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A NEW INSIGHT OF GRAPE SEED EXTRACT IN SKINCARE

by

Maria Leonor Teixeira Pinto de Castro

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A NEW INSIGHT OF GRAPE SEED EXTRACT IN SKINCARE

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfil the requirements of Master of Science degree in Biomedical Engineering

by

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Resumo

A pele é o maior órgão humano e desempenha funções essenciais, incluindo preservação de água, termorregulação e proteção contra radiação solar, trauma e infeções. Devido à sua constante exposição a fatores ambientais, a pele está sujeita ao envelhecimento natural e a diversas patologias. Por isso, para manter a sua saúde, homeostasia e retardar os sinais de envelhecimento, o uso de cosméticos e outros produtos de cuidado com a pele são cruciais. Em particular, o uso de ingredientes naturais derivados de plantas tem sido explorado pela sua bioatividade e enquadramento sustentável. Por exemplo, os subprodutos da uva, como as sementes, o bagaço, e os caules, apresentam várias atividades biológicas como proteção UV, antioxidante, antimicrobiana e anti-inflamatória. A utilização destes subprodutos tem sido importante, uma vez que permite obter moléculas bioativas a partir de matéria orgânica que, em larga escala, pode causar problemas ambientais. No entanto, muitas destas moléculas são instáveis e suscetíveis a degradação, o que limita a sua utilização. Neste sentido, técnicas de encapsulação, como os lipossomas, têm sido exploradas, uma vez que são capazes de aumentar a estabilidade, biodisponibilidade, solubilidade e penetração na pele, entre outros benefícios.

Neste trabalho, foram obtidos dois extratos de grainhas de uva, um de uma casta (GSE-Ov) e outro de uma mistura de cinco castas (GSE-Sv). Estes extratos foram analisados quanto às suas atividades antioxidante e antimicrobiana, e composição e estrutura molecular. O melhor extrato foi obtido a partir de uma só casta de vinho (GSE-Ov) com uma atividade antioxidante, IC_{50}, de 0,079 $mg.mL^{-1}$ e atividade antimicrobiana contra duas estirpes de *Staphylococcus aureus*, uma sensível e outra resistente à meticilina, com concentrações mínimas bactericidas (MBCs) de 3,13 e 6,25 $mg.mL^{-1}$, respetivamente. Além disso, não foi observada qualquer atividade contra S. *epidermidis*, microrganismo típico da microbiota normal da pele, o que assegura o seu equilíbrio. Os principais polifenóis identificados no GSE-Ov foram o ácido gálico, a catequina e a procianidina B1, e a análise FTIR mostrou que o processo de extração foi bem sucedido e o extrato apresentou uma estrutura e integridade molecular preservadas. O extrato biologicamente mais promissor (GSE-Ov) foi encapsulado em lipossomas de lecitina de soja revestidos com pectina, após uma otimização e caracterização, tendo apresentado uma eficiência de encapsulação de 88,8% com uma libertação de polifenóis de 59,4% em 24 h, um potencial zeta de -20,3 mV, diâmetro médio de 13,6 µm, índice de uniformidade de 0,637 e forma esférica. Posteriormente, esse extrato foi avaliado quanto à sua citotoxicidade, atividade anti-inflamatória (produção de IL-1α e IL-6) e produção de pró-colagénio I α1 humano. Os lipossomas carregados com GSE-Ov mostraram-se seguros até concentrações de 5 e 2,5 $mg.mL^{-1}$ para as células HaCaT e HDF, respetivamente. Além disso, demonstraram atividade anti-inflamatória contra IL-1α, quando testados a 2 $mg.mL^{-1}$.

Este trabalho permitiu a valorização de subprodutos da indústria vinícola através da obtenção de um extrato natural que evidenciou atividades antimicrobiana, antioxidantes e anti-inflamatórias muito promissoras para uso em formulações cosméticas.

