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Nanostructured systems containing *Passiflora edulis* extract: A new dermocosmetic alternative as skin antioxidant and depigmentant

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“*You gain strength, courage, and confidence by every experience in which you really stop to look fear in the face”.*

Eleanor Roosevelt

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Abstract

In general, products applied to the skin have great difficulties in crossing the outermost layer of the epidermis, the stratum corneum. Due to this limitation, there has been an increase in research in order to improve the cutaneous penetrability/permeability of drugs and active ingredients, being the use of lipid nanoparticles one of the most promising strategies.

As sustainability is a growing concern, the use of by-products, particularly from the food industry, is essential, which results in a decrease of tons of waste in the ecosystem. As an example, currently, the food industry uses only the passion fruit pulp, being discarded seeds and peel. In this sense, the present work consisted in the use of passion fruit seeds (Passiflora edulis) from Madeira Island to extract the oil used in the production of lipid nanoparticles, specifically nanostructured lipid carriers (NLC), with the objective of developing formulations for cutaneous application, with antioxidant and skin depigmentant effect. Passion fruit oil has an antioxidant effect due to the presence of polyphenols, such as piceatannol and resveratrol. In this work, the extracts obtained by the ultrasound method showed significant amounts of piceatannol and resveratrol when compared with a commercial oil used as a reference. The NLC prepared with Precirol® ATO5, as a solid lipid, and passion fruit oil from Madeira Island, as a liquid lipid, showed good physical and chemical characteristics. In addition, these nanoparticles showed greater tyrosinase inhibitory activity, in relation to the free oil, and good skin penetration, observed by scanning confocal microscopy.

The hydrogel developed from the NLCs, after gelation with Carbopol® 940, showed good stability over one year of storage and promoted the permeation of the active ingredient (piceatannol) in the viable epidermis, which is the target layer for the so-called antioxidant and depigmenting action.

The formulations developed in this work, obtained from a by-product of the Madeira Island food industry, can be considered safe because they do not present any cytotoxicity, in addition to presenting good stability over one year of storage. In fact, from a sustainable point of view, this new application of passion fruit seeds oil can be a strategy for reusing this by-product by the cosmetic industry.

Keywords: passion fruit oil, lipid nanoparticles, piceatannol, antioxidant, depigmenting agents.

Resumo

De um modo geral, os produtos aplicados na pele apresentam grandes dificuldades em atravessar a camada mais externa da epiderme, o estrato córneo. Devido a esta limitação, tem havido uma crescente investigação no sentido de aumentar a penetrabilidade/permeabilidade cutânea de fármacos e ingredientes ativos, sendo a utilização de nanopartículas lipídicas uma das estratégias mais promissoras.

Sendo a sustentabilidade uma preocupação crescente, é fundamental a utilização de subprodutos, nomeadamente da indústria alimentar, o que acarreta uma diminuição de toneladas de lixo no ecossistema. Como exemplo, atualmente, a indústria alimentar utiliza apenas a polpa do maracujá, sendo descartadas as sementes e a casca. Neste sentido, o presente trabalho consistiu no aproveitamento das sementes de maracujá (Passiflora edulis) da Ilha da Madeira para a extração do óleo utilizado na produção de nanopartículas lipídicas, especificamente vetores lipídicos nanoestruturados (NLC), com o objetivo de desenvolver formulações para aplicação cutânea, com efeito antioxidante e despigmentante da pele. O óleo de maracujá apresenta efeito antioxidante devido à presença de polifenóis, como o piceatannol e o resveratrol. Neste trabalho, os extratos obtidos pelo método de ultrassons apresentaram quantidades significativas de piceatannol e resveratrol quando comparados com um óleo comercial usado como referência. As NLCs preparadas com Precirol® ATO5, como lípido sólido, e óleo de maracujá da Ilha da Madeira, como lípido líquido, apresentaram boas características físicas e químicas. Além disso, as nanopartículas apresentaram maior atividade inibitória da tirosinase, em relação ao óleo livre, e boa penetração cutânea, observada por microscopia confocal de varrimento.

O hidrogel desenvolvido a partir das NLCs, após gelificação com Carbopol® 940, apresentou boa estabilidade ao longo de um ano de armazenamento e promoveu a permeação do ingrediente ativo (piceatannol) na epiderme viável, que é a camada alvo para a designada ação antioxidante e despigmentante.

As formulações desenvolvidas neste trabalho, obtidas a partir de um subproduto da indústria alimentar da Ilha da Madeira, podem ser consideradas seguras por não apresentarem qualquer citotoxicidade, além de apresentarem boa estabilidade ao longo de um ano de armazenamento. De facto, do ponto de vista sustentável, esta nova aplicação do óleo de sementes de maracujá pode ser uma forma de reaproveitamento deste subproduto pela indústria cosmética.

Palavras-chave: óleo de maracujá, nanopartículas lipídicas, piceatannol, antioxidante, agentes despigmentantes.

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List of Abbreviations and Symbols

AA	Antioxidant Activity
ABTS	2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid
ANOVA	Analysis of Variance
C*	Chroma Factor
CLSM	Confocal Laser Scanning Microscopy
CPE	Chemical Penetration Enhancers
CORAP	Community Evolutionary Action Plan
CRYO-SEM	Scanning electron cryomicroscopy
DAD	Diode Array Detection
DLS	Dynamic Light Scattering
DMEM	Doubeco´s Modified Eagle´s Medium
DPPH	2,2-diphenyl-1-picryl-hidrazil
ECHA	European Chemicals Agency
EE	Encapsulation Efficiency
FBS	Fetal Bovine Sérum
HET-CAM	Hen's Egg Test-Chorioallantoic Membrane
HPH	High Pressure Homogenization
HPLC	High Performance Liquid Chromatography
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
L*	Luminosity
LD	Laser Diffractometry
NLC	Nanostructured Lipid Carriers
NR	Neutral Red
PBS	Phosphate Buffered Saline
PDI	Polydispersity Index

PFS	Passion Fruit Seeds
PLE	Extraction by Pressurized Liquid
REZ	Resazurin
ROS	Reactive Oxygen Species
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
SCOE	Skin Membrane-covered Oxygen Electrode
SD	Standard Deviation
SLN	Solid Lipid Nanoparticles
SRD	Sulforhodamine B
TEWL	Transepidermal Water Loss
UN	United Nations
US	Ultrasound
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
ZP	Zeta Potential
ζ	Zeta Potential

Aims and Organization of the Thesis

Develop and characterize lipid nanocarriers (NLC) containing passion fruit (Passiflora edulis) seeds oil proceeding from by-products of the food industry from Madeira Island and preparation of hydrogels based on NLC, intended to exert an antioxidant and depigmenting action on the skin.

Specific objectives

- Extraction of oil from passion fruit seeds using several methods and solvents, in a sustainable and efficient manner.

- Evaluation of the antioxidant activity of passion fruit seeds oil.

- Qualitative and quantitative determination of piceatannol and resveratrol content in passion fruit seeds oil by HPLC.

- Preparation of nanostructured lipid carriers (NLC), using passion fruit oil as the liquid lipid.

- Evaluation of the physical-chemical characteristics and cytotoxicity of the developed lipid nanocarriers.

- Preparation and characterization of NLC-based hydrogels.

- Study of the tyrosinase inhibition effect of the developed formulations.

- Evaluation of the stability of the developed formulations after one year of storage.

This thesis is organized in seven chapters. The list of chapters is below:

- Chapter 1 includes the theoretical part about the structure of human skin and penetrability, antioxidant and skin whitening activity, lipid nanoparticles, passion fruit (Passiflora edulis) characteristics and sustainability.
- Chapter 2 describes some aspects related to piceatannol and its benefits on the skin, such as depigmenting, antioxidant, anti-aging, cutaneous wound-healing and anti-acne activity.
- Chapter 3 compares the Soxhlet and ultrasound extraction methods for the sustainable extraction of passion fruit oil using different extraction solvents. Antioxidant activity tests, DPPH and ABTS, were performed in the oils produced and compared with a commercial passion fruit oil.

- Chapter 4 describes the quantitative and qualitative determination of piceatannol and resveratrol in passion fruit extracts produced by Soxhlet and ultrasound methods, using Reverse Phase High-Performance Liquid Chromatography (RP-HPLC), and a comparison with a commercial passion fruit oil.
- Chapter 5 describes two techniques of preparation of nanostructured lipid carriers (NLC), namely HighPressure Homogenization (HPH) and ultrasonication, using different solid lipids. For the characterization of NLC, accelerated stability tests, particle size, zeta potential, PDI, pH analysis, *in vitro* occlusion test and irritability test using HET-CAM, were performed.
- Chapter 6 involves the development and characterization of NLC and NLC-based Carbopol® 940 hydrogels. It also involves long-term stability studies: morphology, encapsulation efficiency, particle size analysis, polydispersity index analysis, zeta potential, pH measurement, colour analysis, rheological studies, and texture analysis. In addition, *in vitro* occlusion tests, *ex vivo* skin penetration study with Confocal laser scanning microscopy (CLSM), tyrosinase inhibition activity, *in vitro* skin permeation experiments and *in vitro* cytotoxicity studies, were also described.
- Chapter 7 presents the main conclusions resulting from the studies carried out in this thesis and refers to the future perspectives.

CHAPTER 1

General Introduction

1. Human Skin

1.1. Structure and Function

The skin is the largest organ of the human body and performs several important and essential physiological functions such as homeostasis, sensory perception, as well as a barrier function against physical agents, chemicals, free radicals, microorganisms and ultraviolet radiation (UV). It also has thermoregulatory and endocrine functions (1,2) and is a source of vitamin D after sun exposure (3).

The layers of the epidermis can be defined according to their position, state of cells differentiation and morphology. In anatomical terms, it is possible to distinguish three layers of skin, namely epidermis, dermis and hypodermis (Figure 1). The deepest layer, hypodermis, is composed essentially of adipocytes and connective tissue, maintains the union between the dermis and the other tissues, serves as a protection against mechanical shock and provides also isolation against cold and heat. The dermis is an internal layer that is subdivided in reticular layer and papillary layer and has nervous, capillaries, sweat and sebaceous glands, as well as hair follicles. In the dermis we also find elastic fibers and collagen, as well as lymphatic vessels (4).

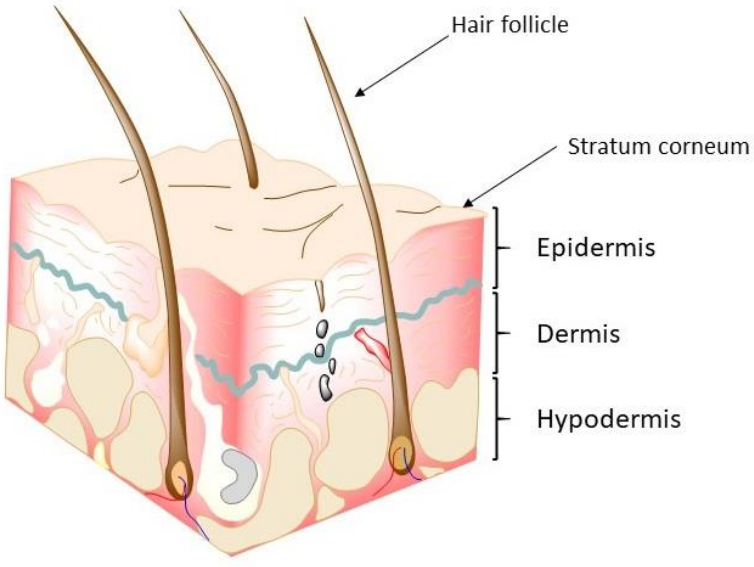


Figure 1. Skin layers representation: epidermis, dermis and hypodermis. Adapted from a template of ChemDraw® Professional 15.0.

The epidermis is characterized for being in constant renewal and for not having any vascularization. It can be organized in several layers: stratum basal (germinativum), stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum (Figure 2). In the epidermis can be found the keratinocytes, but also other cells such as melanocytes, Merkel cells and Langerhans cells (5). Merkel cells are related to the sensory nerves of the skin and Langerhans cells serve as an immunological barrier of the epidermis. Keratinocytes represent around 90% of the cells in the epidermis and are formed in the basal layer, migrating to the surface of the skin and undergoing various differentiations. Besides, it is the epidermis, mainly the stratum corneum, the most superficial layer, which plays an important role on the skin barrier function. The stratum corneum consists of corneocytes, a stacked of dead cells surrounded by free fatty acids, ceramides, phospholipids and cholesterol. Due to the lipid composition, the water permeability of the stratum corneum is decreased, so the barrier function is limited to the lipid composition and water content in the skin. In addition, the stratum corneum has a very important role in the regulation of transepidermal water loss (TEWL) (6,7).

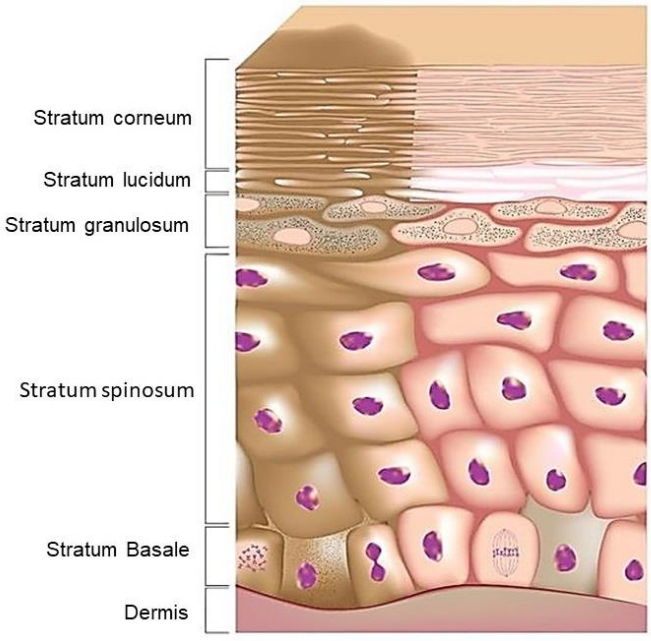


Figure 2. Dermis and layers of the Epidermis: Stratum basale, Stratum spinosum, Stratum granulosum, Stratum lucidum and Stratum corneum. Adapted from a template of ChemDraw® Professional 15.0.

1.2. Skin Permeability

The transport of the active substances through the skin can be carried out in three ways (Figure 3): intercellular lipid route between corneocytes, intracellular (transcellular) route through corneocytes and lipids or transfollicular route (8).

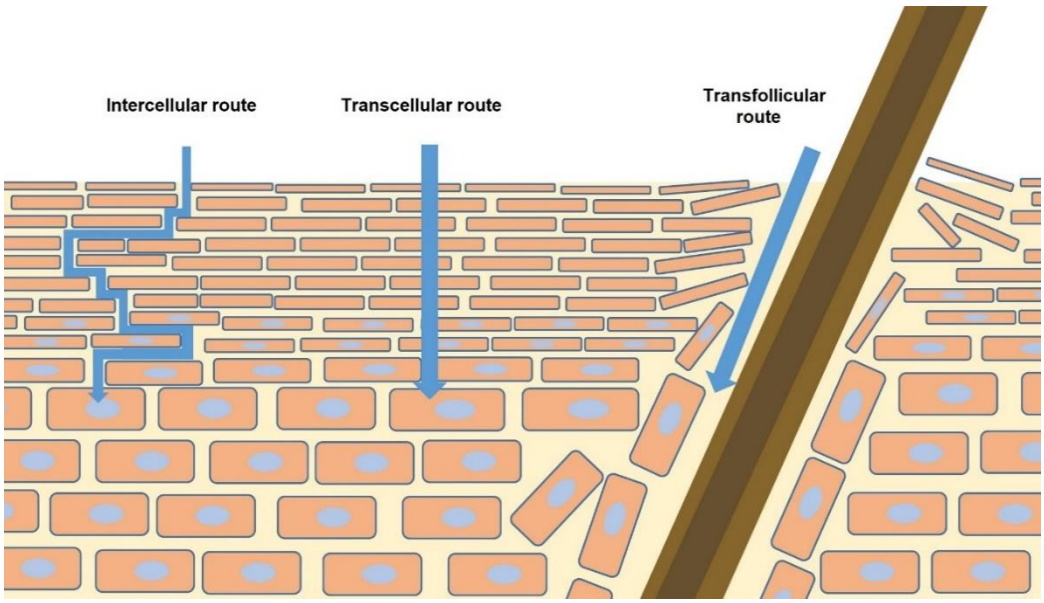


Figure 3. Skin penetration pathways: Intercellular, Transcellular and Transfollicular. Adapted from a template of Biorender®.

A very important factor in the development of cutaneous or transdermal formulations is their ability to cross the stratum corneum in an acceptable amount to have the expected effect at the desired site. Normally small substances (<500 Da), soluble in water and with lipophilic character can diffuse through the stratum corneum, however, there are rare substances that have these characteristics and their permeation tends to be low, so it is advisable that these substances have high activity to achieve the desired effect (9–11).

The effectiveness of a cutaneous product depends on the release of the active substance of the formulation, (either in gel form, cream among others), its diffusion through the different skin layers and finally the activity at the desired location (12). Different strategies can be implemented to avoid the limitations on the low cutaneous penetration of active substances. Chemical penetration enhancers (CPE) can be effective as well as techniques such as iontophoresis and use of microneedles (13,14). More recently, lipid nanostructured systems have been used for these purposes (15,16).

1.3. Antioxidant activity

The skin aging is due to different factors and causes, which can be internal or external. The most common internal causes are biological age, which can affect the efficiency of cellular functions and skin structure. There are many external causes that affect skin aging, such as climate damage, smoking, pollution, solar radiation, although the most important is oxidative stress (17,18).

The oxidative stress on the skin is mainly produced by reactive oxygen species (ROS). These substances are extremely reactive and are formed mainly in the transport chain of electrons found in mitochondria during aerobic metabolism. In this process, the organism uses molecular oxygen to obtain energy. However, around 5% of this oxygen reacts to the formation of ROS. The most common substances are free radicals such as superoxide anion, hydroxyl and peroxyl, although there are other active oxygen species that are not radicals such as hydrogen peroxide or singlet oxygen (17,19).

In normal circumstances, the organism has different mechanisms for the control of these reactive substances, including enzymes (for example peroxyredoxin and glutathione peroxidase, which convert hydrogen peroxide into water), and antioxidants. These latter are substances that react with ROS to form more stable compounds, avoiding oxidative damage. We can distinguish 2 groups of antioxidants, the endogens (produced by the same organism) and the exogenous. As exogenous antioxidants we can mention vitamins C and E, carotenoids and phenolic compounds (20,21).

Even in adverse situations, these protection processes are overcome, producing cellular damages that increase the aging process. In oxidative damage, dysfunction of mitochondria also occurs, changes in intracellular communication, as well as rupture of the extracellular matrix. The exogenous antioxidants can be added to a topical formulation to prevent oxidative stress problems (22,23).

In the cosmetics industry, anti-aging products are one of the main targets, since people are not only concerned about health, but also about appearance. Regarding anti-aging products, antioxidants can be considered innovative skin ingredients (19).

Much of the damage that occurs to the skin, such as oxidative stress, is related to UV radiation and the presence of free radicals, so the presence of antioxidant compounds is important for maintaining healthy skin. In addition, the reactive species are responsible for the increase in melanogenesis, however the presence of antioxidants would help to decrease melanogenesis and eliminate these reactive species (24). Some antioxidants, such as vitamin E and C, also inhibiting tyrosinase and preventing polymerization oxidative

of some melanin intermediates. Consequently, many antioxidants are used as skin whitening agents (25).

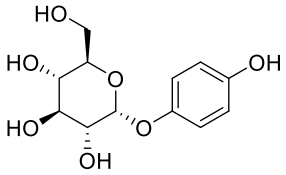
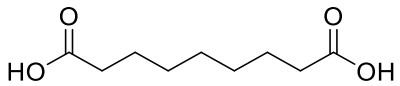
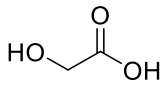
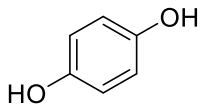
1.4. Skin whitening agents

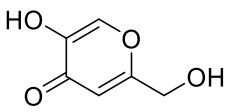
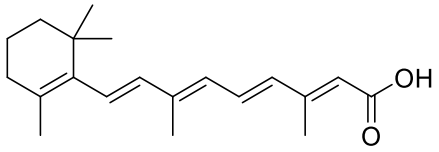
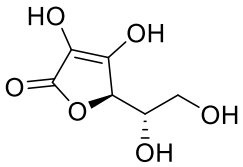
The different colour tones of the skin mainly occur due to alterations in the distribution of melanin in the body, but also include other factors such as the content of carotenoids and water and oxygenation of haemoglobin in capillary vessels. The melanin pigment is synthesized in the basal layer of the epidermis and then absorbed by the keratinocytes of the epidermis (26).

The etiology of melasma is not completely define, but sun exposure can induce the formation of melasma, which is a hyperpigmentation that usually occurs in the frontal and malar areas of the face, in women of fertile age and individuals with Fitzpatrick's phototype IV and V. In addition to exposure to ultraviolet radiation, the genetic predisposition, the use of oral contraceptives, phototoxic drugs and thyroid dysfunctions can affect the appearance of melasmas (27–29).

In Table 1 are identified the main active ingredients used as topical therapies for hyperpigmentation. Hydroquinone (1,4-dihydroxybenzen) is a melanin synthesis inhibitor and one of the most used in the world, which can be used alone or in association with other depigmenting agents. Several studies have proven its depigmentation activity *in vivo* and *in vitro*, however, this substance presents several adverse reactions, namely skin irritation, acne, allergic skin reaction and photosensitization, ochronosis and possible mutagenicity to mammalian cells. As it presents a low degradability and high toxicity, can be classified as a powerful pollutant of the environment, including water (30–34). In addition, hydroquinone is classified by ECHA (European Chemicals Agency) as category 2 carcinogenicity (suspected human carcinogen) and was also included in the Community Evolutionary Action Plan (CoRAP). In the European Union, skin whitening products containing hydroquinone or retinoic acid are regulated as medicines and not as cosmetics (35,36).

Table 1:Active ingredients commonly used for cutaneous hyperpigmentation disorders.

Active ingredients	Structure	Usual Dosage (%)	Possible mode of action	Advantages	Disadvantages	Ref.
Arbutin		10-20	Competitively inhibition tyrosinase activity	Safer, good photostability	Low effectiveness.	(37,38)
Azelaic acid		4- 20	Melanin inhibition	Well-tolerated and safe	Moderate inhibitory effect and local irritation.	(32,39–41)
Glycolic acid		5- 30	Skin turnover acceleration		Burning, epidermolysis and desquamation.	(37,42)
Hydroquinone		2-10	Competitively inhibition tyrosinase activity	Good efficacy	Use only at night; skin irritation and photosensitization, bone marrow toxicity; hypersensitivity appearance of brown spots on the skin, being called ochronosis, mutagenic and carcinogenic potential.	(30–32,43–45)

Kojic acid		1-4	Tyrosinase inhibition	It does not cause skin irritation nor photosensibility and it can be used during daytime	Irritant contact dermatitis; Pregnant restriction and children under 12 years of age restriction.	(46–48)
Retinoic		0.025-0.1	Tyrosinase transcription	Improved efficacy	Most commonly erythema and hypopigmentation.	(49,50)
Vitamin C		1-5	Product reduction and ROS scavengers		Low stability and skin penetration difficulty.	(29,37)

It is estimated that around 15 % of the world population uses skin whitening products, being China and India, respectively, the countries that most use it. With this great demand for skin lightening products, it becomes necessary the research for new products, being the natural ones the most required, because until now the existing skin whitening products present many adverse reactions or are not very effective and have a long treatment period.

There are several natural extracts that show efficacy as depigmenting or whitening agents. As an example, the effect of methanolic extract of jambu (*Acmella oleracea*) on the tyrosinase enzyme was evaluated. In this study it was possible to verify a strong *in vitro* inhibitory effect of the tyrosinase enzyme due to the large amount of spilanthol in the extract, proving to be a possible topical depigmenting alternative (51).

Recently, an *in vitro* study was carried out, evaluating the potential inhibitory effects of *Annona squamosa* leaf extract on melanogenesis using murine B16F10 melanoma cells. In this study it was demonstrated that β-sitosterol is one of the phytochemical compounds that was in greater quantity in the extract and that this has a strong inhibitory effect of tyrosinase in B16F10 cells (52).

