount of EGCG at time } t}{\text{Total amount of EGCG}} \times 100 = \frac{0,920}{2,48} \times 100 \quad \text{ix} \\ &= 36,97\% \end{aligned}$$

The value obtained corresponds to the sum of the value actually released from the nanoparticles at time t but also includes the amount of EGCG that was never encapsulated. Thus, the percentage of EGCG release at 20,7 dias (eq. x):

$$\begin{aligned} \text{EGCG released}(\%) &= \text{free EGCG}(\%) - (100 - EE(\%)) = 36,97 - (100 - 84,2) \quad \text{x} \\ &= 21,2\% \end{aligned}$$

Annex G- Accelerated stability

In Figure G1, G2 and G3 are presented pictures of the samples subjected to the accelerated stability assay.

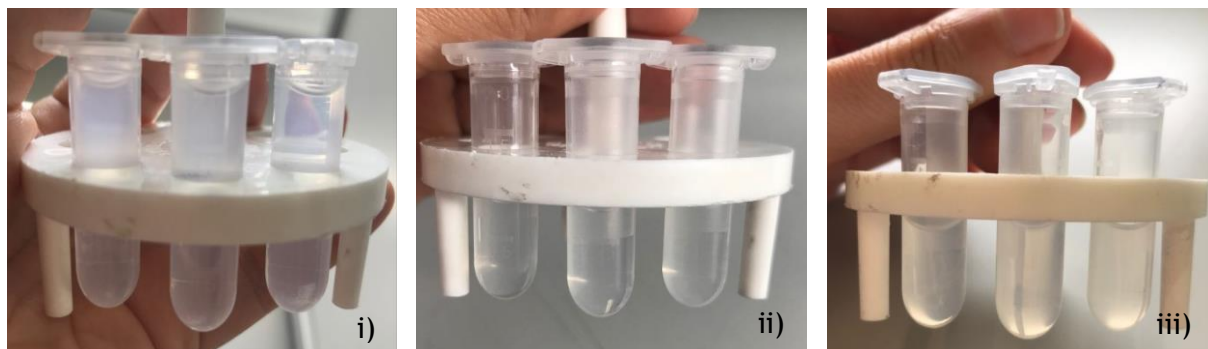


Figure G 1. EGCG-loaded nanoparticles before (i), after 30 min (ii) and 1 h (iii) of centrifugation.

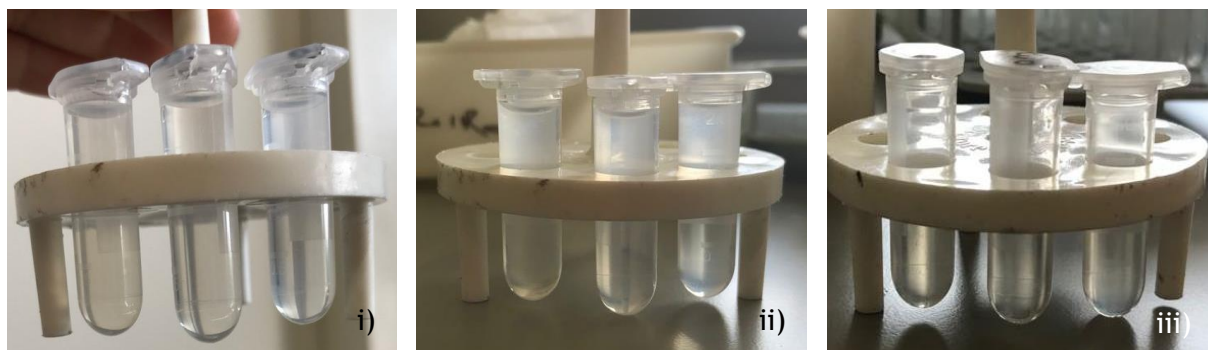


Figure G 2. Resveratrol-loaded before (i), after 30 min (ii) and 1 h (iii) of centrifugation.



Figure G 3. Myricetin-loaded before (i), after 30 min (ii) and 1 h (iii) of centrifugation.

Annex H- Preliminary studies in NLC formulations

Preliminary studies on the use of nanostructured lipid carriers were also developed as future work. In this annex are presented all the materials, methods and results obtained to produced and study NLC in the future.

H.1 Materials

In the following table (Table H1) are presented the liquid lipids used in the nanoparticle optimization process, as well as the respective supplier.

Table H 1. List of liquid lipids used in the nanoparticle optimization process and their supplier.

Liquid lipids	Supplier
Miglyol® 812 (medium-chain triglycerides), Cetylol® V (decyl oleate), Isopropyl myristate, Microcare® (cetyl dimeticone)	Acopharma S.A. (Terrassa, Spain)
Labrafac® WL1349 (medium-chain triglycerides)	Gattefossé (Nanterre, France)

H.2 Methods

H.2.1 Lipid screening

The experimental procedure was based in a process described by Tichota *et al.* (2014) [85]. Firstly, 100 mg of each liquid lipid was mixed with 1 mg of the natural compounds. If the lipid did not dissolve the compound in this proportion, another tentative with 1:200 (w/w) was carried out. The solubility of the natural compound was visually measured at 15-minute intervals, after vigorous magnetic stirring, observing the presence or absence of crystals of the compounds in the lipid.