Palavras-chave: Cosméticos; subproduto(s) da uva; economia circular; *skincare*; lipossomas.

Abstract

The skin is the largest human organ and performs essential functions, including water preservation, thermoregulation and protection against solar radiation, trauma and infections. Due to its constant exposure to environmental factors, the skin is subject to natural ageing and various pathologies. Therefore, to maintain its health, homeostasis and delay the signs of ageing, the use of cosmetics and other skin care products is crucial. In particular, the use of natural ingredients derived from plants has been explored for their bioactivity and sustainable framework. For example, grape by-products, such as seeds, pomace and stems, have various biological activities such as UV protection, antioxidant, antimicrobial and anti-inflammatory properties. The use of these by-products has been important as it allows bioactive molecules to be obtained from organic matter which, on a large scale, can cause environmental problems. However, many of these molecules are unstable and susceptible to degradation, which limits their use. In this sense, encapsulation techniques such as liposomes have been explored, as they are capable of increasing stability, bioavailability, solubility and penetration into the skin, among other benefits.

In this work, two grape seed extracts were obtained, one from a single grape variety (GSE-Ov) and the other from a mixture of five grape varieties (GSE-Sv). These extracts were analyzed for their antioxidant and antimicrobial activities, as well as their composition and molecular structure. The best extract was obtained from a single wine variety (GSE-Ov) with antioxidant activity, IC_{50}, of 0.079 $mg.mL^{-1}$ and antimicrobial activity against two strains of *Staphylococcus aureus*, one sensitive and the other resistant to methicillin, with minimum bactericidal concentrations (MBCs) of 3.13 and 6.25 $mg.mL^{-1}$, respectively. In addition, no activity was observed against *S. epidermidis*, a typical microorganism of the normal skin microbiota, which ensures its balance. The main polyphenols identified in GSE-Ov were gallic acid, catechin and procyanidin B1, and FTIR analysis showed that the extraction process was successful, and the extract had a preserved molecular structure and integrity. The most biologically promising extract (GSE-Ov) was encapsulated in pectin-coated soy lecithin liposomes after optimization and characterization, showing an encapsulation efficiency of 88.8% with a polyphenol release of 59.4% in 24 h, a zeta potential of -20.3 mV, an average diameter of 13.6 µm, a uniformity index of 0.637 and a spherical shape. Subsequently, this extract was evaluated for its cytotoxicity, anti-inflammatory activity (production of IL-1α and IL-6) and production of human pro-collagen I α1. Liposomes loaded with GSE-Ov proved to be safe up to concentrations of 5 and 2.5 $mg.mL^{-1}$ for HaCaT and HDF cells, respectively. In addition, they demonstrated anti-inflammatory activity against IL-1α when tested at 2 $mg.mL^{-1}$.

This work enabled the valorization of by-products from the wine industry by obtaining a natural extract which showed very promising antimicrobial, antioxidant and anti-inflammatory activities for use in cosmetic formulations.

Keywords: Cosmetics; grape by-product(s); circular economy; skincare; liposomes.

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“ Not a single one of us here today has done it alone. We are each a patchwork quilt of those who have loved us, those who have believed in our futures, those who showed us empathy and kindness or told us the truth even when it wasn’t easy to hear. Those who told us we could do it when there was absolutely no proof of that.”