Another plant extract that has demonstrated skin whitening activity is *aloe vera*. The *aloe vera* extract presents several phytochemicals like aloesin and b-sitosterol, among others (53–55).

It has been reported that green tea (*Camellia sinensis*) presents very strong antioxidant and antimelanogenic activity, on mushroom tyrosinase activity *in vitro*, in B16F10 melanoma cells and *in vivo*. This extract presents a competitive tyrosinase inhibitor effect due to the presence of several catechins, among which epicatechin-3-gallate, epigallocatechin-3-gallate and galocatechin-3-gallate (31,56–58).

In order to improve the stability and effectiveness of these extracts, different types of nanoparticles, mainly lipid nanoparticles, have been used for their encapsulation.

2. Lipid Nanoparticles

There are several types of nanocarriers and due to this variety the right choice regarding the desired characteristics such as good stability, bioavailability and release characteristics, becomes a fundamental step. Lipid nanoparticles began to be used in the 1990s to overlap the problems presented by traditional colloid systems, being initially developed in the form of solid lipid nanoparticles (SLN), and only later nanostructured lipid carriers (NLC) were developed (59,60). Regarding the dimensions, these nanoparticles have between 0.1 and 1000 nm (61). The SLN are made with a solid lipid (0.1 - 30% w/w), an aqueous medium

and surfactants. NLCs, in addition to the solid lipid, contain a liquid lipid in their composition. Due to their composition, lipid nanoparticles are considered biocompatible and biodegradable (60).

Lipid nanoparticles present several advantages such as increased chemical stability of encapsulated substances, especially those that are sensitive to light, hydrolysis and oxidation, protecting them against degradation. They also promote a prolonged release of active substances and increase their occlusive properties when applied to the skin, due to their reduced size which allows greater contact with the superficial junctions of the corneocytes (62,63).

NLC have some advantages over SLN, such as encapsulation of high amounts of active substances, as well as a longer encapsulation time of active substances inside them during storage. These advantages may be due to the rearrangement of the NLC components, due to the fact that in its formulations there are solid and liquid lipids, leading to the formation of imperfect crystals, unlike SLN which have a relatively perfect crystalline structure, according to Figure 4 (64,65).

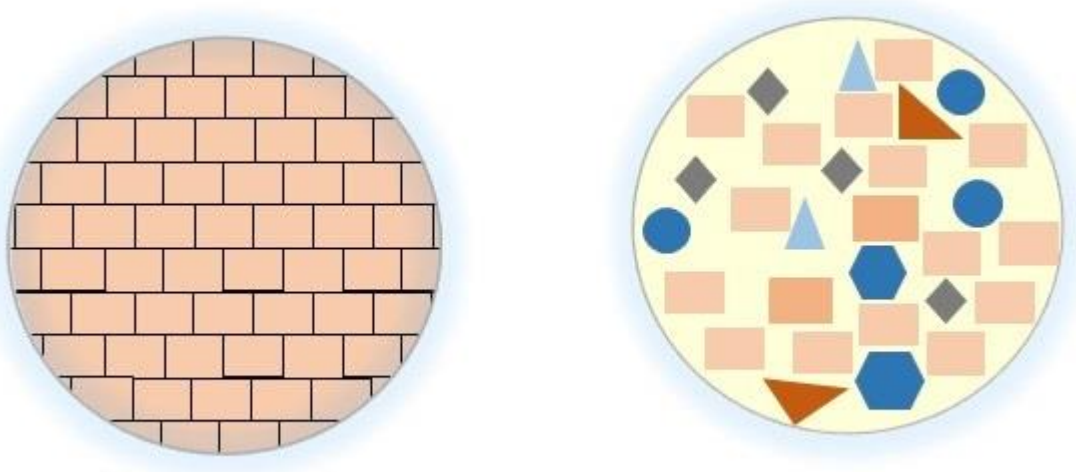


Figure 4. Lipid Nanoparticles: SLN and NLC. Adapted from Pardeike et al. (60).

As can be seen in Table 2, in the literature can be found several plant extracts used as liquid lipids in the composition of NLC.

Table 2: Examples of solid lipids and plant extracts used as liquid lipids in the composition of NLCs.

Liquid lipid	Solid Lipid	Ref.
Centella (*Centella asiatica*) extract	Stearic acid	(66)
Clove (*Syzygium aromaticum*) oil	Carnauba wax and beeswax	(67)
Grape (*Vitis vinifera*) seed oil, St. John'swort oil (*Hypericum perforatum*) and sea buckthorn (*Hippophae rhamnoides*) oil	Cetyl palmitate and glyceryl stearate	(68)
Hibiscus (*Hibiscus sabdariffa*) oil	Hydrogenated coco-Glycerides	(69)
Marigold (*Tagetes erecta Linn*) flower extract	Glyceryl monostearate and stearic acid	(70)
Marigold (*Tagetes patula*) oil	Glycerol monostearate and Cetyl palmitate	(71)
Melaleuca (*Melaleuca alternifolia*) oil	Cetyl palmitate	(72)
Parsley (*Ridolfia segetum (L.) Moris*) oil	Glyceryl palmitostearate	
Pomegranate (*Punica granatum*) seeds oil	Propolis wax and beeswax	(73)
Pterodon pubescens fruit oil	Glyceryl palmitostearate	(74)
Pumpkin (*Cucurbita pepo*) seed oil	Glyceryl palmitostearate	(75)
Sunflower (*Helianthus annuus*), sweet almond (*Prunus dulcis*), olive (*Olea europea*) and coconut (*Cocos nucifera*)	Lauric acid, myristic acid, palmitic acid and stearic acid	(76)
Tumeric (*Curcuma longa*) oil	Glyceryl behenate	(77)
Virgin olive (*Olea europea*) oil	Hydrogenated palm oil	(78)

As NLCs have low viscosity, it is important to increase their viscosity to improve the time of contact with the skin when used topically. One of the strategies to achieve this goal is the incorporation of NLC in a hydrogel, obtaining a semi-solid system designated nanostructured lipid carrier-based hydrogel (79).

For example, recent articles have demonstrated the use of Carbopol® 940 as a gelling agent to improve the viscosity of NLC dispersions (80–83). Carbopol® 940 is a cross-linked

polyacrylate acid polymer widely used in hydrogels preparation because it has the capacity to form gels in aqueous solution after neutralization. Besides improving the rheological characteristics of the formulations, it has good optical clarity, low price and high effectiveness. In addition, it is compatible with several products used in the pharmaceutical, cosmetics and food industries (84,85).

3. *Passiflora edulis*

There are more than 500 species belonging to the *Passiflora* genus, being the most used the purple passion fruit (*Passiflora edulis f. Sims*), Passiflora incarnate L and the yellow passion fruit (*P. edulis f. flavicarpa*). However, P*assiflora edulis* is not included in any Pharmacopoeia, while the other well-known species, *Passiflora incarnata L.,* is officially included in the French and German Pharmacopoeia, the Homeopathic Pharmacopoeia of the United States of America and the British Herbal Compendium. The species belonging to this genus are climbing plants, usually found in Tropical and subtropical regions, including Brazil, Peru, India, Australia, China and Madeira Island, a region belonging to Portugal (86,87).

The passion fruit has been used for many years in traditional medicine as a sedative for anxiety disorder. The main responsible for this effect is the passiflorin, found in the leaves of the passion fruit (88–91). In addition to its medicinal use, passion fruit is widely used in the food industry. Once the production of passion fruit is intended predominantly for the production of juice, only the pulp is used and the seeds are discarded. Since each passion fruit has around 200 seeds inside, this means that large quantities of waste are discarded into nature. Adding value to these by-products of the food industry is of great interest in the scientific, environmental and economic point of view, because these residues can have properties that can be useful for the pharmaceutical, cosmetic and food industries (92,93).

The purple passion fruit from Madeira Island (Figure 5) is a product that has great interest and economic potential with an average annual production of 140 tons. This passion fruit presents good adaptability to the edaphoclimatic conditions of Madeira Island (average temperature of 25 ºC, precipitation 1300 mm, sandy soil with pH 6-7.5) (94). Therefore, this plant has few studies in the literature in comparison with other highly studied passion fruits, such as the yellow passion fruit from Brazil.

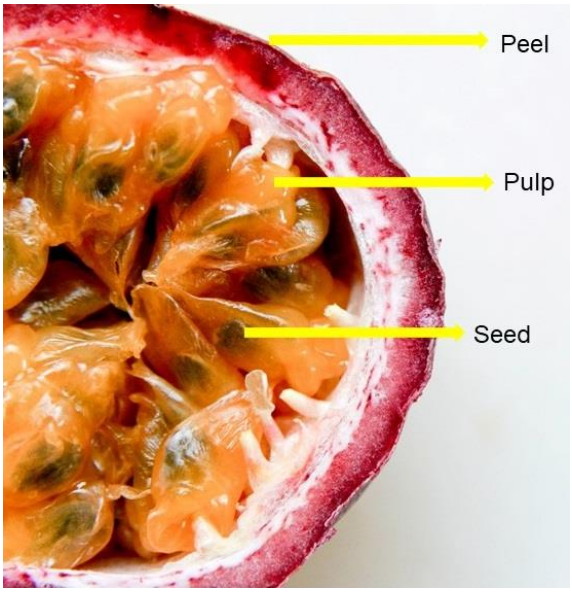


Figure 5. Passiflora edulis fruit: peel, pulp and seeds.

There has been increasing interest in passion fruit seeds, since they can contain large quantities of polyphenols, with various properties such as antioxidant, anti-inflammatory, anti-aging, anti-cancer, among others. Among the main polyphenols found in passion fruit seeds are piceatannol, resveratrol, quercetin, luteolin, gallic acid and rutin, but it is known that these seeds can also contain large amounts of unsaturated fatty acids, vitamin C and vitamin E (95–100).

4. Valorisation of food waste and by-products

Sustainability is based on environmental, economic and social dimensions, and has the objective of satisfying its own needs in the present moment, but without harming the future. In the last few decades, there has been an increase in the number of articles related to food industry by-products or waste. Table 3 presents recent research on food waste valorisation and consequent application.

Table 3: Recently published articles related to food wastes and their pharmaceutical or cosmetic application.

Food waste materials	Biological activities	Ref.
Avocado seed	Antioxidant properties and antimicrobial activity	(101)
Chestnut shells	Anti-aging effect	(102)
Coffee Silverskin	Antioxidant activity	(103)
Macadamia skin	Antioxidant properties	(104)
Orange peel	Antibacterial and anti-inflammatory properties	(105)
Passiflora edulis peel	Reduction in lipid peroxidation in the kidneys	(106)
Pistachio green hull	Antioxidant and antimicrobial activity	(107)
Pomegranate peel	Antimicrobial activity	(108)
Soybean residue (okara)	Strong antioxidant and anti-inflammatory effects.	(109)
Spent coffee grounds	Skin anti-aging and skin whitening	(110)

Currently worldwide we are experiencing a problem in the management of food waste because most of the food waste goes to landfills, to the compost or serve as animal feed. It is estimated that one third of the world's food is currently wasted (111,112). Meanwhile in 2015, it was suggested in the "Circular Economy Action Plan" by the Commission in the European Parliament, the use of food waste and consequent reinjection into the economy. As well, the United Nations (UN) 2030 Agenda for Sustainable Development, appeal to the importance and urgency of the topic. Thus, the reutilization of food waste is of extreme importance, as it affects the ecological, social, economic, and health means (113).

In March 2020, once again the European Commission revealed the importance of the circular economy, reinforcing on the sustainability of the reuse of food waste, thus creating "A new Circular Economy Action Plan for a cleaner and more competitive Europe" (114).

Due to the current situation, with an increase in consumption and the limited amount of resources, it is necessary to seek more sustainable alternatives that reduce the impact on the environment, in addition to boosting the local economy by bringing the food industry closer to the pharmaceutical and cosmetics industries.

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CHAPTER 2

Benefits of skin application of Piceatannol — A minireview

This Chapter was submitted for publication in the journal *Plant Science* as:

“Benefits of skin application of Piceatannol — A minireview”

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Graphical abstract

Abstract

The skin is the largest organ of the human body and has several functions such as barrier against external agents, the maintenance of temperature and homeostatic functions. Skin aging is a natural process that can be influenced by environmental factors, intrinsic skin factors and lifestyle. UV light plays an important role in skin aging, and can cause spots, requiring the use of depigmenting agents. Nowadays there is a great demand for ingredients that prevent skin aging, with natural agents occupying a promising position. Among the natural agents, polyphenols, such as resveratrol and piceatannol, found in grapes, passion fruits and other fruits, have a huge relevance. Great benefits of piceatannol have been reported, so thus this work focuses specifically on a review of the literature regarding the application of this polyphenol in skin care products. This polyphenol can be used in a wound-healing, or as anti-aging, antioxidant, anti-acne and skin whitening, among other effects.

Keywords: piceatannol, antioxidant, skin, polyphenol

1. Introduction

Over the years our skin structure changes, the epidermal layer becomes thinner, wrinkles and spots may appear and there is a major trans epidermal water loss, especially in elderly individuals, resulting in dryness (xerosis). In addition, there is a reduction in skin elasticity, with decreased collagen and elastin. Nowadays there is a great demand for agents that prevent skin aging and skin darkening, and the natural compounds occupy a promising position (1).

Piceatannol (3,3',4',5-trans-tetrahydroxystilbene) (Figure 1) is a stilbenoid compound and a resveratrol analogue (2).

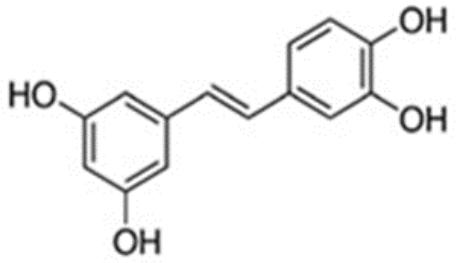

Figure 1. Piceatannol structure.

A search was performed in PubMed from 2010 to 2019 concerning piceatannol and resveratrol (Figure 2). It is possible to verify a growing interest in these phenolic compounds of natural origin. Only 473 articles about piceatannol and 10986 about resveratrol were reported in the literature over the study time. It is possible to verify a much lower number of articles about piceatannol than resveratrol. It is less studied but displays a wide spectrum of biological activity and some studies reveal better activity than resveratrol (2,3).

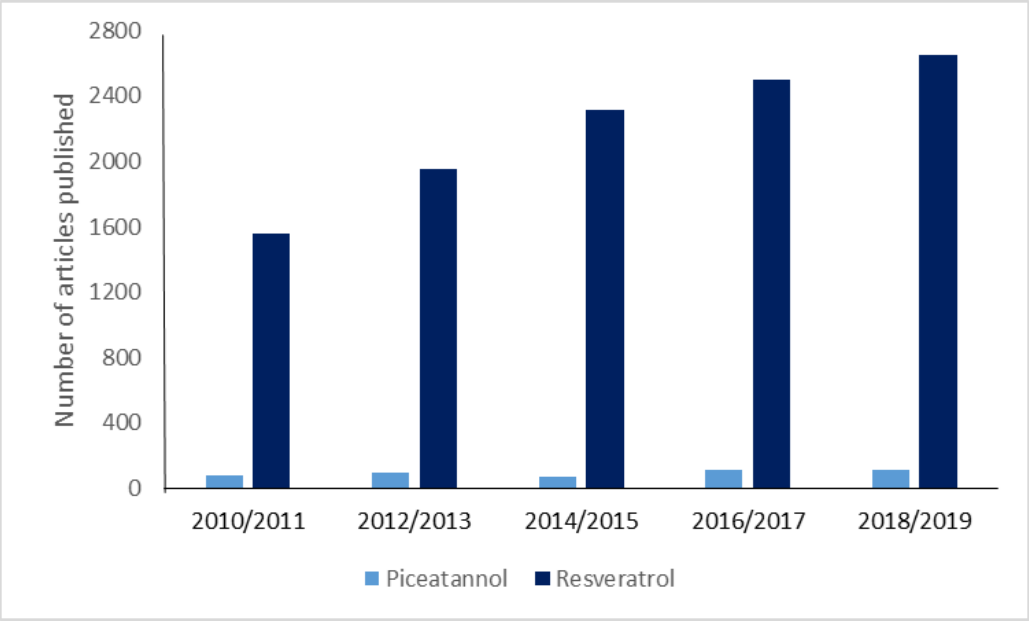

Figure 2. Number of articles published in PubMed about piceatannol and resveratrol from 2010 to 2019.

2. Natural sources of piceatannol

Piceatannol can be found mainly in grapes. However, there are already studies that report the presence of piceatannol in passion fruit, white tea and rhubarb, among others. As showed in Table 1, in some species, piceatannol can be found in seeds, while in other plants it can found in leaves or rhizomes.

Regarding the piceatannol extraction from plants, there are some methods described in literature, such as ultrasound, Soxhlet, maceration, microwave, supercritical fluid extraction. The extraction time, the solvent used and the temperature employed are some of the characteristics that must be taken into consideration in the selection of the piceatannol extraction method from the plant.

Table 1: Natural source of Piceatannol and extraction method.

Natural source	Extraction Method	References
Blueberries (Vaccinium berries)	Frozen berries were mixed with 3 volumes of methanol: acetone: water: formic acid (40:40:20:0.1), kept for 30 min, and ground in a Virtis homogenizer for 2 min.	(4)
Euphorbia lagascae seeds	The air-dried powdered seeds were extracted twice with n-hexane at room temperature.	(5)
Grape stems	Methanolic extracts using Ultrasound Assisted Extraction.	(6)
Grapevine *Vitis vinifera*	Dissolved in Methanol.	(7)
Lophatherum gracile stem and leaves	95% ethanol at a temperature of 80 °C under reflux.	(8)
Moscato bianco grapes	Methanol extract using Ultra-Turrax.	(9)
Passion fruit (*Passiflora edulis*) seeds	The ground seeds were extracted with 70% acetone 3 times, with shaking.	(10)
Passion fruit (*Passiflora edulis*) seeds	Extraction with an organic solvent.	(11)
Passion fruit (*Passiflora edulis*) seeds and seed cake	Supercritical fluid Extraction and Ultrasound.	(12)
Polygonum cuspidatum roots	Crude MeOH extract.	(13)
Rhubarb *(Rhei undulati* rhizome)	The dried rhizome was extracted with methanol at room temperature for 24 hrs.	(14)
The sim fruit (*Rhodomyrtus tomentosa*)	The powdered freeze-dried fruit was mixed with acetone: water: acetic acid (50:49:1; v/v/v) and shaken for one hour at 37 °C.	(15)

3. Skin benefits of piceatannol

Piceatannol has several beneficial effects on the skin, namely skin whitening, antioxidant, anti-aging, cutaneous wound-healing and anti-acne activity, it presents anti-allergic effect, and potential anti-cancer properties, probably due to its ability to suppress the proliferation of a wide variety of tumor cells, including leukemia, lymphoma; breast and lung cancers (3,16–18).

3.1. Skin whitening activity

Human skin color originates from the outermost layer of the skin, the epidermis, where the pigment-producing cells, melanocytes, are localized to produce melanin. The distribution pattern of synthesized melanin by the melanocytes determines the actual color of the skin. Melanin is formed by a process called melanogenesis through a combination of enzymatically catalyzed and chemical reactions. The biosynthetic pathway of melanogenesis has been elucidated, where two types of melanin are synthesized within melanosomes: eumelanin and pheomelanin (19).

Although melanin has mainly a photoprotective function, excess melanin production or abnormal distribution can cause irregular hyperpigmentation of the skin. Exposure to some drugs and chemicals, as well as the existence of certain diseases, can result in hyperpigmentation (20). For this reason, there is a high demand for melanogenesis inhibitors which allowed to reduce or prevent these hyperpigmental disorders.

The skin whitening agents appeared in the 60s of the 20th century with the discovery of hydroquinone to treat freckles, melasmas, senile lentigo, among others (21). As the treatment of melasmas is prolonged, the use of hydroquinone has its drawbacks, as it has various adverse effects, such as irritation and loss of skin elasticity, contact dermatitis, nail pigmentation, sweat with bad odor, as well as possible mutagenicity and carcinogenicity (22).

The studies regarding natural products have increased significantly, including natural depigmenting agents (23–27). Some researchers compared the effect of piceatannol and resveratrol, extracted from passion fruit seeds, on melanogenesis and collagen synthesis using cultivated human melanoma and fibroblast cells. It was verified that there was a significant increase in melanin synthesis inhibition, as well as an increase in collagen production in the samples tested with piceatannol and resveratrol, showing that piceatannol was superior to resveratrol in both cases, possibly due to the structure of the piceatannol, which presents one more hydroxyl group than resveratrol. (10).

The *in vitro* effect of passion fruit extract using human MNT-1 melanoma cells and human SF-TY fibroblast cells was studied for the inhibition of melanogenesis and the promotion of collagen synthesis. It was demonstrated, due to the presence of polyphenols like piceatannol, resveratrol and sircusin B in passion fruit seeds extract, that there was a strong inhibition of melanin synthesis, as well as an increased collagen synthesis (28).

3.2. Antioxidant activity

Antioxidants are regarded as promising agents that reduce skin oxidative stress. In recent years, naturally occurring compounds, such as phenolic acids, flavonoids, and high molecular weight polyphenols have gained considerable attention as beneficial protective agents. *In vitro* antioxidant activity of piceatannol in human fibroblast cells was investigated. It was demonstrated that piceatannol has strong antioxidant activity even at low concentrations and has a certain cytoprotective capacity (29).

Piceatannol has a very strong antioxidant activity, being similar to the antioxidant activity of ascorbic acid and superior than its analogue resveratrol (3,7,30,31). Piceatannol is more active than resveratrol due to the presence of an additional hydroxyl group at 3' position. The presence of an extra hydroxyl group in piceatannol makes it reactive and a more potent antioxidant when compared to resveratrol (3).

Studies using HaCaT-type human keratinocyte cell lines have also been performed, proving the antioxidant effect of piceatannol (32).

Some researchers described the antioxidant effect of some polyphenols, including piceatannol, resveratrol and quercetin, using a porcine skin membrane-covered oxygen electrode (SCOE), an *in vitro* model to identify reactive oxygen species (ROS). The study was based on ROS reactions that occur in the skin after topical application of piceatannol and other polyphenols, showing a great decrease in ROS effects (33).

3.3. Anti-aging activity

A study was performed in Japan with women using capsules containing passion fruit seeds extract, rich in piceatannol (5 mg). The study was conducted for 8 weeks and several measurements were made on the skin of women's cheeks, such as viscoelasticity and transepidermal water loss (TEWL). It was shown a significant increase in skin hydration in the users of the extract in relation to the placebo. About the TEWL, there was a decrease in the group of those who used the passion fruit extract. Finally, the study concluded that due to the high antioxidant activity of piceatannol and increase of the collagen synthesis, skin hydration can be improved, preventing skin aging (34).

3.4. Cutaneous Wound-healing activity

As aforementioned, the skin has the capacity to act as a natural barrier against external agents. Skin damage over a large area of the body can cause serious health problems and even death. Wound healing is a natural and complex process involving several physiological factors such as anti-inflammatory, antioxidant and antimicrobial activities, participating in this process several growth factors, such as the vascular endothelial growth factor (VEGF), the cytokines and several hormones. Annually, many people suffer skin injuries, such as burns and ulcers caused by diabetes mellitus or pressure, having to make use of wound-healing agents (23,35,36).

Studies using grape seeds extract, rich in resveratrol and piceatannol, when tested *in vitro* on HaCaT keratinocyte cells, demonstrated wound-healing activity. This possibly occurred due to the large amounts of antioxidants in the extract (37). *In vitro* tests were also performed with 293T cells demonstrating the effect of polyphenols, including piceatannol, on inhibition of endothelial migration during wound-healing assays (38).

3.5. Anti-acne properties

Piceatannol can also be used against *Propionibacterium acnes (P. acnes).* Acne vulgaris affects many people mainly in adolescence, and around 85% of the population presents this dermatosis (39,40). One of the bacteria belonging to the human face microbiota is P. acnes, gram-positive anaerobic bacteria, which is responsible for acnes vulgaris. Certain cases of acne can be considered serious, causing inflammatory problems, scars and psychosocial problems, so it is necessary to use isotretinoin in the treatment. Due to the serious side effects of isotretinoin, it is necessary to investigate new natural products to treat acne effectively and safely (41).