H.2.2 Liquid lipid-solid lipid compatibility

For NLC preparation, the compatibility between solid lipids and liquid lipids was also addressed. This part was only developed with the liquid and solid lipids that dissolved all three natural compounds and the proportion tested was 50:50 (w/w). The different mixtures of lipids were heated with light magnetic agitation. After this step, the solutions were cooled to room temperature for solidification. To verify the miscibility level between the two lipids, the mixtures were visually analyzed to check for the existence of two phases. If this method was unclear, a portion of the mixture was placed in a filter paper to check for drops of oil, which indicated the non-miscibility between the lipids.

H.3 Results and discussion

H.3.1 Lipid screening

As for liquid lipids, the same strategy as for solid lipids was adopted. In this case, the visualization of the result was facilitated because at room temperature the lipid did not start to solidify as solid lipids often did. In Table H2, it is possible to analyze the results obtained for the EGCG compound, in which the negative sign represents the dissolution of the compound in the lipid and, therefore, no visible crystals of the compound and the positive sign means that there were visible crystals.

Table H 2. Dissolution capacity of EGCG in the liquid lipids available.

	Liquid lipids essay (1:100)			
	15 min	30 min	45 min	60 min
Miglyol® 812	+	+	+	+
Cetylol® V	+	+	+	+
Labrafac® WL1349	+	+	-	-
Isopropyl myristate	+	+	+	+
Microcare® (cetyl dimeticone)	+	+	+	+

As it can be seen from the table above, only labrafac® WL1349 was able to dissolve the EGCG. It should be noticed that microcare® (cetyl dimethicone) also presented a good result, so the experiment was repeated for microcare® (cetyl dimethicone) but in a 1:200 ratio. Despite that, this lipid still failed to completely dissolve the EGCG. In Figure H1, are presented pictures of the results obtained for EGCG in the lipid screening phase.

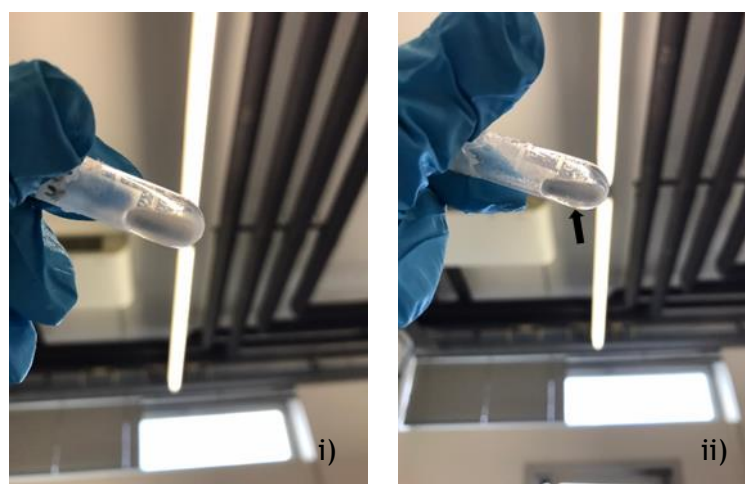


Figure H 1. i) EGCG-Labrafac 1349 (1:100); ii) EGCG-Cetylol V (1:100). Cetylol V did not dissolve EGCG so was excluded from the process.

Then, the only liquid lipid tested with resveratrol was labrafac® WL1349. If this lipid could not dissolve this natural compound, a new attempt would be made for all liquid lipids with EGCG, but in a higher proportion (1:200). The following table (Table H3) shows that labrafac® WL1349 was able to dissolve resveratrol. In Figure H2, is presented a picture of the result of the lipid screening for resveratrol.

Table H 3. Dissolution capacity of resveratrol in the liquid lipid selected.

	Liquid lipids essay (1:100)			
	15 min	30 min	45 min	60 min
Labrafac® WL1349	+	-	-	-

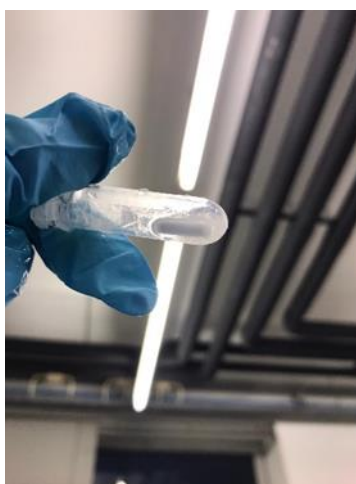


Figure H 2. Resveratrol-Labrafac 1349 (1:100).

Finally, the only liquid lipid tested with myricetin was also labrafac® WL1349, since it was the only one that dissolved the last two natural compounds. Following the same logic as before, if this lipid did not dissolve myricetin then all available liquid lipids would have to be tested with EGCG (in a new ratio) and with resveratrol (in a 1:100 ratio). Table H4 shows that labrafac® WL1349 was able to dissolve myricetin. In Figure H3, is presented a picture of the result of the lipid screening for myricetin.