Taylor Swift

Contents

Introduction

1. Skin: anatomy, functions, aging, and pathologies

The skin is the largest organ of the human body with approximately 2 m^2. Regarding its weight, there are different descriptions among the literature. For instance, it is described as representing approximately 16% of total body weight, which corresponds to approximately 3.6 kg in adults, the double of the weight of the brain **(Gilaberte *et al.*, 2016; McLafferty *et al.*, 2012; Walters and Roberts, 2002)**. This organ is composed by two layers - epidermis (the external layer) and dermis (underneath the epidermis) **(Byrd *et al.*, 2018; McLafferty *et al.*, 2012)**, and has an intricate arrangement of structures (appendages), such as sebaceous, sweat, and apocrine glands, hair follicles, and nails **(Gilaberte *et al.*, 2016; McLafferty *et al.*, 2012; Walters and Roberts, 2002; Vestita *et al.*, 2022)**. The skin enables the body to interact with its environment, providing an essential interface, and carrying out important functions, which can be broadly divided into three categories: sensorial, protective, and homeostasis maintenance. These comprise protection against trauma, solar radiation, toxins, and infections; preservation of water and electrolytes; thermoregulation; water, vitamin D, and fat storage. Furthermore, the skin plays a major role in blood pressure control, and in excretory physiological function **(Gilaberte *et al.*, 2016; Walters and Roberts, 2002)**.

The alterations that take place in the skin over time depend more on how the skin interacts with its environment than with genetic predisposition. Thus, it can be concluded that personal lifestyle has a bearing on how the skin ages **(Silva *et al.*, 2017)**. Aging is the process by which the body's capacity to adjust to the physiological and psychological environment gradually declines and eventually results in death **(Cao *et al.*, 2020)**. This process is induced by both intrinsic (chronological), and extrinsic (environmental) factors, all leading to reduced structural integrity and loss of physiological function. Typical intrinsic factors are cellular senescence (decreased cell dividing ability), increased expression of skin-degrading enzymes, irregular hair growth, and decreased sebum production, which affect all skin areas **(Gu *et al.*, 2020; Kammeyer and Luiten, 2015)**. These factors turn into aging signs typically around the age of 30, when cell renewal is not as fast and the hormone production undergoes changes **(Silva *et al.*, 2017)**. On the other hand, usual extrinsic factors are solar radiation (exposure without protection), cigarette smoke or other pollutants, low air humidity, poor diet, and excess alcohol intake. For instance, photoaging accounts for more than 80% of facial aging, and photo-aged skin is characterized by deep-wrinkling, loss of elasticity, dryness, laxity, rough-texture appearance, telangiectasia, and pigmentation disorders. These factors intensify the intrinsic aging, and, in most cases, it can be reduced by changing one's behaviour **(Gu *et al.*, 2020; Kammeyer and Luiten, 2015; Silva *et al.*, 2017)**. In addition, it has been reported that aged skin has an altered barrier function. The skin degradation by UV radiation is a cumulative process and its rate depends on the frequency, duration, and intensity of solar exposure, and on the natural protection offered by skin pigmentation **(Kammeyer and Luiten, 2015)**. The impact of both types of aging factors is especially prominent when oxidative stress is present **(Peres *et al.*, 2011)**.

Besides aging, skin is also prone to a number of conditions. These diseases have mainly been associated to inflammatory states and skin microbiota dysbiosis **(Duarte *et al.*, 2023)**. For example, acne vulgaris is distinguished by the presence of inflammatory papules, pustules, and nodules **(Lai and Gallo, 2008)**. Simultaneously, it is believed that *Cutibacterium acnes* plays a central role in the progression of this condition **(Dréno *et al.*, 2018; Platsidaki and Dessinioti, 2018)**. Another example is atopic dermatitis, which can be described as an exacerbated immune response in the skin, characterized by flares and remissions **(Robert and Kupper, 1999; Lai and Gallo, 2008)**. For instance, *Staphylococcus aureus* is hypothesized to exacerbate atopic dermatitis and some of its inhibitors generated by skin commensal bacteria were discovered to be lacking in patients with atopic dermatitis **(Nakatsuji *et al.*, 2017; Rozas *et al.*, 2021)**. Furthermore, psoriasis is a chronic inflammatory skin condition marked by peeling, red cutaneous plaques with inflammatory infiltrates, and epidermal hyperproliferation **(Robert and Kupper, 1999; Lai and Gallo, 2008)**. It has also been linked with skin microbiota, being its progression strongly associated to dysbiosis of *Cutibacterium*, *Corynebacterium* and *Staphylococcus* genera **(Chang *et al.*, 2018; Quan *et al.*, 2019; Rozas *et al.*, 2021)**.