A study was conducted for 28 days, with seventeen volunteers with acne, at an average age of 17 years, being applied to the face of a group, twice a day, a gel with berries (*Rhodomyrtus tomentosa*), and in the other group a placebo gel was applied. The berries extract had organic acids, rhodomirtone, piceatannol (500 ppm) and other polyphenols. After the study, it was verified in the volunteers who used the gel with the natural extract a significant decrease in acne symptoms, as well as a decrease in papules and black spots (39).

Some researchers reported the comparison of the in vitro anti-acne activity of purple passion fruit extract (*Passiflora edulis Sims var. edulis)* with clindamycin and erythromycin, two antibiotics that are widely used to treat acne. The extract was obtained from passion

fruit seeds by maceration, containing a large amount of piceatannol. As a result, the passion fruit extract showed the same results as the other two antibiotics used, showing a great inhibitory effect of *P. acnes* (42).

Recent studies have also shown that piceatannol inhibited the proliferation of human keratinocyte cells (HaCaT cell line) induced by *P. acnes* and no *in vitro* cytotoxicity was observed in this cell line (43).

4. Conclusions

This review allows to demonstrate *in vitro* and in vivo the potential of piceatannol, a polyphenol that can be found naturally in some fruits, such as passion fruit and grapes.

Studies indicate that piceatannol presents a strong antioxidant activity, even superior to its resveratrol analogue, possibly due to the fact that piceatannol has one more hydroxyl group than resveratrol. In addition, it can be used topically due to its wound healing, anti-acne and skin whitening properties.

Besides few phytochemical investigations on piceatannol, it was verified that there is much less research on the cutaneous application of piceatannol. It would therefore be an interesting research topic.

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CHAPTER 3

Optimization of extraction parameters on the antioxidant activity of passion fruit waste

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Research Paper

Optimization of extraction parameters on the antioxidant activity of passion fruit waste

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Abstract

The aims of this study were to compare the effectiveness of Soxhlet and ultrasound extraction methods for obtaining *Passiflora edulis* seeds oil from Madeira Island and to evaluate its antioxidant capacity. The effects of two different extraction methods (Soxhlet, ultrasound) and four solvents (acetone, isopropanol, ethanol, and hexane) were investigated in terms of the efficiency of the extraction process. The *in vitro* antioxidant properties were determined using DPPH (2,2-diphenyl-1-picryl-hidrazil) and ABTS (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) methods. In spite of the great efficiency of the Soxhlet method, the results of this study demonstrated that the extraction by ultrasound, using ethanol as biosolvent, allowed obtaining an oil with higher antioxidant activity.

Key-words: Passion fruit, antioxidant activity, extraction methods, piceatannol, natural product.

1. Introduction

The genus *Passiflora* includes over 500 species (1). Purple passion fruit (*Passiflora edulis Sims var. edulis*) is a tropical climbing plant, belonging to the Dicotyledonous class and Passifloreaceae family (2). The seeds of *P. edulis* from Madeira Island are yet to be studied. Currently, only the pulp is used for producing fruit juice. Skin and seeds are not used in the food industry, being considered as waste (3). However, the seeds of passion fruit have been widely used in cosmetic industries due to their antioxidant properties, inhibition of melanin synthesis, and induction of endothelial nitric oxide synthase (4).

Passion fruit contains many beneficial phytochemicals for the skin, such as polyphenolic compounds (piceatannol and resveratrol (Figure 1)), carotenoid and ascorbic acid (5). Piceatannol (3,3',4',5-trans-tetrahydroxystilbene) is a polyphenolic compound abundant in the seeds of passion fruit (*P. edulis*) (6) that can be converted to resveratrol and exhibits strong anticancer activity in tumor cells (7). The aim of this study was to compare the antioxidant activity of passion fruit seeds (from Madeira Island) extracts obtained by Soxhlet and ultrasound using different solvents (acetone, ethanol, isopropanol, hexane).

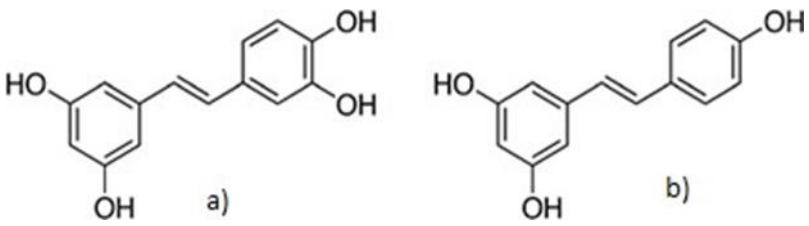

Figure 1. The two polyphenolic compounds: Piceatannol (a) and Resveratrol (b).

2. Materials and Methods

2.1. Materials

Passion fruit seeds were obtained as a by-product of a food industry from Madeira Island. A commercial passion fruit seeds oil was also purchased from Akoma (UK), for comparison with the oil obtained by extraction of the above mentioned by-products obtained from the food industry. Isopropanol was purchased from Emsure Merck (Germany), Acetone from Fisher Chemical (UK), and Ethanol, n-Hexane, ABTS and DPPH from Sigma Aldrich (UK).

2.2. Methods

2.2.1. Soxhlet extraction

The Soxhlet method was selected as a conventional extraction technique. For each extraction, 10 g of passion fruit seeds from Madeira Island were packed in porous cellulose filter thimble and inserted in the Soxhlet extractor. Thereafter, 250 ml of the solvent (Ethanol, Acetone, Isopropanol or n-Hexane) were added and the system was heated until boiling. Reflux was kept for 8 h, then the extraction solvent was eliminated in a rotary vacuum evaporator (Buchi, Switzerland) and the extract was weighed to constant value. The Soxhlet extraction temperature was kept constant five degrees above the boiling point of the solvent in all assays. The assays were conducted in triplicate.

2.2.2. Ultrasound extraction

The ultrasound extraction was performed in an ultrasonic bath (35 kHz/80 W) (Bandelin Sonorex RK100h, Germany) at room temperature. A preliminary study was carried out to choose the best extraction time (5, 10, 15, 30, 60 and 120 min) using a 1:4 (m/v) solid (seeds) to solvent ratio. Then, the mixture was filtered by a vacuum system. Thereafter, the solvent was removed using a rotary vacuum evaporator (Buchi, Switzerland). The results were the mean of three replications.

2.2.3. Extraction yield

For each extraction experiment, the yield was calculated according to Equation (1), in which m_e is the weight of the total extract and m_s is the weight of the seeds that were used in the process:

$$Yield\ (\%) = \frac{me}{ms}\ x\ 100 \qquad \text{Eq. (1)}$$

2.2.4. Antioxidant activity

The *in vitro* antioxidant activity of the passion fruit seeds oil was determined using the DPPH (2,2-diphenyl-1-picryl-hidrazil) method and ABTS (2,2'-azino-bis-(3- ethylbenzthiazoline-6-sulfonic acid) assay. DPPH is known as a stable free radical in solution which possesses a characteristic maximum absorption at 515 nm in ethanol (8,9). The DPPH assay was performed according to the method of Brand-Williams et al. (10) with some modifications. Quartz cuvettes (1 cm) were used for absorbance measurements. Ethyl acetate was used to dissolve the DPPH as reported by Espin et al. (11).

The Antioxidant Activity (AA) was calculated graphically (Equation 2) by plotting the percentage of remaining DPPH·, estimated according to a standard curve, against sample concentrations (10, 25, 50, 75 and 100 mg/mL). TE is Trolox equivalent antioxidant activity and the results were expressed in μmol Trolox equivalents (TE)/ 100g oil:

$$AA\ (\%) = \frac{\text{Abs (DPPH + ethyl acetate)} - \text{Abs (sample)}}{\text{Abs (DPPH + ethyl acetate)}}\ x\ 100 \qquad \text{Eq. (2)}$$

In specification, the ABTS assay is based on the generation of a blue/green ABTS that can be reduced by antioxidants (12). The antioxidant capacity assay was carried out using a UV-VIS Spectrophotometer mini 1240 (Shimadzu, Japan) using the improved ABTS method as described by Re et al. (13).

The stock solution containing ABTS (7 mM) and potassium persulfate (2.450 mM) was kept at room temperature for 16 h in a light protection vessel. Before use, the solution was diluted in ethanol to obtain an absorbance of 0.700 ± 0.200 at 750 nm using a UV-VIS Spectrophotometer mini 1240 (Shimadzu). In the assay, 20 μL of samples were mixed with the ABTS solution (180 μL), individually. The absorbance at 750 nm was determined after 6 min of mixing using the microplate reader. The ability to scavenge ABTS was calculated. Ascorbic acid standard solution in 80% ethanol was prepared and assayed under the same conditions. The absorbance of the resulting oxidized solution was compared to that of ascorbic acid standard solutions. The % inhibition can be calculated with the formula below (Equation 3). All determinations were performed in triplicate and the results were expressed as μmol ascorbic acid equivalent/100 g oil. A calibration curve was made in the range of 0.02 to 0.50 mg/mL.

$$\%\ Inhibition = \frac{\text{Abs (ABTS + ethanol)} - \text{Abs (ABTS sample)}}{\text{Abs (ABTS +ethanol)}}\ x\ 100 \qquad \text{Eq. (3)}$$

2.2.5. Data Analysis

The influence of the extraction method (Soxhlet and Ultrasound) and the use of different solvents (acetone, ethanol, isopropanol, n-hexane) on extraction yield of passion fruit seeds oil, as well as Antioxidant activities of oils by DPPH and ABTS methods were evaluated by Levene`s test for homogeneity of variances and one-way analysis of variance with IBM SPSS Statistic 25® software package. Tukey`s test was used for *post hoc* comparisons. Significance was tested at the 0.05 level of probability.

3. Results and Discussion

Since the highest oil extraction yield was obtained at 60 min of ultrasonication, the studies were followed by using this time of extraction. Similar results were obtained in studies with passion fruit, pomegranate, and pumpkin seeds oil (14,15).

The Soxhlet method allowed obtaining a high production of passion fruit seeds oil. As shown in Figure 2, using the ultrasound method, ethanol was the solvent that showed the best results (22.95%), followed by acetone (11.46%). The extraction with isopropanol (9.72%) and n-hexane (10.47%) was lower than with the other solvents. The extraction yield using the Soxhlet method with acetone (34.54%) was higher than with ethanol (33.84%), isopropanol (28.52%) and n-hexane (24.39%). The values of the yield of passion fruit seeds oil using the ultrasound and Soxhlet methods were compared with the analysis of variance. Therefore, it can be concluded that there are no statistically significant differences between the two methods ($p<0.05$). The Tukey`s test showed that, for the ultrasound method, there were significant differences between the ethanol and the other solvents. For the Soxhlet method, there were no significant differences between the use of ethanol and acetone, and between isopropanol and n-hexane.

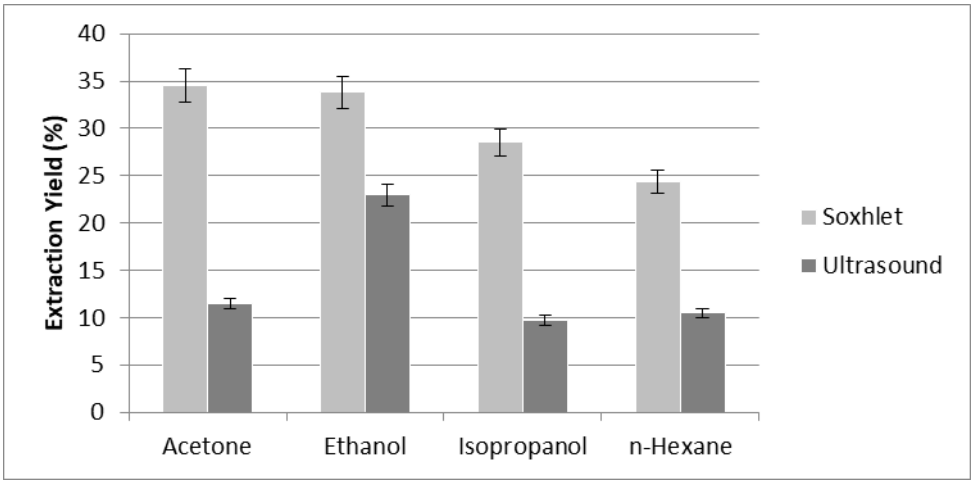

Figure 2. Extraction yield of passion fruit seeds oil using Soxhlet and Ultrasound methods.

Thus, one of the main advantages of using the ultrasound method is that it may provide higher selectivity. Furthermore, it significantly reduces sample processing time because the ultrasound time is relatively short (60 min) as compared with the 8 h required in the Soxhlet extraction method. A general overview of the results indicates that the ultrasound method is faster, more efficient and more selective for polyphenols than the Soxhlet method as verified by other researchers (16).

It can be seen (Figure 3) that the commercial oil showed lower antioxidant activity as compared with the PFS oils obtained from Madeira Island using the ultrasound method. For example, in Figure 4, the sample obtained with ethanol using the ultrasound method exhibited the strongest anti-radical inhibition (300 µmol/100g oil), which was 50-fold higher than that obtained with n-hexane (6 µmol/100g oil).

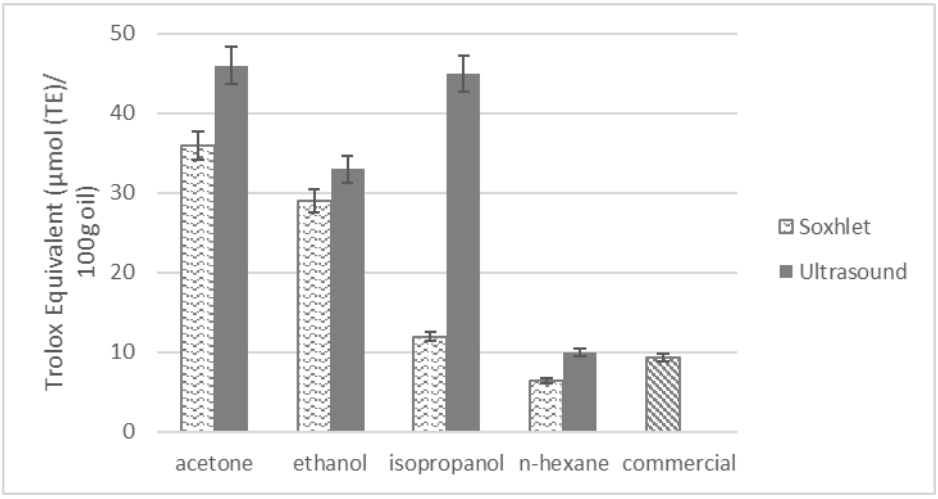

Figure 3. Antioxidant activities of PFS oil obtained using Soxhlet and Ultrasound extraction methods, based on their abilities to scavenge DPPH free radicals.

Through the statistical analysis, it was verified that regarding the antioxidant activity obtained with DPPH, there were significant differences between the commercial oil and samples obtained from the Soxhlet and ultrasound methods. Similar results were obtained in other studies with the ultrasound method (17).

By comparing the results shown in Figures 3 and 4, in general, it can be seen that PFS oils obtained with the ultrasound method showed higher values of Trolox Equivalent Antioxidant Capacity and Ascorbic Acid Equivalent Antioxidant Capacity. *In vitro* antioxidant activity assessed by the free radical scavenging activity (DPPH) method for PFS oil obtained by the ultrasound method using acetone provided better results than those obtained using the Soxhlet method.

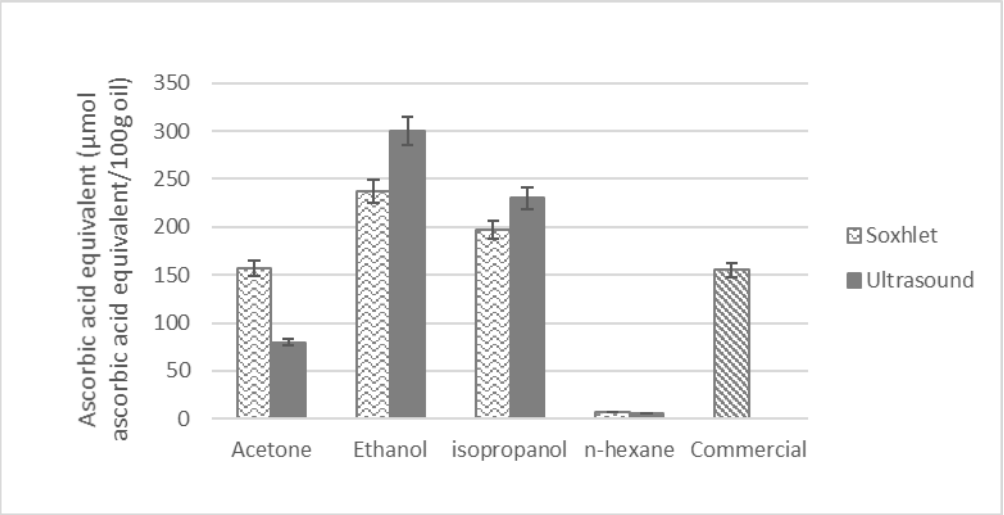

Figure 4. Ascorbic acid equivalent from PFS oil found in Soxhlet and ultrasound extraction with ABTS.

There were statistically significant differences between values of the antioxidant activity obtained using the ABTS method among the studied oils ($p < 0.05$).

On the other hand, the lower antioxidant activity observed in the samples obtained using the Soxhlet extraction method may be partially caused by thermal degradation due to the high temperatures and long extraction times used in this method. Thus, the longer extraction times and the temperature employed in this technique possibly increased yields, but the occurrence of thermal degradation reduced the concentration of important compounds in the final sample (16,18).

4. Conclusions

In spite of the great efficiency of Soxhlet, the results of this study demonstrated that the extraction by ultrasound using ethanol and acetone allowed obtaining an oil with greater antioxidant activity. These suggest the possibility of green production using ultrasound technology on pilot and industrial scales in cosmetic industry or pharmaceutical industries. Therefore, further analysis of the chemical composition of passion fruit seeds oil needs to be carried out in order to identify other compounds with antioxidant properties.

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CHAPTER 4

Identification and Quantification of Stilbenes (Piceatannol and Resveratrol) in *Passiflora edulis* By-Products

pharmaceuticals

MDPI

Communication

Identification and Quantification of Stilbenes (Piceatannol and Resveratrol) in *Passiflora edulis* By-Products

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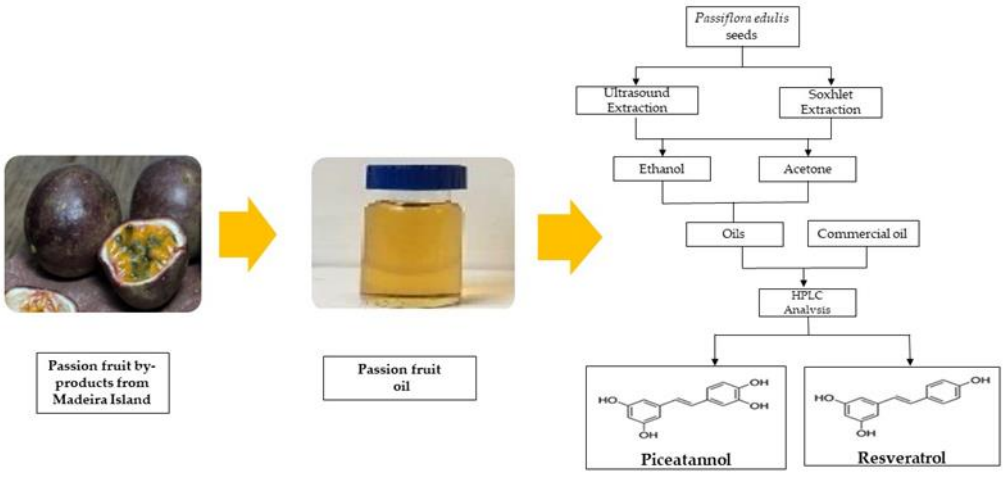

Graphical abstract

Abstract

Recently, studies on the by-products from the food industry, such as passion fruit seeds, have significantly increased, as these can have an added value, due to their properties, such as potential antioxidant activity. This study was conducted to determine the presence of piceatannol and resveratrol in various extracts of passion fruit (*Passiflora* edulis) seeds from Madeira Island and a commercial passion fruit oil was used as reference. The commercial oil and the extracts that were obtained by traditional Soxhlet method with ethanol and acetone did not reveal the presence of the two stilbenes, piceatannol and resveratrol. However, the extracts that were obtained by the ultrasound method showed significant amounts of piceatannol and resveratrol when compared with the commercial oil. The presence of these compounds indicates that this oil could have potential application in cosmetic and pharmaceutical industries, due to their proven antioxidant and anti-aging properties.

Keywords: stilbenes; *Passiflora* edulis; by-products; piceatannol; resveratrol

1. Introduction

Madeira Island is a Portuguese territory that is located in the Atlantic Ocean, which has a temperate tropical climate, which allows for the cultivation of various species of passion fruit. The purple passion fruit (*Passiflora* edulis) is one of the species used in the production of juices by the food industry. Only the passion fruit pulp is used in the production of the juice, and the discarded seeds generate thousands of tons of waste every year (1–3). The generation of waste has high costs in its treatment and, based on this, the use of this waste in other processes that can produce value-added products results in great interest for the society and scientific community (4–6).

It is mentioned in the literature that the purple passion fruit seeds oil has antioxidant, anti-inflammatory, and skin lightening, among others (7–9). The oil is rich in stilbenes, vitamins, and catechin. It is described in the literature the presence of piceatannol and resveratrol in passion fruit from Japan and Brazil (10).

Several studies highlight the efficiency of resveratrol due to its antioxidant activity, anti-aging potential, neuroprotective, and anti-cancer properties, particularly in cases of leukemia, and in cancers of the breast and colon (11–19).

The benefits of piceatannol have not been studied as extensively as in the case of resveratrol (20). Piceatannol (3,30,40,5-trans-tetrahydroxystilbene) is a polyphenolic compound that has been found in some plants, including grapes, passion fruit, white tea, rhubarb, peanuts, berries, and some mushroom species (10,21,22).

Stilbenes are compounds that are considered phytoalexins, because they protect plants against fungi and toxins. The presence of an additional hydroxyl group in the piceatannol structure gives it greater antioxidant activity when compared to its prodrug, resveratrol (21,23). Piceatannol also promotes collagen production, preventing skin damage and inhibiting melanin synthesis (24).

Yokozawa and Kim studies (25) have shown that piceatannol has a better inhibitory activity of the tyrosinase enzyme, as well as decreases melanin production, better than resveratrol and kojic acid, a potent skin whitening agent.

In previous studies, we evaluated the antioxidant activity of passion fruit seeds extracts, which were obtained by two methods, Soxhlet and Ultrasound, using various solvents. Extracts that were obtained by both methods using ethanol and acetone were chosen, since they showed the highest antioxidant capacity (8).

In view of the abundance of passion fruit waste as by-products in Portugal, sustainable management of these by-products is necessary. In this context, the objective of this work

was to identify and quantify stilbenes, piceatannol, and resveratrol, by High Performance Liquid Chromatography (HPLC), in *Passiflora edulis* seeds oil from Madeira Island and compare with commercial passion fruit seeds oil, in order to evaluate the potential of these antioxidant compounds for further applications by the pharmaceutical and cosmetic industries.

2. Results and Discussion

2.1. Standard Samples

Using the RP-HPLC method, piceatannol and resveratrol peaks were detected with retention times of 35.66 and 36.40 min., respectively. Figure 1 shows the chromatograms and UV spectra of piceatannol and resveratrol. The identification of both stilbenes was confirmed with this HPLC-DAD. Two calibration curves, one for piceatannol and the other for resveratrol, were obtained with R2 = 0.999 and R2 = 0.996, respectively.