Table H 4. Dissolution capacity of myricetin in the liquid lipid selected.

	Liquid lipids essay (1:100)			
	15 min	30 min	45 min	60 min
Labrafac® WL1349	-	-	-	-

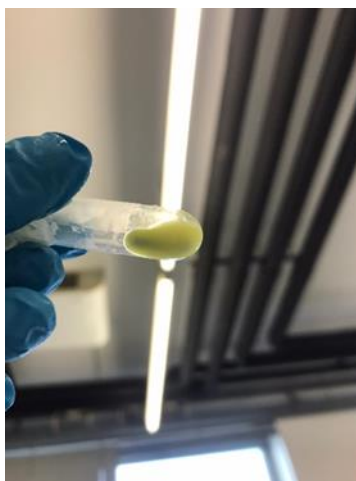


Figure H 3. Myricetin - Labrafac 1349 (1:100).

H.3.2 Liquid lipid-solid lipid compatibility

Apart from studying the dissolution capacity of natural compounds in the two types of lipids, it was also necessary to understand whether the three selected lipids were compatible, meaning that there was no visible phase separation. Therefore, the compatibility of the two solid lipids that dissolved the three natural compounds, gelucire[®] 50/13 and compritol[®] HD5 ATO, with the only liquid lipid that dissolved the three compounds, labrafac[®] WL1349, was analyzed. The first and only proportion that was tested was 50:50 (solid lipid: liquid lipid). Table H5 shows the results of this compatibility test after 1 h of heat and agitation, in which the negative sign means that the lipids are miscible, and the positive sign means that the lipids are not miscible.

Table H 5. Lipid compatibility between the three selected lipids.

Lipid compatibility (50:50)	
	Labrafac [®] WL1349
Gelucire [®] 50/13	-
Compritol [®] HD5 ATO	-

To confirm that the lipids were miscible, the samples were analyzed before and after the solidification of the mixture. As mentioned, only one proportion was tested, and it worked at the first attempt since no oil droplets were observed when the two samples solidified. If that did not happen, other proportions would have to be tried in which the solid lipid would increase and the liquid lipid decrease. In Figures H4 and H5, images of the samples are presented before and after solidification

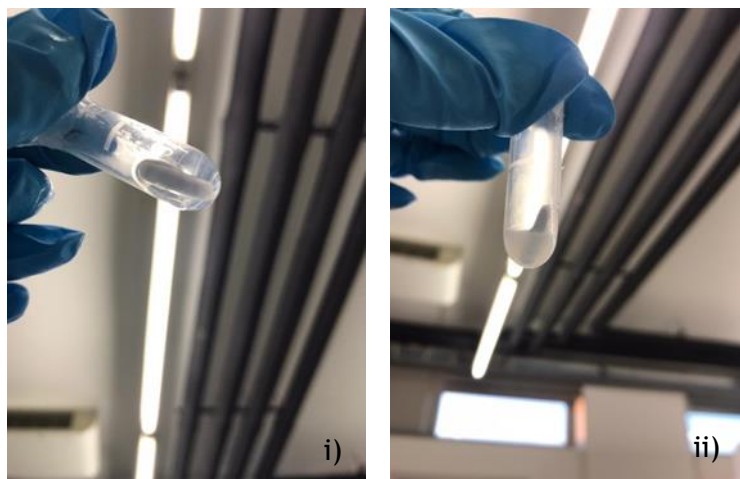


Figure H 4. i) Gelucire 50/13- Labrafac 1349 (50:50) liquid state; ii) Gelucire 50/13- Labrafac 1349 (50:50) solid state.

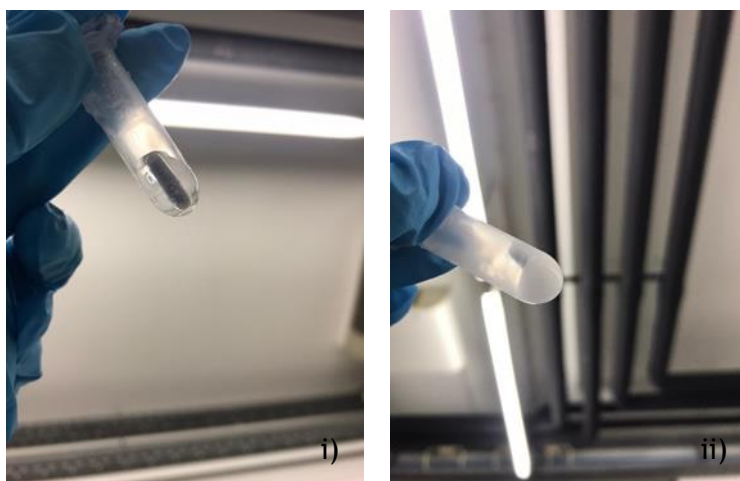


Figure H 5. i) Compritol HD5 ATO- Labrafac 1349 (50:50) liquid state; ii) Compritol HD5 ATO- Labrafac 1349 (50:50) solid state.