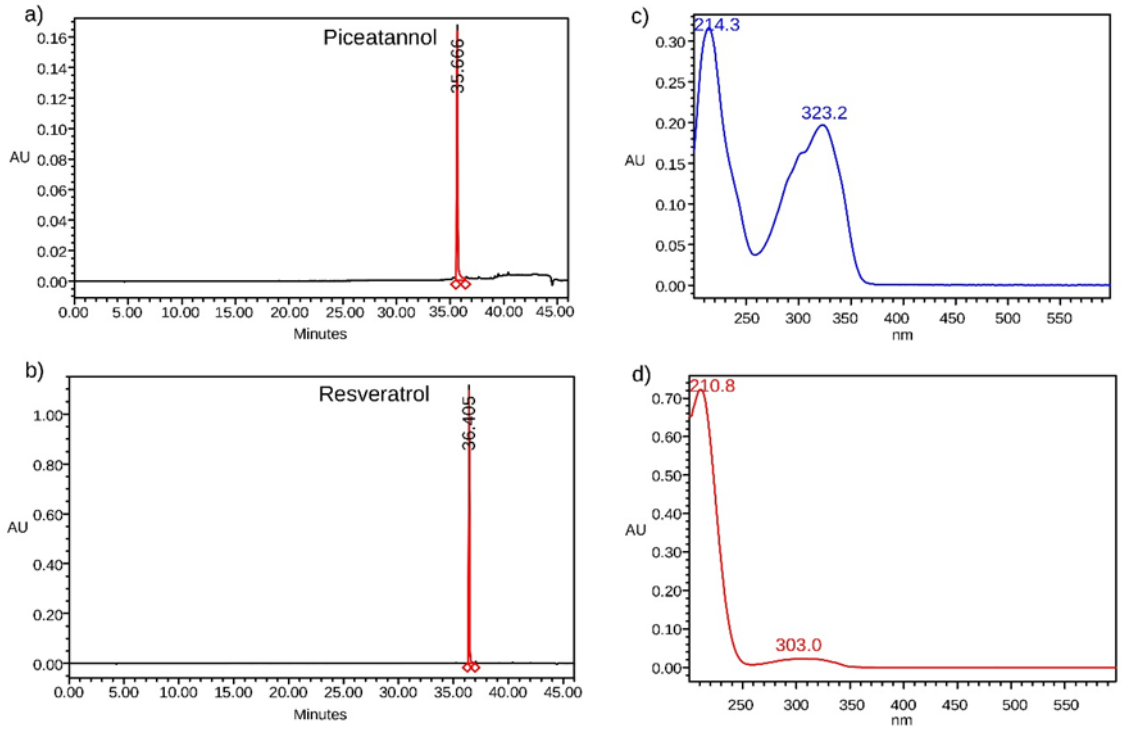

Figure 1. High-performance liquid chromatography with diode array detection (HPLC-DAD) chromatograms of the standard solutions: (a) piceatannol; (b) resveratrol. UV spectra of the standard solutions: (c) piceatannol; and (d) resveratrol.

2.2. Soxhlet Extraction

There was no evidence of the presence of piceatannol and resveratrol in both extracts that were obtained by the Soxhlet method. The chromatograms showed unknown peaks, although some of the peaks obtained the same retention time, they did not absorb at the same UV wavelength as piceatannol and resveratrol. Because piceatannol and resveratrol are sensitive to high temperatures, these compounds may have been degraded during the eight-hour extraction of the Soxhlet method. Although the Soxhlet is a method with excellent

extraction performance, in the case of polyphenols, due to the solvent heating at boiling temperatures for several hours, the degradation of phenolic compounds might occur (26).

Similar results have recently been described by Viganó and collaborators (27). The study described by these authors demonstrated that the amount of piceatannol in the extracts of passion fruit bagasse obtained by the Soxhlet method was lower than the amount of this stilbene in the extracts that were obtained by maceration and extraction by pressurized liquid (PLE).

2.3. Ultrasound Extraction

The HPLC-DAD method allowed for separating piceatannol and resveratrol in a single run, as can be seen in the Figure 2. In HPLC chromatograms two peaks were identified as piceatannol and resveratrol by comparison of the ultraviolet (UV) spectra and retention time.

There are no significant differences regarding the amounts of piceatannol in the samples obtained with ethanol and acetone, according to Figure 3. However, there is a higher amount of resveratrol in samples extracted with acetone. When comparing the amount of piceatannol and resveratrol found in the extracts, it was verified that the extracts obtained using ethanol showed small differences of stilbenes content, whereas in the case of extracts that were obtained with acetone, the amount of resveratrol was significantly higher than the amount of piceatannol. These results corroborate other previous studies, in which the amount of resveratrol was higher than the amount of piceatannol found in plants (28–30). Some authors showed that the amount of resveratrol in grapes was approximately four times higher than that of piceatannol (0.78 µg/g and 3.18 µg/g, respectively) (31).

Ultrasound extraction is a widely used method, since it is low cost, simple, and generally presents better results than conventional extraction methods (32). This improvement in efficiency might be justified, because ultrasound is based on the energy of sound waves, promoting a good penetration of the solvent into the sample, thus increasing the contact surface as well as the acoustic cavitation produced, facilitating the release of contents (33,34).

A general overview of the results that were obtained by other authors allows for concluding that the ultrasound method is faster, more efficient, and more selective for polyphenols than the Soxhlet method (35).

A recent study investigated and quantified the polyphenol content in *Passiflora subpeltata* pulp from India by UPHLC-MS analysis. Significant amounts of epicatechin, ferulic acid, and protocatechuic acid were detected (36). Rimando and collaborators detected up to 422

ng/g of piceatannol in species of blueberries from Mississippi, North Carolina. Significant amounts of resveratrol were also found in this fruit (37).

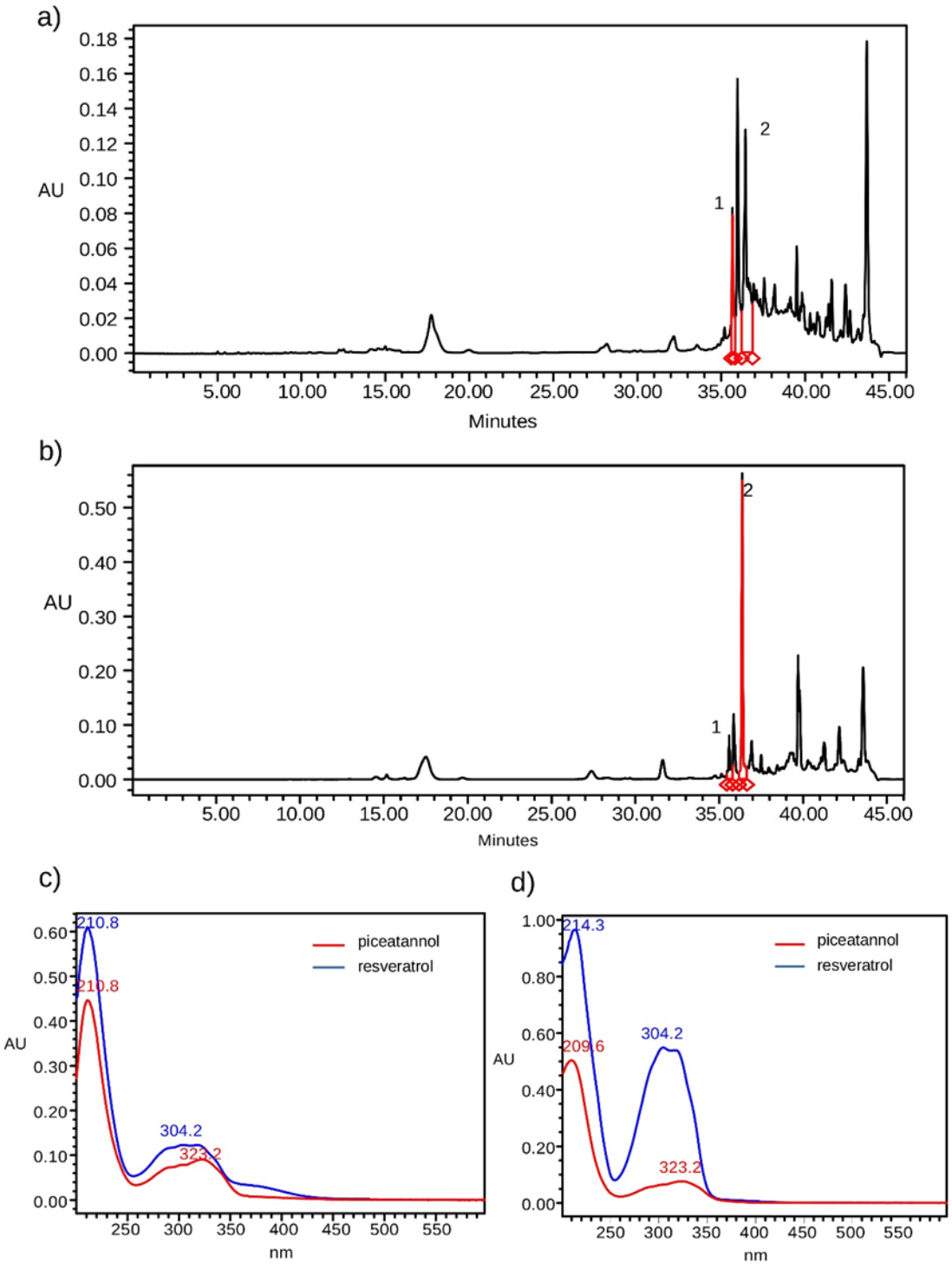

Figure 2. HPLC-DAD chromatograms (320 nm) of the extract obtained by the ultrasound method with ethanol (a) and with acetone (b). Number 1 and 2 corresponds to piceatannol and resveratrol, respectively. The UV spectra of piceatannol (red) and resveratrol (blue) detected on ethanol (c) and acetone (d) extracts.

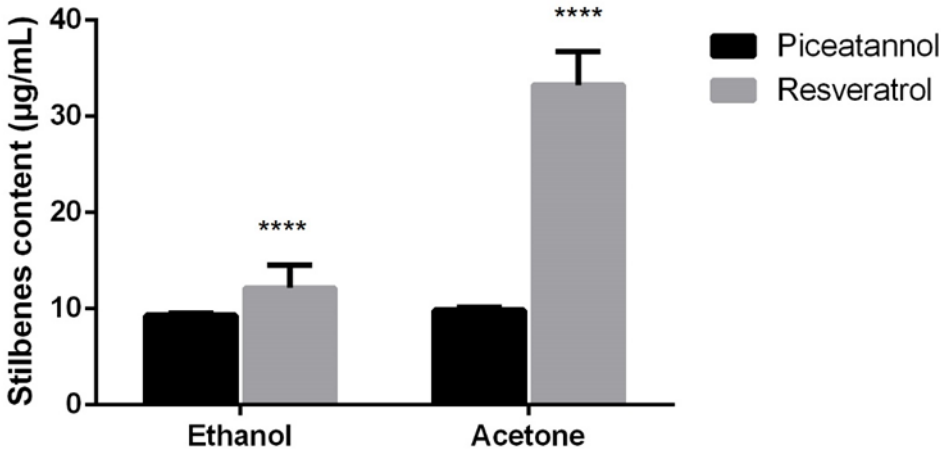

Figure 3. Piceatannol and resveratrol content (µg/mL) in ethanol and acetone extracts obtained with ultrasound method. Results are expressed as Mean ± SD. Statistical comparisons were made using one-way ANOVA, followed by the Tukey's multiple comparisons test. Values significantly different from piceatannol (**** $p < 0.05$).

1.3. Commercial Oil

In Figure 4, it can be observed that the commercial passion fruit oil had no piceatannol or resveratrol. However, there were other unknown peaks.

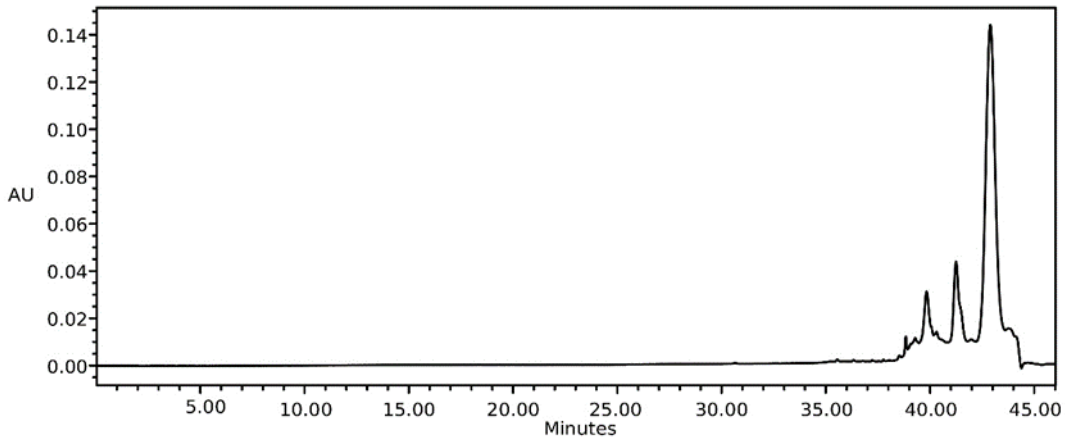

Figure 4. Chromatograms of the commercial passion fruit oil.

In a previous study, the same commercial oil presented lower antioxidant activity in relation to the extracts that were obtained by ultrasound using acetone and ethanol as solvents. This activity was determined while using DPPH (2,2-diphenyl-1-picryl-hidrazil) and ABTS (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) methods [8]. Despite obtaining

antioxidant activity, it might be due to the presence of many other compounds of the passion fruit oil, such as vitamin C and gallic acid.

3. Materials and Methods

3.1. Samples Preparation

Passion fruit seeds were obtained from the food industry of Madeira Island. These seeds were then dried in a stove and, after that, the extracts were prepared. These extracts were prepared according to Krambeck and collaborators (8). The extracts were prepared using ethanol and acetone, with two preparation methods: Soxhlet and ultrasound.

3.2. Chemicals and Standards

Piceatannol and resveratrol standards, as well as ethanol and formic acid, were obtained from Sigma Aldrich (London, UK). Acetone was purchased from Fisher Chemical (Loughborough, UK). Methanol was purchased from VWR Chemicals (Vila Nova de Gaia, PT).

The stock solutions containing 1mg/mL of piceatannol and the same concentration for resveratrol in ethanol were prepared. All the solutions were stored at −4°C. Subsequently, for the calibration curve, standard solutions with concentrations ranging from 1.25–20 µg/mL for piceatannol and 0.625–35 µg/mL for resveratrol were prepared.

3.3. Methods

The flow diagram for the extraction of the two stilbenes, piceatannol and resveratrol, can be seen in Figure 5. Briefly, after preparing the extracts, these were compared with a commercial oil from Akoma (London, UK), regarding the content of the stilbenes studied through HPLC analysis. In addition to determining the presence of stilbenes, the content of these elements in the extracts were also quantified.

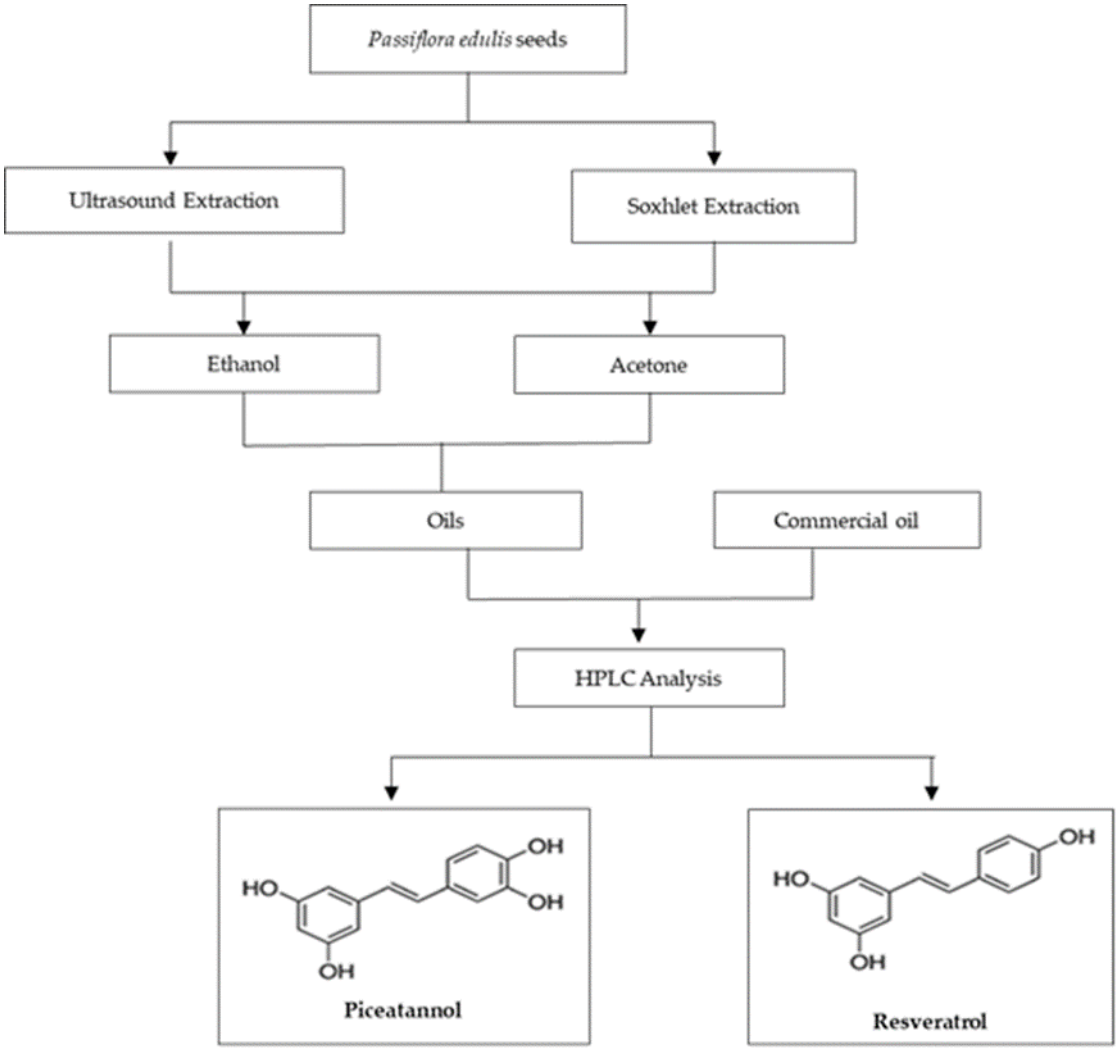

Figure 5. Flow diagram for the extraction and separation by HPLC of piceatannol and resveratrol.

For the extracts that were obtained by Soxhlet, each selected solvent was heated to its boiling point, and the reflux was maintained for eight hours. For the extracts that were obtained by ultrasound, an ultrasound bath (35 kHz/80 W) (Sonorex RK100h, Bandelin, Germany) was used. The extraction time was 60 min at room temperature. At the end of all tests, the solvents were removed while using a rotary vacuum evaporator R-300 (Buchi, Flawil, Switzerland).

3.3.1. Reverse Phase High-Performance Liquid Chromatography (RP-HPLC)

RP-HPLC was carried out with some modifications, according to Lai and collaborators (38), for the simultaneous determination of two polyphenols: piceatannol and resveratrol. Analyses were carried out using a high-performance liquid chromatography (HPLC)Waters

2690 Separations Module, with photodiode array detector (PDA-Waters 996), and a 20 µL aliquot of the extract was injected onto a Waters ACE Equivalence C18 column (250 × 4.6 mm i.d.; 5 µm particle size). The mobile phases consisted of (A) water with 0.1% formic acid and (B) methanol with 0.1% formic acid. The total run time was 46 min., being 0–10 min., 0–15% B; 10–20 min., 15% B; 20–30 min., 15–35% B; 30–35 min., 35–100% B; 35–40 min., 100% B; 40–41 min., 100–0% B; 41–46 min., 0% B. All the measurements were carried out at a flow rate of 0.8 mL/min., using a wavelength of 320nm. Peaks corresponding to piceatannol and resveratrol were analyzed by comparison with the retention times and UV spectra of their respective standard solutions and then quantified through calibration curves. The results were expressed in µg/mL oil. All of the analyses were carried out in triplicate.

3.4. Statistical Analysis

The results were statistically evaluated by one-way analysis of variance (ANOVA), in which significant differences at the 5% level were analyzed by the Tukey's test. SPSS Software (Version statistic 26, IBM SPSS, Chicago, IL, USA) was used for the statistical analysis in this study.

4. Conclusions

In this study, resveratrol and piceatannol were not detected either in the extracts of by-products of *Passiflora edulis* that were obtained by the Soxhlet method or in the commercial oil.

Extracts obtained by the ultrasound method using ethanol or acetone showed significant amounts of stilbenes such as piceatannol and resveratrol. Passion fruit by-products can be used in cosmetic and pharmaceutical industries having an added value, in addition to reducing the environmental pollution, avoiding the burning or landfill of waste.

The obtained results also suggest the possibility of production of Passiflora edulis seeds oil with green solvents and the potential interest of this product to industries, as it represents a low-cost ingredient.

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CHAPTER 5

Lipid nanocarriers containing *Passiflora edulis* seeds oil intended for skin application

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Lipid nanocarriers containing *Passiflora edulis* seeds oil intended for skin application

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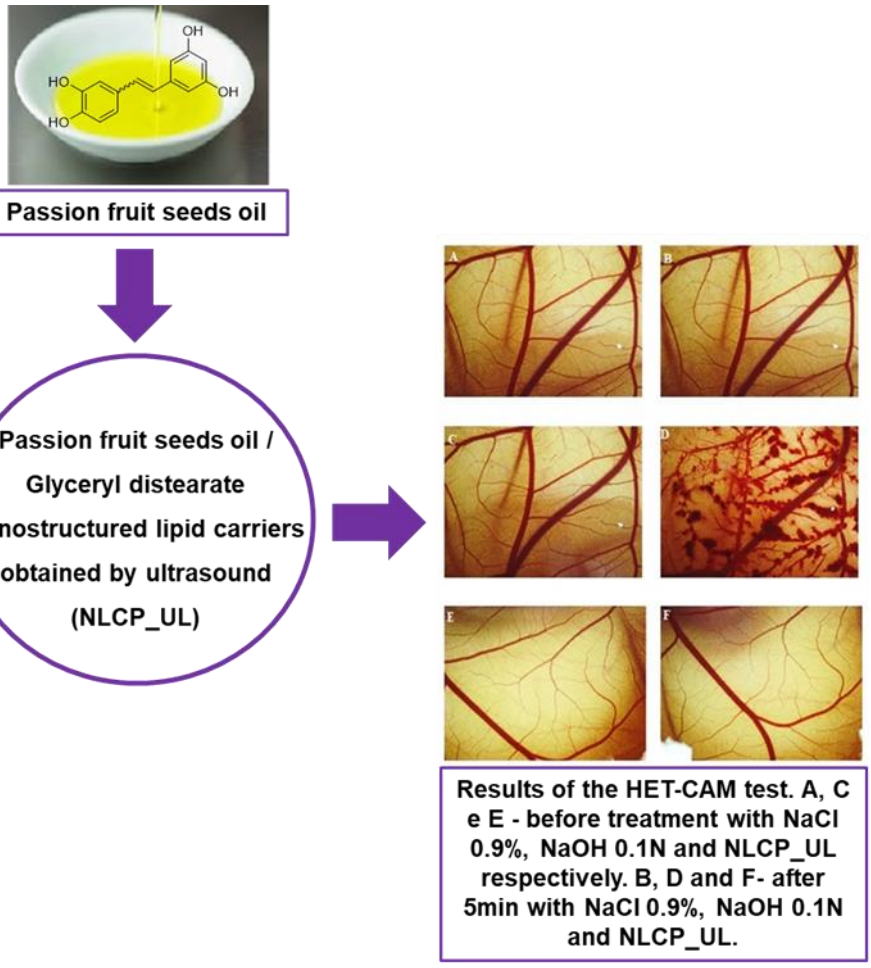

Graphical Abstract

Abstract

Nanostructured lipid carriers (NLC) have been studied for over 20 years, constituting the second generation of lipid nanoparticles. These nanosystems were introduced to overcome the drawbacks of solid lipid nanoparticles (SLN). Passion fruit seeds oil have a high antioxidant potential and also skin whitening properties. The objectives of this work were to prepare NLC by two methods (ultrasonication and High pressure homogenization) using different solid lipids (Glyceryl Distearate, Glyceryl Dibehenate and Cetyl Palmitate) and passion fruit seeds oil as liquid lipid. The nanoparticles prepared with glyceryl distearate, using the ultrasonication method showed better characteristics, since these nanosystems presented smaller particle sizes and polydispersity index, and higher zeta potential. Besides that, these nanoparticles showed a high occlusion factor and non-irritant potential in HET-CAM assay. Based on the results obtained, it may be suggested that the prepared NLCs can be applied to the face, since they did not cause any irritation, and represent a potential strategy for further use in topical formulations with antioxidant activity.

Keywords: Nanostructured lipid carriers; *Passiflora edulis*; HET-CAM; Oil; Antioxidant; Nanoparticles; Polyphenols**;** By-product

1. Introduction

Nanostructured lipid carriers (NLC) constitute the second generation of lipid nanoparticles and these were introduced to overcome the drawbacks presented by Solid lipid nanoparticles (SLN). One of the differences between SLN and NLC is that NCL have a nanostructured matrix capable of increasing encapsulation capacity, as well as preventing the expulsion of the active substance during storage. Another difference is that NLC are composed of solid lipids and liquid lipids, whereas SLN are only composed of solid lipids (1–4). NLC have been proposed as nanocarriers for various drugs and cosmetic ingredients since 2005 (5).

According to the method employed, the active substances can be encapsulated in nanoparticles in different ways. Briefly, there are several methods of obtaining nanoparticles, such as high pressure homogenization, ultrasonication, coacervation, spray-drying, among others (6–8).

Nowadays, the interest on vegetable extracts and phytochemicals has increased considerably, as evidenced by the large number of scientific articles (9–15). However, there are few articles describing passion fruit seeds oil from Madeira Island in cosmetic formulations.

Madeira Island has a particular climate which favors the cultivation of tropical plants such as passion fruit (Passiflora edulis). Only the pulp is used by the food industry, and the seeds are discarded (16). From this by-product, it is possible to extract an oil rich in polyphenols, such as resveratrol (trans 3,4′,5-trihydroxystilbene) and piceatannol (trans 3,4,3′,5′-tetrahydroxystilbene) (17,18).

Piceatannol, as a resveratrol metabolite, is a stilbene that shows better antioxidant activity than resveratrol, which could be due to the additional hydroxyl group (19,20). Stilbenes are naturally produced in some plants like grapes, passion fruits, green tea and others (21,22). Piceatannol can inhibit the melanin synthesis, prevent and treat skin cancer (melanoma), has anti-inflammatory and antioxidant properties, promotes collagen production, and prevents UV damage (16,23–26).

Despite the interesting properties of polyphenols, their use has several disadvantages such as low solubility, bioavailability and stability. In this study, NLC have been proposed to load extracts of Passiflora edulis seeds in order to improve the bioavailability of the above mentioned polyphenols and to increase the chemical stability of actives sensitive to light oxidation and hydrolysis. The objectives of this work were to prepare NLC by two methods (Ultrasonication and High pressure homogenization) using different solid lipids and using passion fruit seeds oil as liquid lipid. Then, to compare the nanoparticles produced by both

methods regarding their particle size, zeta potential, polydispersity- index (PDI), pH, occlusion potential and irritant properties.

2. Materials and methods

2.1. Materials

Tween® 80 (polysorbate 80) was supplied from Acofarma (Spain). Glyceryl distearate (Precirol® ATO5), Glyceryl dibehenate (Compritol® 888 ATO) and Cetyl Palmitate were a kind gift from Gattefossé (France). Cetrimide® was purchased from José M. Vaz Pereira, SA (Portugal). The ultrapure water was obtained from a Direct-Q® Ultrapure Water Systems Merck (Germany).

Passion fruit seeds oil (*Passiflora edulis*) used in this study was previously prepared by ultrasound according to Krambeck et al. (17). In a previous study, stilbenes such as piceatannol and resveratrol were identified and quantified in *Passiflora edulis* seeds oil, by HPLC (18).

2.2. Methods

2.2.1. NLC preparation

NLCs (NLCP, NLCC, NLCEE) composition is shown in Table 1. As solid lipids were used three lipids, Glyceryl distearate (Melting range 50–60 °C), Glyceryl Dibehenate (Melting range 65–77 °C) and Cetyl Palmitate (Melting point 54 °C) were used as solid lipids, while passion fruit seeds oil was used as liquid lipid in all formulations. The NLCs were prepared by two methods: ultrasonication (UL) and high pressure homogenization (HPH).

In both methods, the lipids were heated at 10 °C above the melting point of the solid lipid. Then the water phase was heated at the same temperature and added to the lipid phase under vigorous stirring using Ultra-Turrax (T25D, IKA, Germany), at 8500 rpm for 5 min, and a pre-emulsion was formed. From this step, the methods differed. For the ultrasound method, the obtained pre-emulsion was subjected to the action of a 6mm ultrasound probe (Sonics Vibra-Cell VCX130, USA), with an amplitude of 70 % for 15 min. Then, the hot dispersion was cooled in an ice bath for 30 min to generate NLC. For the hot HPH method, the pre-emulsion was subjected to two cycles, using a Pressure Cell Homogenizer (Stansted SPCH-10, UK) with a pressure of 500 bar, and a temperature of 70 ± 0.5 °C. All the samples were prepared in triplicate.

Table 1: Composition (%, w/w) of NLCs containing Passion fruit seeds oil.

Formulations	Ingredients (%, w/w)						
	Glyceryl distearate	Glyceryl Dibehenate	Cetyl Palmitate	Tween® 80	Passion fruit Oil	Cetrimide®	Purified water
NLCP	7.0	_	-	2.5	3.0	0.1	87.4
NLCC	_	7.0	-	2.5	3.0	0.1	87.4
NLCE	_	-	7.0	2.5	3.0	0.1	87.4

2.2.2. Particle-size, polydispersity-index (PDI) and zeta-potential (ZP)

One of the most important parameters regarding the physical stability of lipid nanoparticles is the zeta potential. A lower value, both negative and positive, means that the formulation is more unstable (27). Other important tests are particle size measurement and PDI evaluation. The stability of NLC depends on the nature of the oily phase and the balance of the emulsifiers at the oil/water interface. A reduction in the particle size of NLC can increase stability, viscosity and translucency. To provide prolonged stability, the PDI values should be 0.1−0.25. Values above 0.5 indicate polydisperse size populations with low physical stability (28,29).

Particle size and PDI of nanoparticles were analyzed by Dynamic Light Scattering (DLS), using the ZetaPALS Particle Sizing Software, (Brookhaven Instruments,USA) and zeta potential analyzer (ZetaPALS, Brookhaven Instruments, USA).

2.2.3. Accelerated stability

It is necessary to carry out stability tests in a short period of time, in order to predict any changes that may occur in the formulations during its storage. Physical stability can be assessed in an accelerated manner by centrifugation (mechanical stress), and the volume of supernatant obtained can be related to the stability of heterogeneous.

systems. If a colloidal dispersion of lipid nanoparticles changes after the accelerated stability test by centrifugation (formation of precipitates, occurrence of phase separation, coalescence, among others) it means that probably will not be stable during storage and should be discarded (30).

To evaluate the accelerated stability, 5 mL of each NLC dispersion were submitted to two cycles of centrifugation, during 30 min at 3500 rpm, using a centrifuge (Eppendorf, AG 5804 Germany). The formulations were observed after centrifugation regarding their appearance,

phase separation, sedimentation or any change, which may be predictive of sample instability. This test was performed in triplicate for each NLC formulation.

2.2.4. pH analysis

Topically applied NLCs should have an intermediate pH value, neither too acidic nor too basic, in order to avoid adverse effects (4). The pH was determined using the pHmeter (Crison Basic 20, Spain) Measurements were carried out in triplicate.

2.2.5. *In vitro* occlusion test

Lipid nanoparticles are able to adhere to the surface of the skin, leading to the formation of a film that exerts an occlusive effect. The *in vitro* occlusion factor was determined by the Vringer method (31). In 100 mL beakers were added 40 mL of water, and covered with filter paper. Under the filter paper were applied 200 mg of each of the

prepared NLCs. Beakers covered with filter paper with no sample, were considered as reference. The determination of the mass of each of the beakers was performed at zero time, and at the end of 24 h and 48 h of incubation in an incubator at 34 °C with 50–55 % of relative humidity. Every experiment was carried out in triplicate. The occlusion factor was calculated using the Eq. (1).

$$F = \frac{A-B}{A} * 100 \qquad \text{Eq. (1)}$$

Where A is water loss without sample and B is water loss with sample (NLC). The occlusion factor scale ranges from 0 to 100 and the closer values to 100 indicate higher occlusion of the sample (32).

2.2.6. Irritant potential assay

The irritant potential assay or Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) assay was performed as described in the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). Fertile Broiler chicken eggs were used to perform the test HET-CAM. The eggs were rotated in an Egg incubator (YZ56S, HT-56S, China) at 38±0.5 °C for 8 days. On the 9th day the rotation was stopped and the test was performed. After opening the eggs (performed with a tiny drill Dremel®), 0.3 ml of each formulation was

added and the eggs chorioallantoic membrane was directly visualized by a CAM Nikon D7000 (Japan) with AF-S Micro Nikkon 40mm 1:2.8 G lens for 5 min. A 0.9 % NaCl solution was used as negative control and a 0.1 N NaOH solution as positive control. The irritant potential on the chorioallantoic membrane was assessed and the irritation scored (0–21). It was considered non-irritant if the score was between 0 and 0.9 and severe irritant with values above 9 (33,34).

2.2.7. Statistical analysis

Statistical analysis were carried out by IBM SPSS Statistics26 (SPSS IBM, Japan). The tests utilized were the Levene as Test of Homogeneity of variances, then the one-way ANOVA and Tukey and Duncan as post hoc tests. All the results are mean values ± standard deviation (SD) of at least three samples. The level of significance was 95 % ($p < 0.05$).

3. Results and discussion

3.1. Particle-size, polydispersity-index (PDI), and zeta-potential measurements

In Table 2 are presented the results of particle size, polydispersity index and zeta potential of different NLC formulations.

Table 2: The particle size, Zeta potential and Polydispersity Index (PDI) of different NLC formulations. The results are expressed as mean ± SD (n=5).

Formulation	Particle size (nm)		Zeta Potential (mV)		PDI	
	HPH	UL	HPH	UL	HPH	UL
NLCP	156.0±1.3	140.2±2.8	-30.20±1.40	-33.78±0.68	0.27±002	0.17±0.01
NLCC	249.0±1.8	259.7±3.7	-25.07±1.06	-21.76±1.12	0.347±0.03	0.39±0.03
NLCE	469.0±2.0	570.0±2.6	-23.03±0.75	-16.80±2.60	0.44±0.09	0.49±0.02

The NLCs prepared with glyceryl distearate as solid lipid using ultrasonication (NLCP_UL) and high pressure homogenization (NLCP_HPH) presented smaller particle sizes in comparison with the other formulations prepared by the same methods. It can also be observed that the ZP was higher in the formulations prepared with glyceryl distearate. ZP obtained for NLCP were above −30 mV, indicating that this formulation may have good physical stability. Besides, the polydispersity index of the NLCP prepared with both methods was lower than 0.20 showing that this NLCs can be considered homogeneous regarding their particle size.

NLCs prepared with cetyl palmitate (NLCE), using both methods, showed larger particle sizes and PDI, and lower ZP, suggesting that with this solid lipid it was not possible to obtain lipid nanoparticles with good physical characteristics and stability.

In a study of Puglia and collaborators (35), NLCs and nanoemulsions with UV-filters were compared. In this study, octyl methocycinnamate loaded NLCs with Compritol® 888 ATO as solid lipid and Miglyol® 812 as liquid lipid, prepared by ultrasonication presented particle sizes greater than 200 nm (318.8±25.4 nm) and mean PDI values of 0.25±0.02. However, the unloaded NLC studied had particle sizes of 250.6±10 nm and PDI values of 0.29±0.04. These results were similar to those obtained in our study for NLCs prepared with Compritol® 888 ATO.

3.2. Accelerated stability

NLCP and NLCC formulations obtained by both methods, ultrasonication and HPH, showed no visible modification, phase separation or sedimentation. However, phase separation was observed in the formulation prepared with cetyl palmitate obtained by ultrasonication and HPH. NLCs prepared with cetyl palmitate (NLCE) were eliminated from this study due to their low stability.

3.3. pH analysis

As can be seen in Figure 1, all the formulations studied showed pH values between 5.33 and 5.89, which is in accordance with what is described in the literature about the suitable pH of products for skin application. There were significant differences between the samples, and NLCP_UL showed the highest pH values.

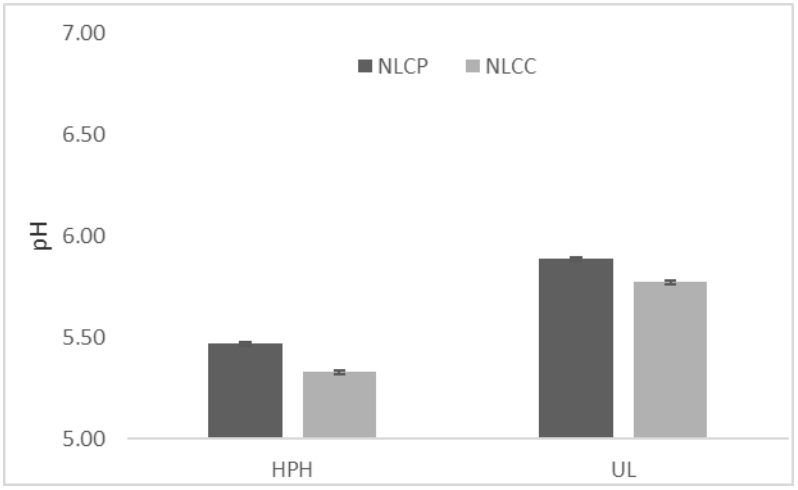

Figure 1. pH values of NLCP and NLCC prepared by HPH and UL.

3.4. *In vitro* occlusion test

According to the results related to the *in vitro* occlusion factor (Figures 2 and 3), it was possible to verify significant differences between the samples. NLC prepared with glyceryl distearate by ultrasonication (NLCP_UL) presents the higher values of the occlusion factor. NLCC showed the lowest values of the occlusion factor.

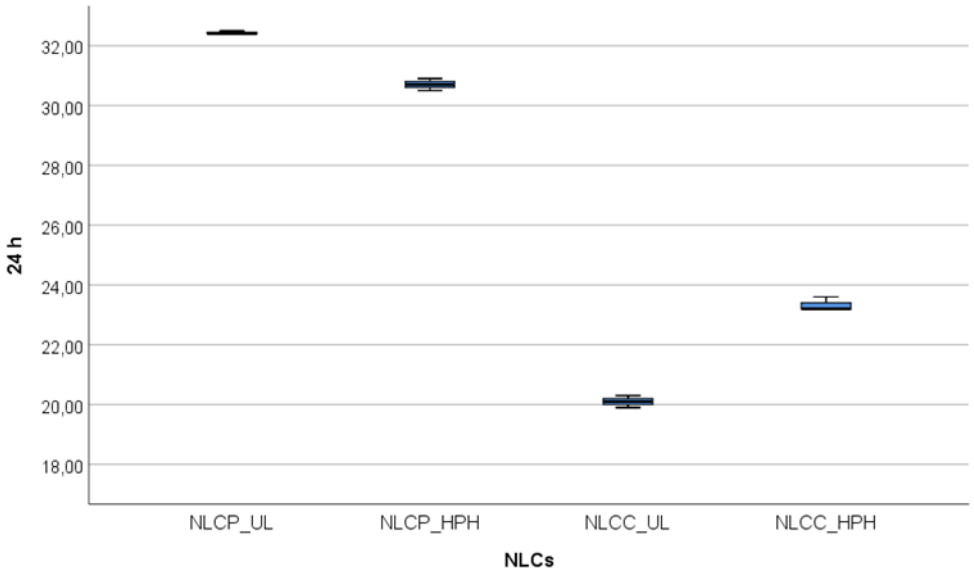

Figure 2. Occlusion factor (F) for NLCP and NLCC prepared by high pressure homogenization (NLCP_HPH and NLCC_HPH) and ultrasonication methods (NLCP_UL and NLCC_UL) at 24 h.

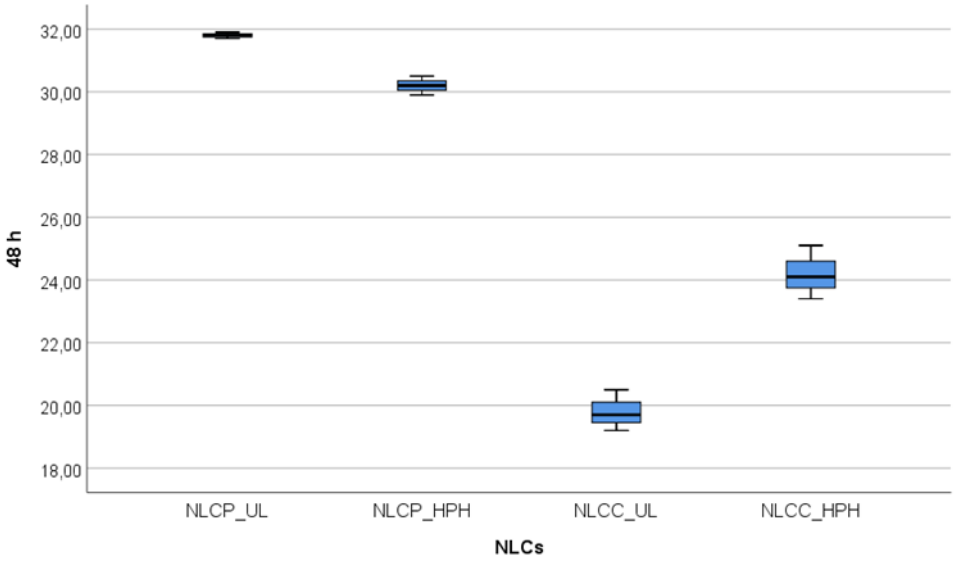

Figure 3. Occlusion factor (F) for NLCP and NLCC prepared by high pressure homogenization (NLCP_HPH and NLCC_HPH) and ultrasonication methods (NLCP_UL and NLCC_UL) at 48 h.

Similar results of occlusion factor were obtained in studies regarding NLC with clotrimazole prepared by the hot high pressure homogenization technique (5).

Lipid nanoparticles applied to the skin induce the formation of a lipid film that results in an increase of the occlusive effect, thus reducing the transepidermal water loss (TEWL) (1).

Previous studies (29) confirm that the occlusion factor is inversely proportional to the particle size, that is, the smaller the particle size the greater the occlusive effect. In addition, the use of lipids with a low melting point in the preparation of nanoparticles results in a higher occlusion in the skin (36). Golmohammadzadeh and co-workers (37) also observed that the occlusion factor depends on the size of the particles. Besides, according to Teeranachaideekul and co-workers (38), NLCs with high content of liquid lipids presented less occlusive effect.

3.5. Irritant potential assay

To evaluate the irritant potential of the formulation NLCP_UL, the Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) assay was performed. The HET-CAM is a simple and reliable test, which reduces animal suffering. It is one of the oldest alternative methods to the Draize test. Through the observation of changes that occur in the chorioallantoic membrane of the eggs after being exposed to formulations for 5 min, the irritant potential of the compounds is evaluated. This assay evaluates the appearance vascular lysis (blood vessel disintegration), coagulation (intra- and extra- vascular protein denaturation) and haemorrhage (bleeding from the vessels) (34,39–41).

According to Figure 4, NLC containing passion fruit seeds oil (NLCP_UL) had no irritant potential after the study time (5 min), thus obtaining a value of 0, similar to the negative control (NaCl 0.9 %). The positive control (NaOH 0.1 N) presented a score of 20, being possible to verify coagulation, lysis and haemorrhage.

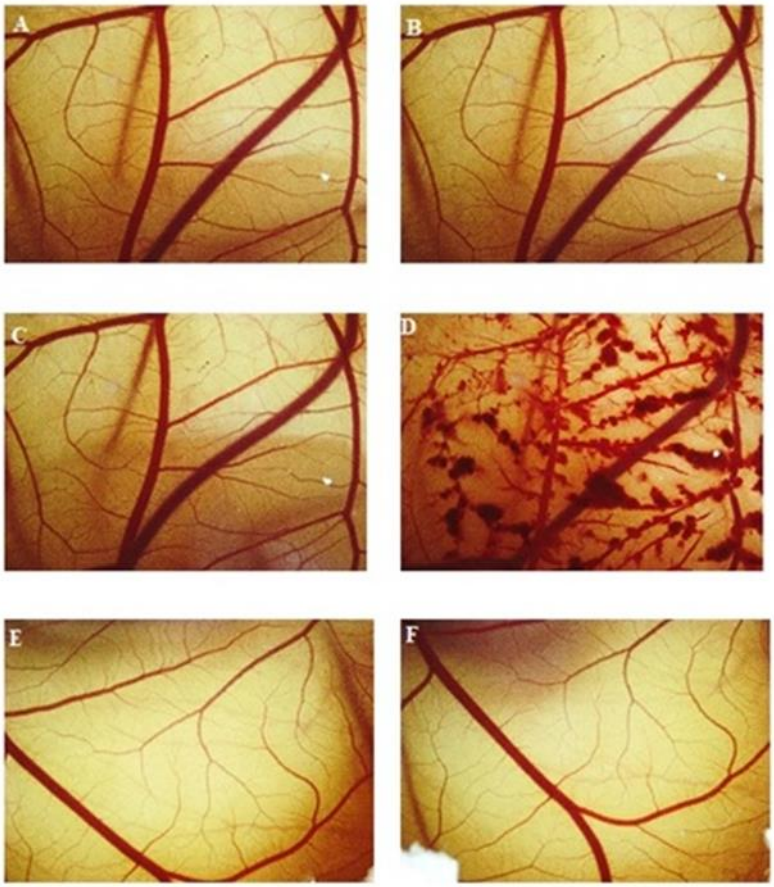

Figure 4. Results of the HET-CAM test. A, C e E= before treatment with NaCl 0.9%, NaOH 0.1N and NLCP_UL respectively. B, D and F= after 5min with NaCl 0.9%, NaOH 0.1N and NLCP_UL.

Several studies used HET-CAM assay to evaluate the irritant potential of formulations. Felippi and co-workers (42) showed that nanoparticles with a blend of Retinyl palmitate; *Linum usitatissinum* Seed Oil, *Vitis vinifera* Seed Oil and Ubiquinone were non-irritant and the nanoparticles suspension did not cause any lysis, coagulation or haemorrhage.

4. Conclusions and future prospects

In this study it was verified that it is possible to obtain lipid nanoparticles containing passion fruit seeds oil as liquid lipid and glyceryl distearate as solid lipid, with good characteristics for skin administration. These nanostructured lipid carriers showed suitable particle size and pH for cutaneous application, as well as absence of irritation potential proven by the HET-CAM test.

This study also showed that nanoparticles containing passion fruit seeds oil and cetyl palmitate are not suitable due to their high particle size and eventual physical instability demonstrated by the test of accelerated stability.

In future studies the nanoparticles with passion fruit seeds oil and glyceryl distearate should be incorporated in a semisolid formulation to improve their skin application.

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CHAPTER 6

Design and characterization of Nanostructured lipid carriers (NLC) and Nanostructured lipid carrier-based hydrogels containing *Passiflora edulis* seeds oil

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Design and characterization of Nanostructured lipid carriers (NLC) and Nanostructured lipid carrier-based hydrogels containing *Passiflora edulis* seeds oil

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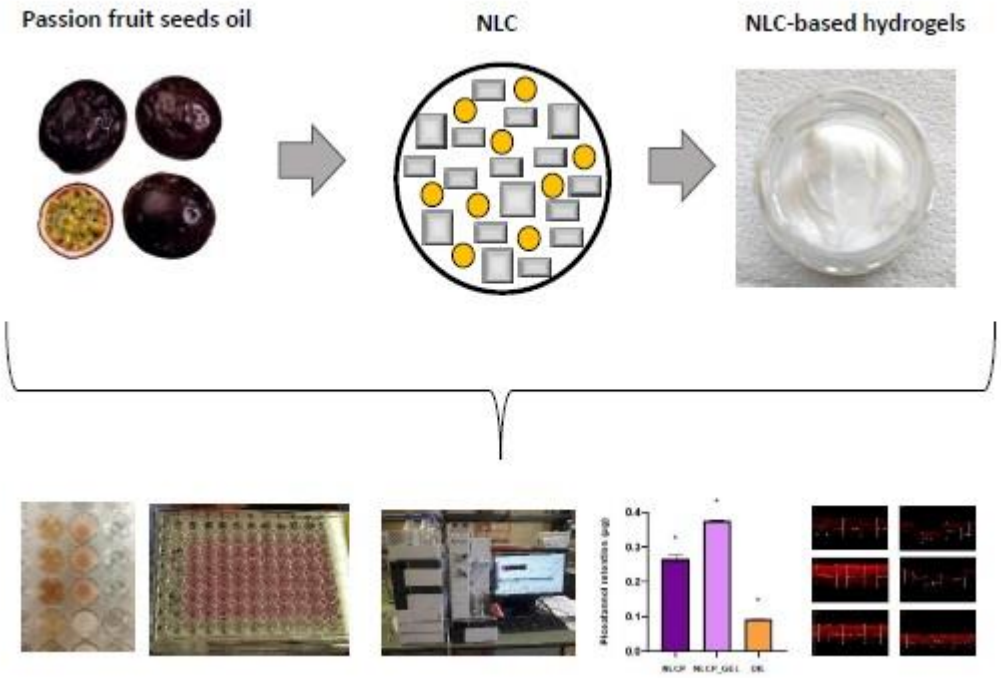

Graphical abstract

Abstract

This study aims to design and characterize Nanostructured lipid carriers (NLC) and Nanostructured lipid carrier-based hydrogels with *Passiflora edulis* seeds oil, a by-product from Madeira Island food industry. NLC were prepared by the ultrasonication technique, using passion fruit seeds oil as a liquid lipid and glyceryl distearate as a solid lipid. These NLC were then gelled with Poly (acrylic acid). Long-term stability studies were conducted with NLC and NLC-based hydrogels stored for 12 months. The following tests were performed: morphology, encapsulation efficiency, particle size analysis, polydispersity index analysis, zeta potential, pH measurement, color analysis, viscosity studies, texture analysis, *in vitro* occlusion test, ex vivo skin penetration study, tyrosinase inhibition activity, *in vitro* skin permeation experiments and *in vitro* cytotoxicity studies. The developed NLC had spherical shape and narrow particle sizes distribution with mean sizes in the range of 150 nm and PDI below 0.3, Zeta potential values around −30 mV and high Encapsulation efficiency. The tyrosinase inhibitory activity and skin retention of the nanoparticles was superior to that of the non-encapsulated oil. The developed formulations did not show cytotoxicity towards HaCaT cells and presented suitable viscosity and texture properties for skin application, proving to be good candidates as depigmenting agent.

KEYWORDS: Nanostructured lipid carriers; Passion fruit oil; nanoparticles; hydrogels; tyrosinase.

1. Introduction

Over the years, the skin acquires spots, wrinkles and other effects of exposure to solar radiation. In this sense, the search for treatments to attenuate these effects is increasingly desired. Nowadays, there is an increased concern about skin pigmentation disorders. One of the causes of skin colour disorders are dyschromia, which can be darker spots, so-called hypercromia, or lighter spots (hypocromia). Hypercromia, is related to several factors such as genetics, hormonal changes, exposure to solar radiation, use of some drugs, aging, among others. These factors are responsible for the increase in melanogenesis, that is, the synthesis of melanin that occurs in melanosomes by the melanocyte found in the deeper layers of epidermis (1).

The highest incidence of melasmas occurs in women with Fitzpatrick skin types IV and V and usually in the center of the face. It is a disease that affects women's quality of life and which is difficult to treat. There are several products that can be used for skin whitening; however, most of them have no success, several adverse reactions or contraindications, namely for pregnant women (2,3). The most popular depigmentants are hydroquinone (1, 4-dihydroxybenzene) and kojic acid (5-hydroxy-2-(hydroxymethyl) 4-pyrone) (4,5). However, there is an interest in studies of natural depigmentants (6) like stilbenes, in particular piceatannol, due to few studies on this when compared to resveratrol (7).

Piceatannol is a resveratrol analogue, which has strong antioxidant activity and can be found naturally in grapes, passion fruits, peanuts, sugar cane, some berries and other plants (8–10). Some studies have shown the presence of considerable amounts of piceatannol in the oil of purple passion fruit (Passiflora edulis) seeds from Madeira Island, Portugal (11).

Nanocarriers have been a worldwide trend, especially nanostructured lipid carriers (NLC). NLC have great benefits in their use, such as increased therapeutic effect, increased cutaneous hydration, greater stability of the encapsulated active ingredients, among others. A mixture of solid lipids and liquid lipids, plus surfactant(s), are used in the preparation of NLC (12–14).

In this study, passion fruit seeds obtained as by-products of the food industry from Madeira Island, Portugal, were used. This industry uses only the pulp of the passion fruit in the production of juices, and tons of seeds are discarded annually. Thus, the objective of this work was to develop and characterize NLC containing passion fruit seeds oil (NLCP), which were later transformed into a nanoemulgel (NLCP_GEL), to increase the consistency of the final formulation and, therefore, its physical stability. The preparations were characterized regarding the morphological aspect (Cryo-SEM), encapsulation efficacy, hydrodynamic particle size, zeta potential, polydispersity index, pH, color, viscosity tests, texture, *in vitro*

occlusion, ex vivo skin penetration study with confocal laser scanning microscopy (CLSM), *in vitro* penetration and retention in the skin, tyrosinase inhibition activity, and *in vitro* cytotoxicity (resazurin reduction, neutral red uptake and sulforhodamine B assays).

2. Materials and methods

2.1. Materials

Glyceryl distearate (Precirol® ATO5) was received as a gift from Gattefossé (France). Tween® 80 (polysorbate 80) was purchased from Acofarma (Spain). Alkyltrimethylammonium bromide (Cetrimide®) and Poly (acrylic acid) (Carbopol® 940) were acquired from José Vaz Pereira, SA (Portugal). Piceatannol, resveratrol, tyrosinase from mushroom, L-tyrosine, rhodamine B, ethanol and acetonitrile were purchased from Sigma-Aldrich (Germany). Triethanolamine was obtained from Merck (Germany). Passion fruit seeds were a by-product of the food industry from the Madeira Island, Portugal, and an oil was extracted from these seeds as described by previous studies (11).

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, antibiotic (10,000 U/mL penicillin, 10,000 µg/mL streptomycin) and phosphate buffer solution with or without calcium and magnesium [PBS (+/+) or PBS (-/-), respectively] were obtained from GibcoTM (Thermo Fisher Scientific, Alfagene, Portugal). Resazurin (REZ), neutral red (NR) solution, sulforhodamine B (SRB), Triton™ X-100 detergent solution and Trizma® base were obtained from Sigma-Aldrich (Germany). All the reagents used were of analytical grade or of the highest grade available.

2.2. Preparation of NLC

The lipid nanoparticles were produced by the ultrasonication method according to Krambeck and co-workers (15). The dispersions of lipid nanoparticles (NLCP) were prepared by mixing solid lipid (glyceryl distearate) and liquid lipid (passion fruit seeds oil) in a ratio of 7:3 (w/w). Briefly, the oil phase and the aqueous phase containing a surfactant (polysorbate 80, 2.5%), a preservative (alkyltrimethylammonium bromide, 0.1%) and 87.4% of purified water were heated at 70 °C, then the aqueous phase was added the oily phase followed by constant stirring for 5 minutes at 8500 rpm using an Ultra-Turrax (T25D, IKA, Germany). Thereafter, the pre-emulsion formed was subjected to ultrasonication (Sonics Vibra-Cell VCX130, USA), using a 6 mm probe with 70% amplitude, for 15 minutes. Finally, the dispersion was cooled in an ice bath for approximately 30 minutes. This method allows to decrease the size of nanoparticles and also to improve their stability and uniformity (16).

2.3. NLC Characterization

2.3.1. Organoleptic characteristics

The organoleptic characteristics of NLCP were evaluated by visual and also olfactory perception.

2.3.2. Encapsulation efficiency (EE)

EE was evaluated according to the ultrafiltration-centrifugation method. For the purpose, 1 mL of the NLCP dispersion was added in a test tube along with 4 mL of water. After centrifugation the sample was filtered with filter paper and then 1 mL of the filtrate was removed, 4 mL of ethanol was added and submitted to centrifugation (Eppendorf Centrifuge AG 5804, Hamburg, Germany) at 3500 rpm, for 30 minutes. The sample was filtered with 0.22 µm Millipore® filter and after that was analysed by High Performance Liquid Chromatography (HPLC).

Piceatannol was used as a marker for the quantitative analysis of passion fruit seeds oil. The amount of free drug was detected in the supernatant and the amount of incorporated piceatannol was determined as a result of the initial drug minus the free drug. The encapsulation efficiency was calculated by the equation (1):

$$EE\ (\%) = \frac{W\ in}{W\ all} * 100 \qquad \text{Eq. (1)}$$

Where W_{all} was the weight of piceatannol in system, W_{in} was the weight of entrapped piceatannol in supernatant after centrifugation of the dispersion.

The amount of free piceatannol was evaluated in the supernatant by reversed phase HPLC (model CBM-20A, Shimadzu, Kyoto, Japan) equipped with a photodiode array UV-VIS detector (model SPD-M20A, Shimadzu, Kyoto, Japan) and using a Accucore™ Polar Premium HPLC C18 column (2.6 µm particle size; 150 x 4.6 mm i.d.). The injection was performed with a volume of 20 µL of aliquot. The mobile phase consisted of 30% acetonitrile and water with 0.1% formic acid. All of the measurements were carried out at a flow rate 0.8 mL/min using a wavelength of 320 nm. Data were collected and processed by LC Realtime Analysis Program (Shimadzu, Kyoto, Japan). The amount of piceatannol incorporated in NLCP was determined having into account the initial amount of this compound minus its free amount evaluated in the supernatant.

2.3.3. Particle size analysis

Particle size analysis was performed to ensure the presence of particles with colloidal sizes using a laser diffractometry (LD, Mastersizer® 3000E, Malvern, UK). All experiments were replicated five times and were taken on the day 0, 30, 60, 90, 180 and 365 after samples be stored at 4 °C and 25 °C.

2.3.4. Zeta Potential (ζ, ZP) and polydispersity index (PDI)

A Zeta Potential Analyser (ZetaPALS, Brookhaven Instruments, USA) was employed to measure the surface charge of particles and PDI of nanoparticles was analyzed by Dynamic Light Scattering (DLS), using the ZetaPALS Particle Sizing Software (Brookhaven Instruments, USA). A ζ potential above 30 mV or below 30 mV is required for electrostatic stabilization, avoiding agglomeration of particles (17). Both ZP and DPI were analyzed in samples stored at 4 °C and 25 °C, on day 0 and after 365 days of storage. All samples were diluted in Milli-Q® water to optimize signal strength. All experiments were replicated six times.

2.3.5. *Ex vivo* skin penetration by Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) has been widely used to visualize the distribution of dyes or fluorescent drugs incorporated into the skin and hair follicles after incorporation into nanostructured systems. It also provides valuable morphological information complementary to that obtained by conventional microscopy (18,19).

CLSM was carried out to see the depth of penetration of the NLCs. To achieve this aim rhodamine-B dye loaded nanoparticles were prepared. Rhodamine-B solution (0.05%, w/v) in water was used as control.

The *ex vivo* skin penetration test of ear pig was carried out using rhodamine-B to simulate the process of drug penetration into the skin, and the frozen section of the skin was observed by CLSM (Leica TC-SP2 Confocal System, Leica Microsystem Srl, Milan, Italy).

CLSM was used to visualize the distribution and penetration depth of NLCP through the skin for 1, 2 and 24 hours.

2.3.6. Tyrosinase Inhibition Activity

To estimate the tyrosinase inhibitory action of samples was used the method previously described by Gholamhoseinian and Razmi (20), with a minor modification. In the 96-well plate, a volume of 110 µL was used, being 50 µL PBS 50 mM (pH 6.5), 25 µL substrate (L-tyrosine 1 mM), 25 µL NLCP, 10 µL mushroom tyrosinase aqueous solution (1000 units). After this, the plate was placed in the Epoch2 microplate reader (BioTeck Instruments, Vermont, USA). It was shaken for 2 minutes and the absorbance was measured at 492 nm, after 30 minutes at 25 °C.

All experiments were done in triplicate. The percentage of inhibition of the tyrosinase enzyme activity was calculated using the following equation (2):

$$\mathrm{I}\ (\%) = \frac{(\mathrm{B1}-\mathrm{B2})-(\mathrm{S1}-\mathrm{S2})}{(\mathrm{B1}-\mathrm{B2})} * 100 \qquad \text{Eq. (2)}$$

Where: I (%) is the percentage of inhibition of tyrosinase enzyme activity, B1 is the reference with enzyme (PBS, L-tyrosine and tyrosinase enzyme); B2 is the reference (phosphate buffer and L-tyrosine); S1 is the NLCP with the enzyme; S2 is the NLCP without enzyme.

The higher the percentage of tyrosinase enzyme inhibition, the greater the whitening effect of the sample.

2.3.7. *In vitro* Cytotoxicity studies

The cytotoxicity of the NLCP and nanoparticles without the Passion fruit seeds oil (SLN) (0-500 µg/mL) was evaluated in HaCaT cells, an immortalized human keratinocyte cell line, by the REZ reduction, neutral red NR uptake and SRB binding assays, 24 hours after exposure. The cells were routinely cultured in 75 cm2 flasks using DMEM with 4.5 g/L glucose and GlutaMAX™, supplemented with 10% heat inactivated FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. HaCaT cells were maintained at 37 °C, in a 5% CO2 - 95% air atmosphere, and the medium changed every 2 days. Cultures were passaged weekly by trypsinization (0.25% trypsin / 1 mM EDTA). For the cytotoxicity studies, the cells were seeded in 96 well plates at a density of 20,000 cells/well and exposed, 24 hours after seeding, to the NLC and SLN formulations (0-500 µg/mL). Triton™ X-100 (1%) was used as positive control. The cells used in all experiments were taken between the 40th and 50th passages.

2.3.7.1. Resazurin reduction assay

The REZ reduction assay is based on the ability of living cells to reduce the oxidized blue REZ dye into a pink and fluorescent resorufin product. For that purpose, after the 24 hours exposure of HaCaT cells to the NLC and SLN formulations (0-500 µg/mL), the cell culture medium was removed, replaced by cell culture medium containing REZ (10 µg/mL), and the cells incubated for 90 minutes, at 37 °C, in a humidified 5% CO2 - 95% air atmosphere. The resorufin fluorescence was then read in a multiwell plate reader (PowerWaveX BioTek Instruments, Vermont, USA) at excitation and emission wavelengths of 560 nm and 590 nm, respectively. The percentage of REZ reduction relatively to that of the control cells (0 µg/mL) was used as the cytotoxicity measure. Six independent experiments were performed in triplicate.

2.3.7.2. Neutral red uptake assay

The NR uptake assay provides a quantitative estimation of the number of viable cells in culture, being based on the ability of living cells to incorporate and bind the supravital dye NR in the lysosomes (21–24).

The NR uptake assay was performed as previously described (25). Briefly, 24 hours after exposure of HaCaT cells to the NLC and SLN formulations (0-500 µg/mL), the cell culture medium was removed, replaced by fresh cell culture medium containing NR (50 µg/mL), and the cells incubated for 90 minutes, at 37 °C, in a humidified 5% CO2 - 95% air atmosphere. The cell culture medium was then removed and the NR dye absorbed only by viable cells extracted [absolute ethanol/distilled water (1:1) with 5% acetic acid]. The NR absorbance was then 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 µg/mL) was used as the cytotoxicity measure. Six independent experiments were performed in triplicate.

2.3.7.3. Sulforhodamine-B binding assay

The SRB binding assay provides an estimation of the total protein mass, which is related to cell number in culture, being based in the binding of the SRB dye to the basic amino acids of cellular proteins under mild acidic conditions. For that purpose, 24 hours after exposure of HaCaT cells to the NLC and SLN formulations (0-500 µg/mL), the cell culture medium was removed, the cells washed with PBS (+/+) and fixed overnight with a methanolic solution of 1% acetic acid (v/v), at -20 °C. The fixing medium was then removed and the

cells incubated with a 0.05% SRB solution (prepared in 1% acetic acid), at 37 °C, for 60 minutes. The SRB solution was then removed, the cells washed with 1% acetic acid (v/v) to remove the unbound dye, and the bound SRB extracted with a Tris base solution (10 mM, pH 10.5). The absorbance of the bound SRB was measured, at 540 nm, in a multiwell plate reader (PowerWaveX BioTek Instruments, Vermont, USA). The percentage of SRB binding relatively to that of the control cells (0 µg/mL) was used as the cytotoxicity measure. Six independent experiments were performed in triplicate.

2.4. Preparation of NLC-based hydrogels

One of the most important parameters for the acceptance of a topical product is its easy application, apart from the appearance and sensory characteristics conferred to the skin, such as shine, stickiness and amount of residue. However, lipid nanoparticle dispersions are liquid systems and do not have adequate consistency for application in the skin. Thus, their transformation in semisolid systems becomes fundamental so that their characteristics are the desirable. In addition to their ease of application, an increase in the physical stability of nanoparticles has been described in the literature, due to the decrease in the possibility of particles aggregation (26,27). Thus, a hydrogel based on NLC (NLCP_GEL) was developed. For the preparation of the NLCP-based hydrogel, 0.5% of Poly (acrylic acid) has been added to 99.5% of the NLCP colloidal dispersion which contains 10% of the lipid phase (7% Glyceryl distearate and 3% Passion fruit seeds oil) and subsequent neutralization with triethanolamine (TEA) for the gel formation.

2.5. NLC-based hydrogels characterization

2.5.1. Scanning electron cryomicroscopy (Cryo-SEM)

The CRYO-SEM exam was performed using a high-resolution Scanning Electron Microscope with X-Ray Microanalysis and CryoSEM experimental facilities: (JEOL JSM 6301F/ Oxford INCA Energy 350/ Gatan Alto 2500). The specimen was rapidly cooled (plunging it into sub-cooled nitrogen – slush nitrogen) and transferred under vacuum to the cold stage of the preparation chamber. The specimen was fractured, sublimated (‘etched’) for 120 s at -90 °C, and coated with Au/Pd by sputtering for 50s, with a 12 mA current. The sample was then transferred into the SEM chamber. The samples were studied at a temperature of -150 °C. The conditions in which images and spectrum were obtained are in the respective labels. NLCP and NLCP_GEL were visualized by this method.

2.5.2. pH measurement

Formulations to be applied in the skin must have a compatible pH. Measurements were performed using a previously calibrated digital pHmeter (Basic 20 pHmeter, Crison Instruments SA, Spain). The pH of NLCP_GEL was evaluated after 0, 30, 60, 90, 180 and 365 days of storage at 4 °C and 25 °C. All measurements were performed at room temperature and in triplicate.

2.5.3. Color Analysis

The color analysis aims to detect color changes resulting from any alteration occurring during the storage of the formulations. According to the literature, lipids undergo degradation processes resulting from oxidation reactions which may modify their coloring (28). This analysis was performed with the colorimeter Chroma Meter® CR-400 (Konica Minolta, Japan) and the color parameters L*a*b* were determined.

The value of L* is an indication of luminosity, resulting from the amount of reflected light, which can vary from 0 (black) to 100 % (white). With the values of a* and b* the Chroma factor (C*) can be calculated, which reveals the color of the formulation through the following equation (3):

$$C* = \sqrt{a*^2 + b*^2} \qquad \text{Eq. (3)}$$

Where, C* is Chroma Factor, a* varies from red to green and b* varies from yellow to blue. The luminosity and Chroma Factor of NLCP_GEL stored at 4 °C and 25 °C were evaluated in triplicate after 0, 30, 60, 90, 180 and 365 days.

2.5.4. Viscosity Test

The study of the viscosity allows to estimate whether a formulation is suitable for cutaneous application, with the pseudoplastic behaviour with thixotropy being the most accepted, since a decrease in apparent viscosity occurs with an increase in the shear rate and with the time, respectively 29. Viscosity experiments were performed using a rotational viscometer Thermo HAAKE Viscotester VT 550 (Thermo Scientific, USA), with SV DIN coaxial cylinder sensor. This study consisted in determining the viscosities from 0.1 to 500 s-1 and from 500 to 0.1 s-1, at a constant temperature of 20 °C. Rheological experiments were performed with NLCP_GEL after 0, 30, 60, 90, 180 and 365 days of storage at 4 °C and 25 °C.

2.5.5. Texture analysis

The texture corresponds to the physical characteristics perceived by the sense of touch that are related to the deformation caused by a force and which are measured in terms of force, distance and time. In the development of preparations for topical application it is necessary to take into consideration certain attributes that contribute to the acceptance of the product and the improvement of its effectiveness. These attributes include mechanical properties such as adhesiveness and spreadability (30). Usually, for texture analysis the penetration test is performed, in which the probe penetrates the sample at a certain speed and at a predefined distance, then returning to a position at a distance above the predefined sample (31).

In this study, the texture properties of the NLCP_GEL were performed with the Texture Analyser TA-XT2i (Stable Micro systems, UK), with a 25 mm diameter acrylic probe, a penetration distance of 5 mm, a test speed of 3 mm/s and the Trigger force of 0.049 N. On the graph of force versus distance, the positive peak force represents the firmness of the sample, and the negative area corresponds to the adhesiveness. This test was performed in NLC-based hydrogels after 0, 30, 60, 90, 180 and 365 days of storage at 4 °C and 25 °C. Measurements were performed in triplicate.

2.5.6. *In vitro* occlusion test

The *in vitro* occlusion property of NLCP and NLCP_GEL was evaluated using a previously reported method (32). For this test, 200 mg of each sample were weighed and spread on a filter paper placed over a beaker containing 40 mL of water. As a control a beaker coated with filter paper but without any sample was used. The beakers were incubated at 34 °C, with 50-55% relative humidity. The initial weight of each beaker was determined, as well as the weights after 24 and 48 hours. The occlusion factor was calculated using the equation (4) and there is a scale from 0 to 100, where 100 corresponds to maximum occlusion. All determinations were made in triplicate.

$$F = \frac{A-B}{A} * 100 \qquad \text{Eq. (4)}$$

Where A is the water loss of the control and B is the water loss corresponding to the beaker with the sample.

2.5.7. *In vitro* skin permeation study by Franz diffusion cell

The test with the Franz diffusion cell was performed according to Scognamiglio and co-workers (33) with some modifications. Pig ear skin was freshly obtained from a slaughter house in Porto, Portugal. The excised skin from pig ear was immersed in PBS (pH 7.4) at 60 °C for 2 minutes. The thickness of the pig skin was 0.91 ± 0.12 mm. Franz diffusion cells with a diffusion area of 0.784 cm^2, containing a receptor volume of 5 ml were used to study skin permeation in vitro. A phosphate buffer solution (PBS) pH 7.4 was used as the receptor medium. The agitation speed during the experiment time was 600 rpm and the diffusion system was coupled to a water bath at 37 °C. On the skin surface, being the dermis directed to the receiving solution, 2 g of the studied samples (NLCP, NLCP_GEL) and 0.06 g of *Passiflora edulis* seeds oil were applied (corresponding to a concentration of 9.85 µg/mL of piceatannol). At predefined times (0.5, 1, 2, 4, 6 and 24 h), 1 mL of the receiving solution was removed and, to keep the volume constant, 1 mL of PBS solution was added to the receiving compartment. The amount of piceatannol that permeates the skin was determined by HPLC. The calculations of the cumulative amount of piceatannol were corrected taking into account the dilution resulting from the replacement of the sample volume collected by the PBS solution.

To perform the skin retention test, after finishing the *in vitro* permeation study the pig ear skin was removed and cleaned. Then, 5 mL of Acetonitrile:Water (3:1) was added, shaken vigorously in an Ultra-Turrax for 5 minutes and then placed on ultrasound for 10 minutes for a complete rupture of the cells. Then it was filtered by a syringe with a Millipore® filter and the filtrate was analysed using the same HPLC method described above.

2.6. Statistical analysis

All statistical calculations were performed using the GraphPad Prism 6 for Windows (GraphPad Software, San Diego, CA, USA). For the cytotoxicity data, Two-way ANOVA was used to perform the statistical comparisons, followed by the Tukey's multiple comparisons test (for each formulation, for comparisons between concentrations) or by the Sidak's multiple comparisons test (at each concentration, for comparisons between formulations). Details of the performed statistical analysis are described in the figure legends. Differences were considered significant for p values lower than 0.05.

3. Results and Discussion

3.1. Organoleptic characteristics

The organoleptic characteristics of NLCP were evaluated by visual and olfactory perception. As for appearance, all samples maintained their integrity, the initial appearance in storage conditions, no coalescence or phase separation occurred. With respect to color and odor, it could be seen that all samples, independent of the storage temperature, had the same color and no distinct odor.

3.2. Encapsulation Efficiency (EE)

Through the EE it was possible to verify the percentage of piceatannol inserted in the nanocarriers, namely NLCP. Encapsulation efficacy was found to be 94.91%. This value was obtained by calculating the amount of piceatannol present in the supernatant of the colloidal dispersion after filtration and centrifugation at 3500 rpm for 30 minutes. The main factors that influence the encapsulation efficiency are the concentration of the surfactant used and the lipids, as well as the solubility of the compounds in the oil phase. Previous studies have shown that active substances with high lipid solubility have relatively high EE, above 80% (34).

3.3. Particle size analysis

The particle size of NLCP was assessed on days 0, 30, 60, 90, 180 and 365 of storage at 4 ºC and 25 ºC, as can be seen in Figure 1.

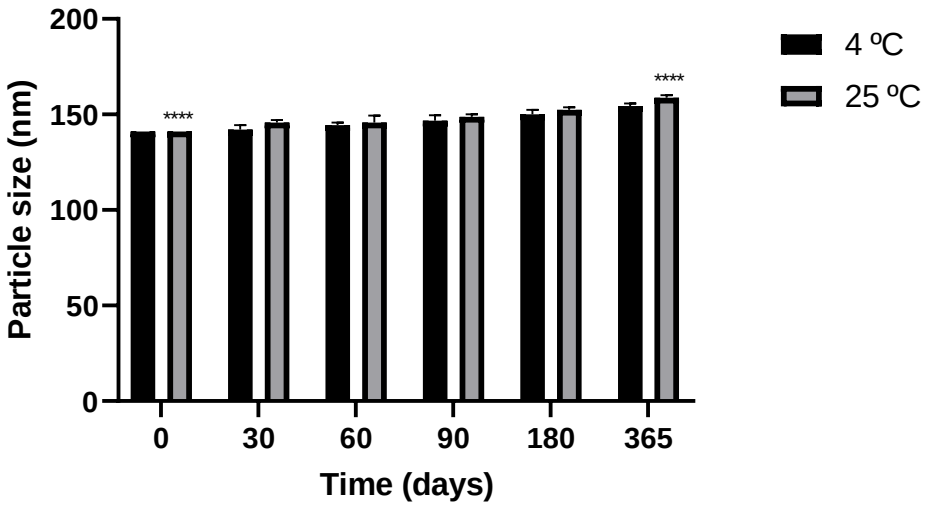

Figure 1. Particle size analysis of NLCP, measured on days 0, 30, 60, 90, 180 and 365 (mean values, n=3) of storage at 4 and 25 °C. (**** $p < 0.05$. In all cases, p values < 0.05 were considered significantly differents).

The mean particle size of NLCP prepared with passion fruit oil and glyceryl distearate, maintained at 4 and 25 °C, slightly increased over 365 days of storage. However, after 365 days samples stored at 25 °C showed a mean particle size of 158 nm, which is acceptable for NLC. These results are in accordance with those obtained by Averina and co-workers (35) with Siberian pine.

3.4. Zeta Potential (ZP) and polydispersity index (PDI)

In Table 1 it can be seen that NLCP stored at 4 and 25 °C presented ZP values between -33.78 and -30.86 mV, which can contribute to an electrostatic stabilization of the nanoparticles. These values may also suggest that the temperature difference did not have a considerable influence in the stability of the NLCP. According to the literature, ZP values between -30 and +30 mV are considered ideal for colloidal dispersions (17). Another conclusion is that after one year of storage at the two studied temperatures, NLCP remained stable predicting good long-term stability.

Table 1: Mean values of Zeta Potential (ZP) and polydispersity index (PDI) of NLC at 0 and 365 days.

Temperature	Time (days)			
	0		365	
	ZP ± S.D (mV)	PDI ± S.D	ZP ± S.D (mV)	PDI ± S.D
4 °C	-33.78±0.68	0.17±0.01	-31.10 ± 1.5	0.26 ± 0.07
25 °C	-33.78±0.68	0.17±0.01	-30.86 ± 1.9	0.28 ± 0.08

It can also be seen that the PDI values of NLCP stored at both temperatures were below 0.28, even after one year of storage, indicating a narrow and uniform size distribution. The polydispersity index (PDI) can indicate the homogeneity of the particle size, and the smaller its value, the more homogeneous is the particle size distribution.

3.5. *Ex vivo* skin penetration by confocal laser scanning microscopy

Confocal laser scanning microscopy was used to give some information about the depth and uniformity of the penetration of nanoparticles into pig ear skin. Thus, NLCP were marked with Rhodamine to improve the visualization of product distribution on the skin. These samples were compared with a rhodamine solution without NLCP. NLCP samples and Rhodamine solution can be observed in Figure 2 after 1, 2 and 24 hours with CLSM.

Regardless of the time of study, it is possible to verify that NLCP have penetrated more than the rhodamine solution, which suggests an improvement in skin penetration with the use of nanoparticles.

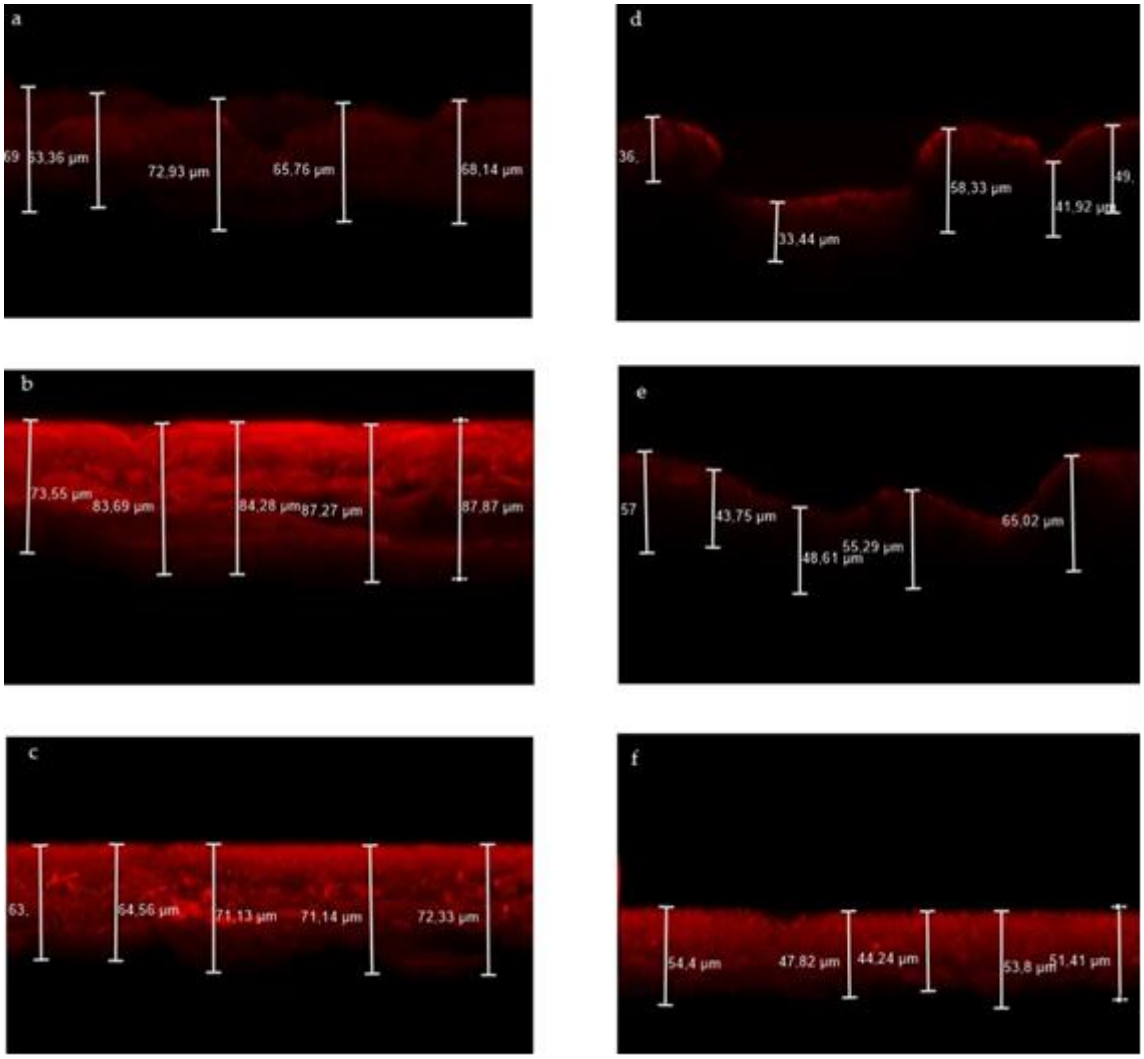

Figure 2. Confocal microscopic images of pig skin: (a, b and c) Penetration of NLCP with Rhodamine applied on pig ear skin models at 1, 2 and 12 hours, respectively; (d, e and f) Penetration of Rhodamine solution applied on pig ear skin models at 1, 2 and 12 hours, respectively.

In summary, the upper part of the skin is called the stratum corneum, with approximately 10-20 µm. After the stratum corneum, we have the viable epidermis, where we find the keratinocytes and melanocytes. This layer measures approximately 80-100 µm. Some of the most important factors that influence skin penetration are particle size, hydrophobicity, pH, surface load, raw materials, viscosity, among others. However, NLC penetration can occur in two ways: intercellular and intrafolicular. Lipidic nanoparticles can penetrate faster through hair follicles than other routes (36).

The CLSM images showed that NLCP have a higher accumulation in the skin. It can be seen in the images that after 24 hours of study, NLCP had an average penetration of 68.63 µm, which means that they have penetrated up to the viable epidermis, and this result was

desirable, since it is intended that the product developed in this study has an effect on this layer. Cosmetic products must not penetrate to the deepest layers of the skin. Furthermore, cosmetics containing lipidic nanoparticles should release and retain their active ingredients in the surface layers of the skin.

3.6. Tyrosinase Inhibition Activity

The tyrosinase inhibition activity of passion fruit seeds oil was compared with the activity of NLCP.

Figure 3 represents the percentage of tyrosinase inhibition obtained with passion fruit seeds oil and NLCP. There was a significant difference between the samples, with NLCP showing a higher tyrosinase inhibition compared to the oil.

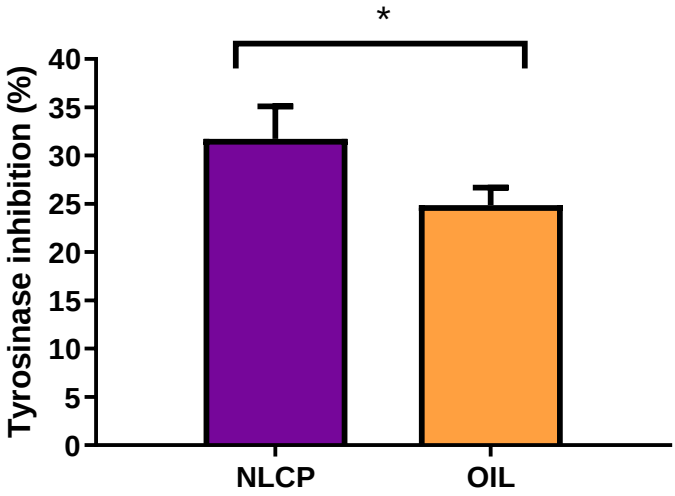

Figure 3. Tyrosinase inhibition activity of NLCP and Passion fruit seeds Oil. Data presented as mean ± SD (n=3). (* $p < 0.05$). In all cases, p values < 0.05 were considered significantly different.

3.7. *In vitro* Cytotoxicity studies

The cytotoxicity of the NLCP and SLN (0-500 µg/mL) was evaluated, 24 hours after exposure, by the REZ reduction, NR uptake and SRB binding assays. As can be seen in Figure 4, a slight but significant decrease in REZ reduction was detected for concentrations ≥ 150 µg/mL, when compared to control cells (0 µg/mL). Indeed, REZ reduction significantly decreased to 90.6, 89.4, 81.5, 80.6, 79.2 and 77.3 %, when compared with control cells, after treatment with 150, 200, 250, 300, 400 and 500 µg/mL of NLCP, respectively.

Concerning the SLN formulation, REZ reduction significantly decreased to 90.1, 88.8, 79.6, 79.0, 78.3 and 77.9 % of control cells, 24 hours after exposure to 150, 200, 250, 300, 400 and 500 µg/mL, respectively. It is also important to mention the lack of significant differences in REZ reduction between the NLCP and SLN formulations, at each tested concentration (Figure 4).

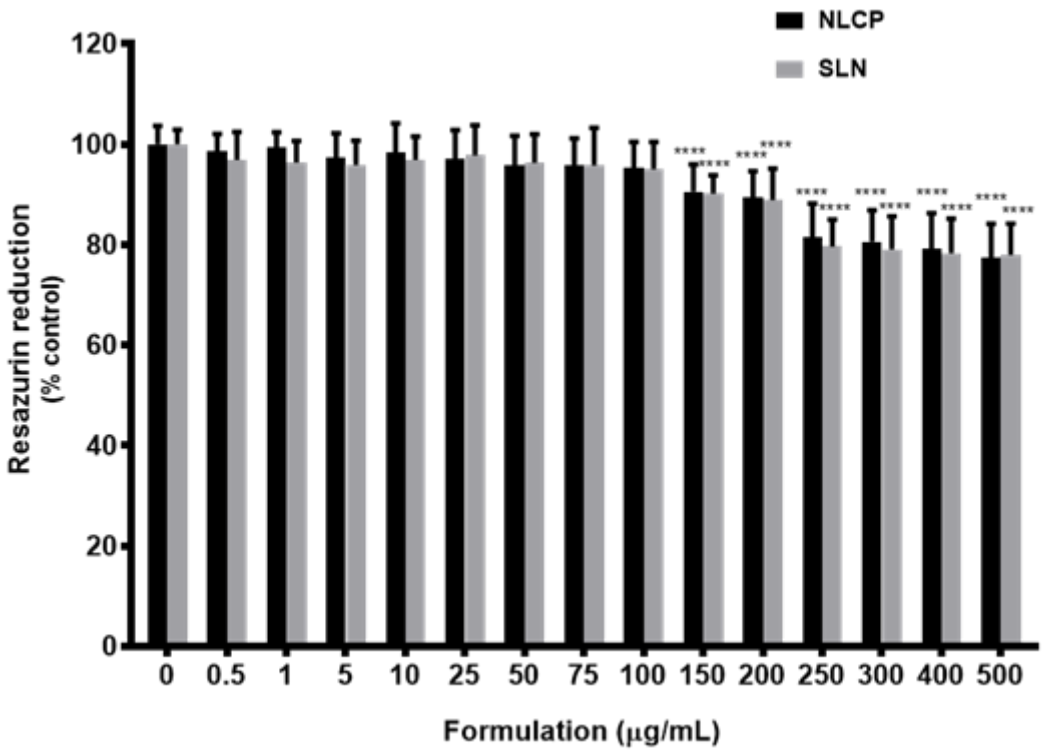

Figure 4. Cytotoxicity of the NLCP and SLN formulations towards HaCat cells, evaluated by the resazurin reduction assay 24 hours after exposure. Results are expressed as Mean ± SD from 6 independent experiments, performed in triplicate. Statistical comparisons were made using Two-way ANOVA, followed by the Tukey's multiple comparisons test (for each formulation, for comparisons between concentrations) or by the Sidak's multiple comparisons test (at each concentration, for comparisons between formulations) (****$p < 0.0001$ vs. 0 µg/mL, for each formulation). In all cases, p values < 0.05 were considered significantly different.

A similar cytotoxicity profile was observed in the NR uptake assay, with a significant decrease in dye uptake being observed for concentrations ≥ 150 µg/mL, when compared to control cells (0 µg/mL) (Figure 5). In fact, NR uptake significantly decreased to 92.8, 90.7, 83.6, 83.2, 81.2 and 79.8 % of control cells, 24 hours after exposure to 150, 200, 250, 300, 400 and 500 µg/mL NLCP, respectively, and to 92.0, 87.9, 80.1, 80.1, 79.3 and 77.8 %, after exposure to 150, 200, 250, 300, 400 and 500 µg/mL SLN, respectively. Again, no

significant differences were detected in the NR uptake between the two tested formulations, at each tested concentration.

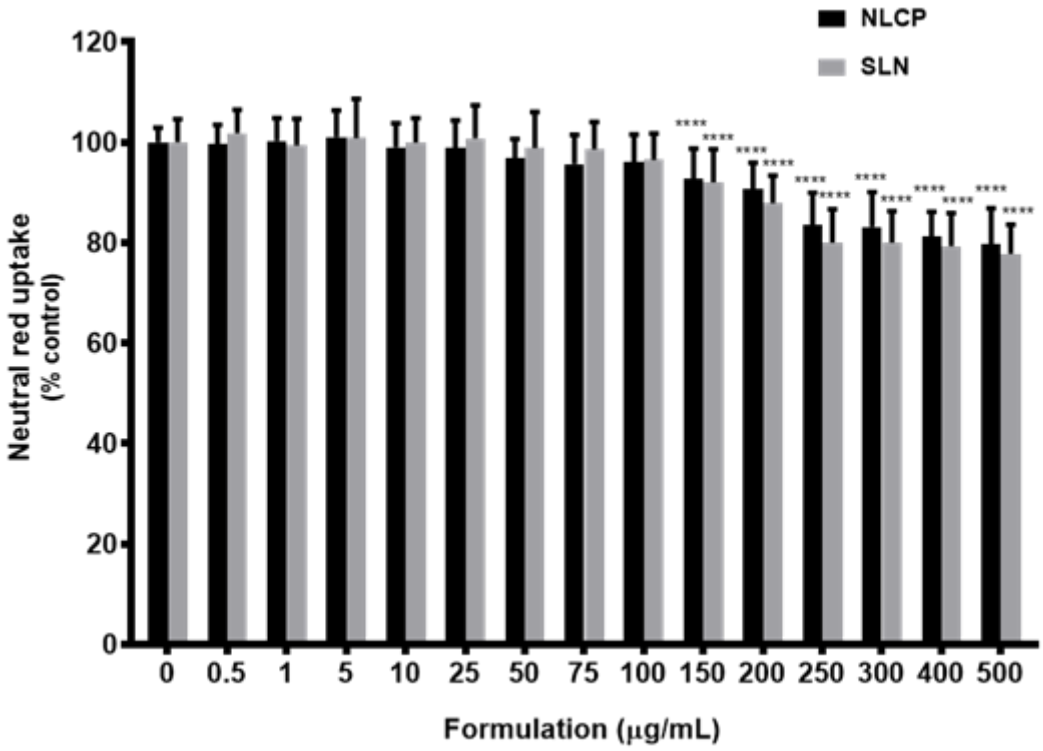

Figure 5. Cytotoxicity of the NLCP and SLN formulations towards HaCat cells, evaluated by the Neutral red uptake assay 24 hours after exposure. Results are expressed as Mean ± SD from 6 independent experiences, performed in triplicate. Statistical comparisons were made using Two-way ANOVA, followed by the Tukey's multiple comparisons test (for each formulation, for comparisons between concentrations) or by the Sidak's multiple comparisons test (at each concentration, for comparisons between formulations) (****p < 0.0001 vs. 0 µg/mL, for each formulation). In all cases, p values < 0.05 were considered significantly different.

Lastly, and in accordance with the previous cytotoxicity assays, a significant reduction in SRB binding was detected 24 hours after exposure of HaCaT cells to concentrations ≥ 150 µg/mL of the NLCP and SLN formulations, with no significant differences being detected between the two formulations, at each tested concentration (Figure 6).

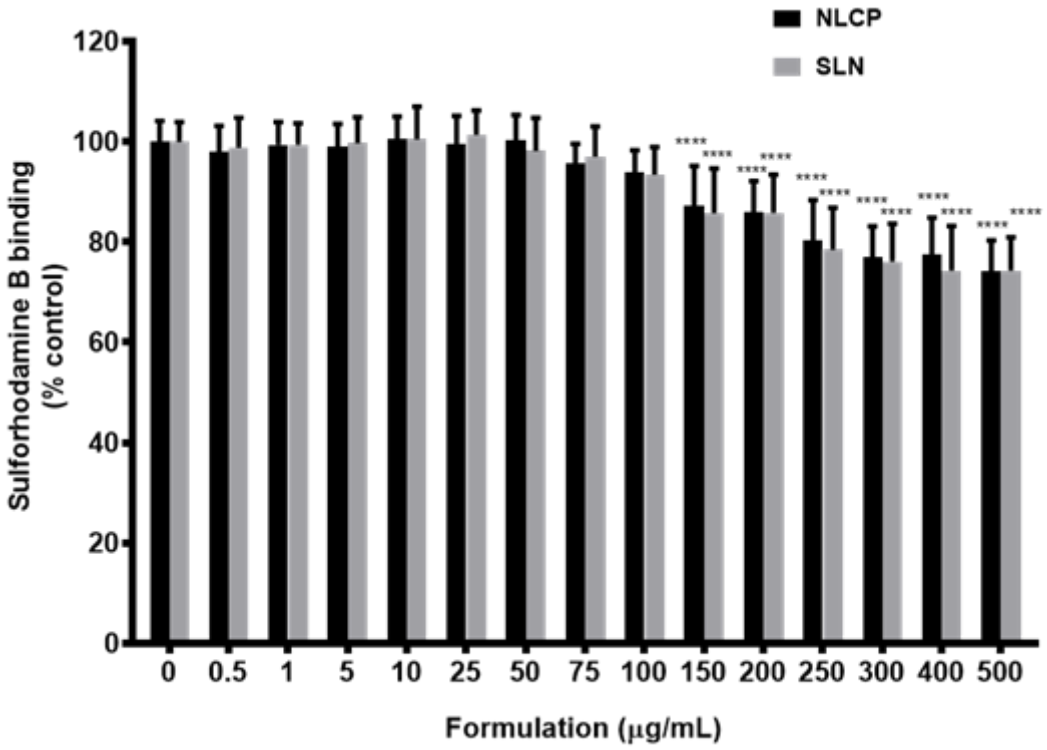

Figure 6. Cytotoxicity of the NLCP and SLN formulations towards HaCat cells, evaluated by the SRB assay 24 hours after exposure. Results are expressed as Mean ± SD from 6 independent experiences, performed in triplicate. Statistical comparisons were made using Two-way ANOVA, followed by the Tukey's multiple comparisons test (for each formulation, for comparisons between concentrations) or by the Sidak's multiple comparisons test (at each concentration, for comparisons between formulations) (****$p < 0.0001$ vs. 0 µg/mL, for each formulation). In all cases, p values < 0.05 were considered significantly different.

3.8. Scanning electron cryomicroscopy (Cryo-SEM)

In order to clarify the general morphology and internal structure of the NLC particles, Cryo-SEM analyses were performed. Figure 7 (a) shows the typical structure of NLC, with an almost spherical shape and a smooth surface. Spherical particles can promote a prolonged release as well as being able to protect natural compounds (37), such as passion fruit oil.

In Figure 7 (b) can be seen a typical network of hydrogels and the presence of NLC within this network, marked by green arrows, so we can suggest that the incorporation of NLC into the gel was effective.

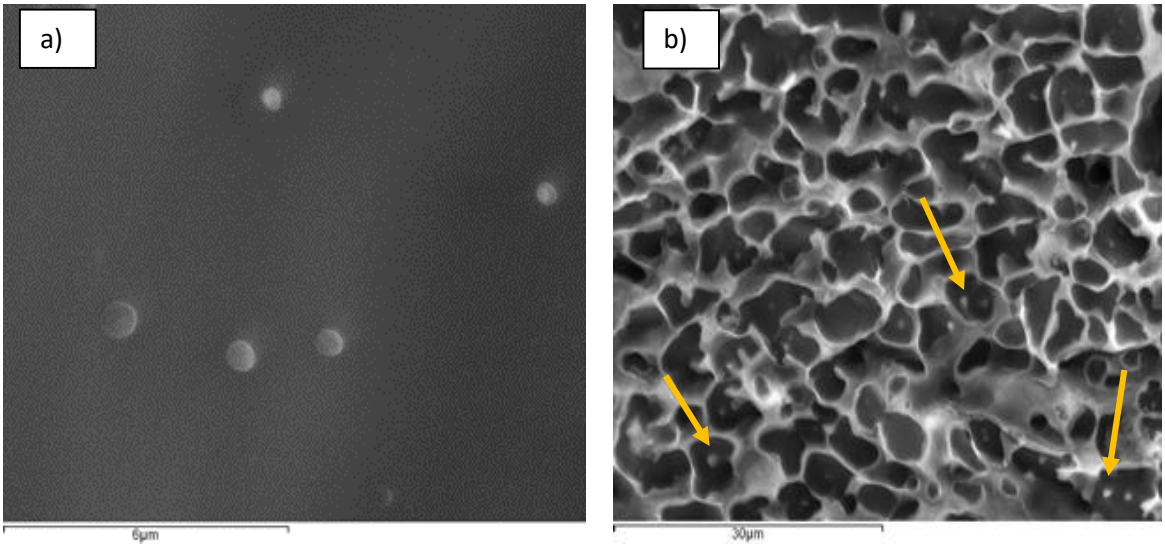

Figure 7. Cryo-scanning electron microscopy images of the (a) NLCP and (b) NLCP_GEL (magnification 40.000×).

3.9. pH measurement

The pH value is an important parameter for monitoring the stability of samples, as changes may indicate the occurrence of chemical reactions that could compromise the quality of the final product. A decrease in pH values may be due to hydrolysis of the fatty acid esters that generate free fatty acids (38).

In this study, no significant differences were observed regarding pH values of both samples stored at 4 and 25 ºC (Figure 8). It can be stated that NLCP_GEL, throughout the study period, showed pH values suitable for cutaneous application.

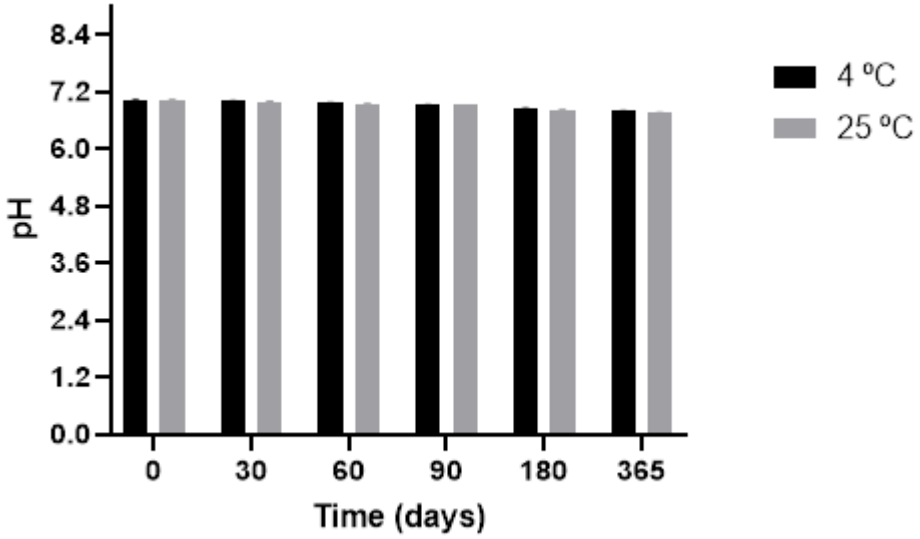

Figure 8. pH values of NLCP_GEL stored at 4 and 25 °C, over 365 days.

3.10. Color analysis

Regarding the analysis of the color of NLCP_GEL it can be seen that the luminosity (L*) of samples stored at 4 °C only had a small decrease throughout the study, although the Chroma Factor (C*) slight increased. However, there is no statistical difference, which means that the product maintained the white coloration throughout one year of study, suggesting that there was no lipid oxidation. It can also be inferred that the presence of passion fruit oil maintained the formulations stability, since it has antioxidant activity, due to the presence of phenolic compounds such as piceatannol and resveratrol.

Color determination of NLCP_GEL stored at 4 °C and 25 °C are summarized in Figure 9.

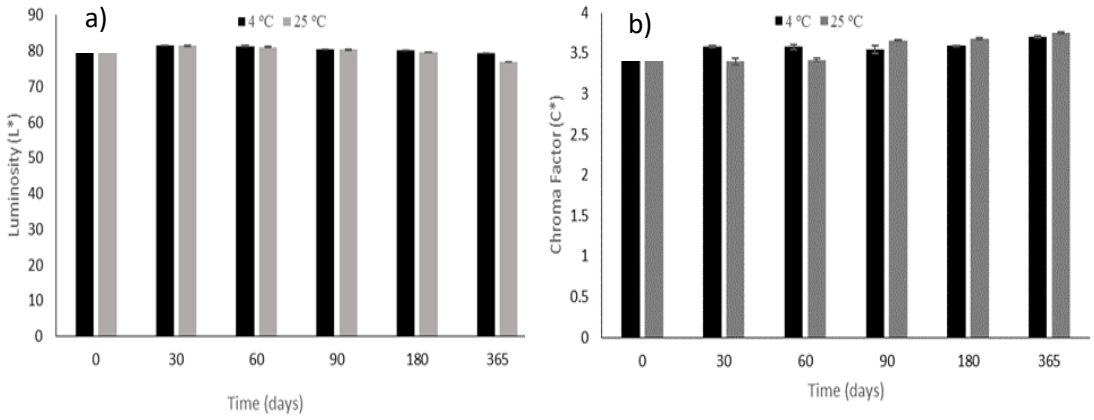

Figure 9. Color determination of NLCP_GEL on days 0, 30, 60, 90, 180 and 365 of storage at 4 and 25 °C. a) Luminosity (L*) and b) Chroma Factor (C*).

NLCP_GEL stored at 25 °C showed a higher variation of L* and C* values throughout the study, although visually it maintained the same color.

3.11. Viscosity test

NLC dispersions typically have low viscosity, which makes them difficult to apply, reducing skin retention. To facilitate the application of the product, and consequently increase its time of contact with the skin, the dispersions of NLC can be incorporated in semi-solid bases such as hydrogels (39).

In both rheograms, the viscosity of the NLCP_GEL decreased with the increase in the shear rate, due to a rupture in the internal structure of the gel, so the molecules aligned in the direction of the flow, offering less resistance to it. Based on the graphs of Figure 10, we can suggest that the samples stored at 4 (Figure 10.a) and 25 °C (Figure 10.b) present a non-Newtonian, rheofluidificant behaviour, since the apparent viscosity decreased with the increase of the shear rate. The viscosity values and the rheological behaviour of samples stored at both temperatures remained unchanged over time.

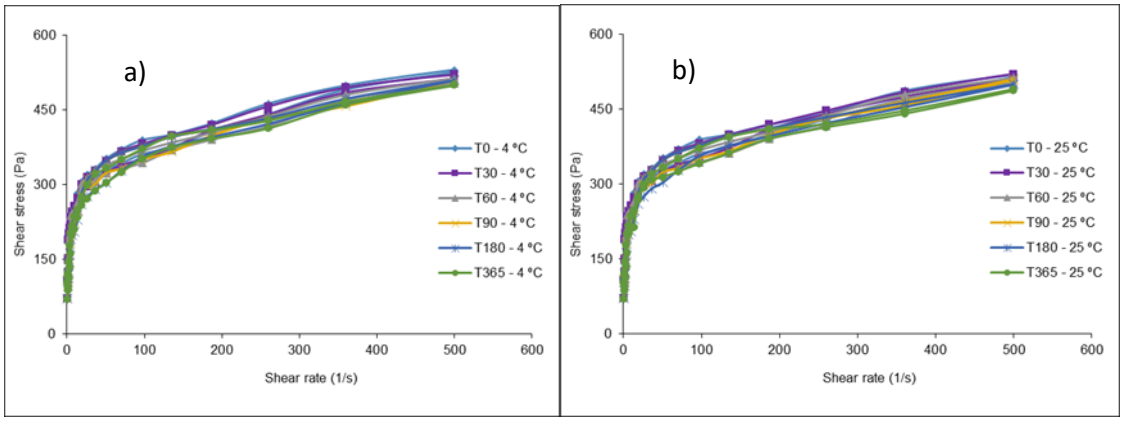

Figure 10. Rheograms of NLCP_GEL after 0,30,60,90,180 and 365 days of storage, a) at 4 °C (left) and b) at 25 °C (right).

3.12. Texture Analysis

The maximum force applied to obtain the deformation of the gels is known as firmness, and this property is a means of expressing the ease of application of the product to the skin (40). Through the analysis of Figure 11 it can be seen that samples stored at 4 and 25 °C presented a slight decrease of firmness. With the results, it is possible to suggest that the gel presents firmness values suitable for cutaneous application.

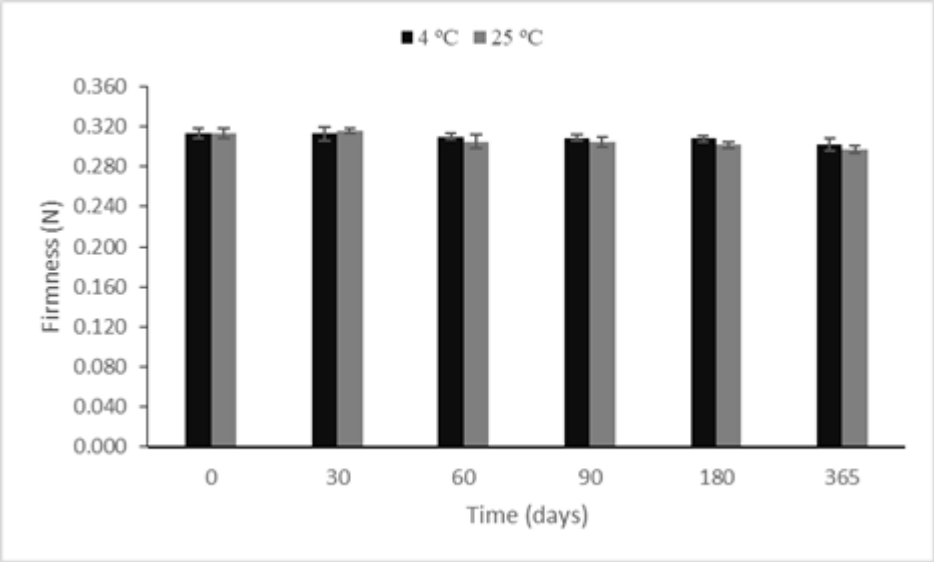

Figure 11. Firmness (N) of NLCP_GEL after 0, 30, 60, 90, 180 and 365 days of storage at 4 and 25 °C.

Adhesiveness is the work required to overcome the attractive forces between the sample surface and the probe surface. It is possible to observe in Figure 12 that the adhesiveness of NLCP_GEL stored at 25 °C increased after 60 days of storage.

It can be seen that NLCP_GEL stored at 4 °C showed a constant adhesiveness for 365 days, being more stable than NLCP_GEL stored at 25 °C.

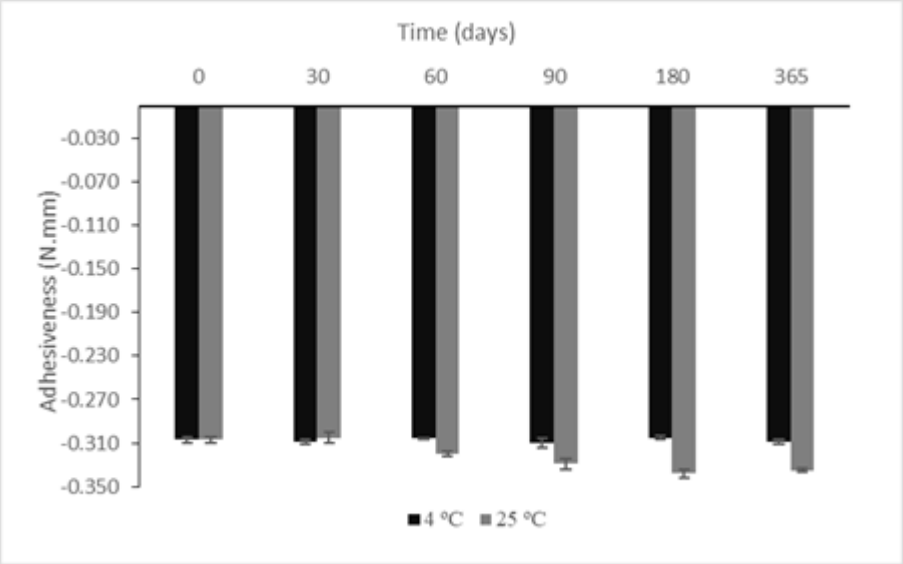

Figure 12. Adhesiveness (N.mm) of NLCP_GEL after 0, 30, 60, 90, 180 and 365 days of storage at 4 °C and 25 °C.

3.13. *In vitro* occlusion test

The occlusion of the skin can have some benefits. In addition to the soothing effect on wrinkles, occlusion can promote penetration of the active ingredients. When NLC are applied to the skin, these nanoparticles form a lipid film on the surface of the stratum corneum which causes an increase in the occlusive effect. Furthermore, the trans epidermal water loss (TEWL) decreases due to this increased occlusive effect, enhancing skin hydration even further (41,42).

Figure 13 represents the graph of the occlusion factor (%) of NLCP and NLCP_GEL. There was a significant difference between the occlusion factor values of the two samples studied, after 24h and 48h at 34 ºC. The occlusion factor of NLCP_GEL was almost two-fold the obtained with NLCP.

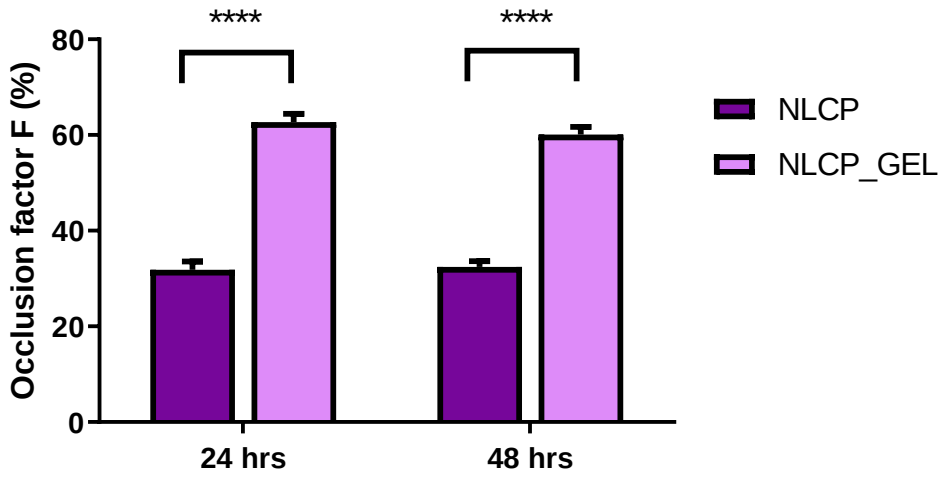

Figure 13. Occlusion factor (F) for NLCP and NLCP_GEL after 24 and 48 hours at 34 ºC. (**** p< 0.05. In all cases, p values < 0.05 were considered significantly different).

It can be concluded that NLC-based hydrogel greatly increases the occlusion factor, thus potentially increasing the hydration of the skin. The greater occlusion properties of NLCP-gel comparing with NLCP was due to the high viscosity of the former, which has the ability to form a film that prevents water loss through evaporation.

3.14. *In vitro* skin permeation by Franz diffusion cell

Release rates and skin retention of samples were evaluated using Franz-type diffusion cells, which are usually used for skin permeation assays, using pig ear skin as a membrane. Pig ear skin is widely used in these permeation studies since it is similar to human skin in terms of permeability and composition (43,44).

Some studies have concluded that nanoparticles may not permeate as deeply into the skin, however they serve as a reservoir of the active substance in the upper layers of the skin (45,46).

Skin retention of piceatannol released from NLCP, NLCP_GEL and *Passiflora edulis* seeds oil is shown in Figure 14. The best results were obtained with NLCP_GEL since it allowed to deliver a significantly higher ($p < 0.05$) amount of piceatannol, followed by NLCP and seeds oil, respectively. Furthermore, NLCP_GEL presented adhesive properties that enhanced the contact time with the skin.

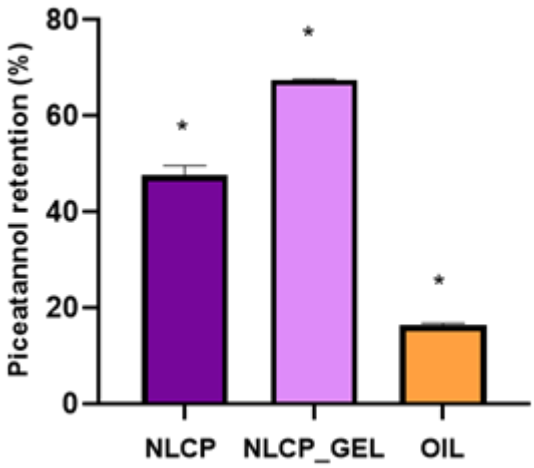

Figure 14. Skin retention of piceatannol (%) released from NLCP, NLCP_GEL and passion fruit seeds oil after 12 hours of permeation through the skin. Data represent the Mean ± SD (n = 3). (* $p < 0.05$. In all cases, p values < 0.05 were considered significantly different.)

As expected, it was possible to verify that Piceatannol was mostly located in viable epidermis but did not permeate into the receptor fluid. Therefore, no systemic side effects should occur after the cutaneous application of the developed formulations. The administration of cutaneous products may have a topical or systemic action. For topical action products, such as cosmetics, a greater retention of the product in the skin is required, with little or no permeability in the skin (44,47).

These results are in accordance with the results obtained in the *in vitro* occlusion test, in which the NLCP_GEL sample showed greater occlusion than the NLCP.

4. Conclusions

The NLC developed in this study, which contained Passion fruit seeds oil (NLCP), a by-product of the Madeira Island food industry, showed good physical and chemical characteristics throughout the storage. Besides, the nanoparticles showed an excellent piceatannol encapsulation efficiency and a higher tyrosinase inhibitory activity, compared to the free oil, suggesting a greater advantage of its incorporation. In addition, a good cutaneous penetration was observed by confocal laser scanning microscopy (CLSM).

The NLC-based hydrogel (NLCP_GEL) developed from NLC containing passion fruits seeds oil (NLCP), showed good stability over 1 year of storage at 4 and 25 °C, showing no significant changes in pH, color, texture, and viscosity. The incorporation of passion fruit seeds oil in nanoparticles and their gelation with Poly (acrylic acid) allowed the permeation of the active ingredient studied (piceatannol) into the viable epidermis, which is the target layer for the designated antioxidant and depigmentant action. In addition, the hydrogel maintains greater contact with the skin.

The cytotoxicity of the nanoparticles was also studied, revealing that the developed formulations are not cytotoxic to human immortalized keratinocytes, the HaCaT cells, for concentrations up to 100 µg/mL, as evaluated by the three different cytotoxicity assays performed (REZ reduction, NR uptake and SRB binding assays). Furthermore, the agreement between the cytotoxicity data obtained in all cell viability assays provides high confidence in the obtained results. The developed formulations obtained from a by-product of the food industry can thus be considered safe, as well as having good stability throughout a year of storage. Indeed, from a sustainable point of view, this new application of passion fruit seeds oil could provide a way to reuse this product as depigmenting agent by the cosmetic industry.

As a future perspective, other tests may be performed for a more complete characterization of the systems developed in this study, namely *in vivo* efficacy studies.

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CHAPTER 7

Conclusions and Future Perspectives

Conclusions and Future Perspectives

Nowadays the choice of natural products for cosmetic use has increased, as well as the use of by-products of the food industry with added value, making the economy circulate. Therefore, it is interesting to use passion fruit waste, such as seeds, to obtain products with potential benefits for cutaneous application.

Scientific studies indicate an extensive research related to the use of nanocarriers for the improvement of efficacy of formulations intended for skin application, being the lipid nanoparticles, specifically NLCs, the ideal candidates for these purposes. In addition, NLCs can increase the occlusion of the skin and prolong the release time of the active ingredients.

Piceatannol is a stilbene, which can be found in some fruits like grapes and passion fruits. This compound has antioxidant, anti-acne, skin healing, anti-aging and skin whitening effects. In our research we could conclude that piceatannol is much less studied in comparison with its analogue, resveratrol. There are few published articles about its use in skin formulations.

The first experimental chapter (chapter 3) describes the processes for obtaining passion fruit extracts from passion fruit seeds from Madeira Island using Soxhlet and ultrasound methods, and acetone, ethyl alcohol, isopropanol and n-hexane as solvents. These extracts were submitted to different tests to determine the antioxidant activity, namely DPPH and ABTS. The extracts obtained using ethanol and acetone as solvents had the highest results regarding the antioxidant activity, so these were chosen to continue the study.

In chapter 4, we can conclude, from the HPLC analysis, that the extracts obtained using the Soxhlet method, did not present any of the stilbenes, piceatannol and resveratrol. The commercial passion fruit oil, used for comparison, also showed no evidence of the presence of piceatannol and resveratrol, which may suggest the presence of other antioxidant compounds such as gallic acid, vitamin C and E. However, the amount of piceatannol found in the extract obtained using ultrasounds with ethanol and acetone was practically the same, but the amount of resveratrol was higher in the extract obtained with acetone. The yield of the alcoholic extract was almost double that of the extract with acetone, as well as the antioxidant activity determined by the ABTS method, which was six times higher compared to the acetone extract. Therefore, passion fruit by-products can be used in cosmetic and pharmaceutical industries having an added value, in addition to reducing the environmental pollution, avoiding the burning or landfill of waste. The obtained results also suggest the possibility of production of *Passiflora edulis* seeds oil with green solvents and the potential interest of this product to industries, as it represents a low-cost ingredient.

In chapter 5, NLCs containing Glyceryl distearate (Precirol® ATO5) as the solid lipid, prepared by both methods, High pressure homogenization (HPH) and ultrasonication showed smaller particle sizes, higher occlusion factor and the pH was considered acceptable for cutaneous application. The HET-CAM irritation test performed on NLCs obtained with Glyceryl distearate, prepared by the ultrasonication method, revealed that these nanoparticles showed no sign of irritability and could be used in skin products, even in the eye contour area. Besides, the cytotoxicity test revealed that these nanoparticles are not cytotoxic to HaCaT cells in the three different tests performed (resazurin reduction, Neutral red uptake and Sulforhodamine B assay), even in high concentrations.

In chapter 6, NLCs containing Precirol® ATO5, as solid lipid, and Passion fruit seeds oil from Madeira Island, as liquid lipid, showed good physical and chemical characteristics throughout the storage. Besides, these nanoparticles showed a higher tyrosinase inhibitory activity, compared to the free oil, suggesting a greater advantage in its incorporation. In addition, a good cutaneous penetration of the developed nanoparticles was observed by Confocal laser scanning microscopy (CLSM).

The NLC-based hydrogel developed from NLC containing passion fruits seeds oil, showed good stability over one year of storage at 4 °C and 25 °C, showing no significant changes in pH, colour, texture, and rheological behaviour. The incorporation of passion fruit seeds oil in nanoparticles and their subsequent gelation with Carbopol® 940 allowed the permeation of the active ingredient (piceatannol) into the viable epidermis, which is the target layer for the designated antioxidant and depigmenting action. In addition, the hydrogel allows to maintain a greater contact of the active ingredient with the skin.

The developed formulations obtained from a by-product of the food industry of Madeira Island can be considered safe as they did not have any cytotoxicity, as well as having good stability throughout a year of storage. Indeed, from a sustainable point of view, this new application of passion fruit seeds oil may constitute a strategy to reuse this product as depigmentant agent by the cosmetic industry.

In the future, *in vivo* studies, approved by the Ethics Committee of Faculty of Pharmacy, should be carried out in order to better assess the depigmenting efficacy of the formulations developed in this study.