2. Cosmetic products and markets

The term ‘cosmetics’ is described in the literature as having several origins. For instance, **Chaudhri and Jain (2009)** reported that Roman slaves whose job was to perfume men and women were the first to use the term “cosmetae”, and that men and woman in Egypt began using scented oils and ointments to clean and soften their skin, as well as to conceal body odour, as early as 10 000 BC. On the other hand, **Halla and colleagues (2018)** report that the term is originally derived from the Greek “Kosm tikos”, which means ‘having the power to arrange, skilled in decoration’.

In this line, cosmetic product is defined by the Council of European Union Regulation as “any substance or mixture intended to be placed in contact with external parts of the body (epidermis, hair system, nails, lips, and external genital organs), or with the teeth and the mucous membranes of the oral cavity, with a view to clean, perfume, protect, change the appearance, or correct body odours”. Cosmetic products can be classified based on their use, application, function, form of preparation, consumer’s age, or gender as: 1) personal cleansing (soaps, shampoos); 2) skin, hair, and integument care (toothpastes); 3) embellishment (perfumes, lip sticks); 4) protection (solar and anti-wrinkles products); 5) correction (beauty masks, hair dyes); 6) maintenance (shaving cream); and 7) active cosmetics (antiseptics, fluoridated toothpastes) **(Halla *et al.*, 2018)**.

Why is the skin’s appearance so relevant? Human skin is the body's outermost tissue. Because of this, people are very sensitive to, and aware of, its appearance **(Igarashi *et al.*, 2007)**. With the improvement in human living standards, more attention is paid to skin aging. Cosmetics and medications for the prevention and treatment of skin aging account for a sizeable portion of the regular expenses of many people, particularly among women **(Cao *et al.*, 2020)**, but, more recently, also among men. Skin with brighter complexion and smoother surface is typically

regarded as healthier, more attractive and linked to a fresher appearance **(Igarashi *et al.*, 2007)**. The enormous demand for this aesthetics is what fuels ongoing research into anti-aging strategies for the skin, among others **(Cao *et al.*, 2020)**.

According to a study performed by Fortune Business Insights, the global cosmetics market size was 299.77 billion USD in the year 2022. However, the demand across all regions has witnessed a negative impact during the COVID-19 pandemic period. Based on this study, the global market exhibited a decrease in growth in 2020, when compared to 2019. Still, the market is projected to grow from 313.22 billion USD in 2023 to 417.24 billion USD in 2030, corresponding to a compound annual growth rate (CAGR) of 4.2%. This rise in CAGR is the consequence of the market´s demand and growth, returning therefore to pre-pandemic growth levels **(Fortune Business Insights, 2023a)**.

Segmenting the cosmetics market by categories, skincare products have a prominent share **(Fortune Business Insights, 2023a)**. As expected, and just like the global cosmetics market, the skincare market was also negatively impacted during the pandemic. According to another study by Fortune Business Insights, skincare market growth suffered a decrease in 2020, and it is projected to grow from 109.71 billion USD in 2023 to 167.22 billion USD in 2030 at a CAGR of 6.21% **(Fortune Business Insights, 2023b)**. According to Transparency Market Research Inc., this growth trend is confirmed with the market projected to grow from 101.34 billion USD in 2021 to 154.7 billion USD in 2031, with a CAGR of 4.8% **(Transparency Market Research, n.d.)**. Thus, the main determining factors for the expected growth of this industry are the increased attention to personal care in all age groups, the increased demand for sustainable and clean label products, the increasing willingness of men to incorporate skincare products in their daily routine **(Fortune Business Insights, 2023b)**, the growing longevity coupled with the rapid aging of the population **(Transparency Market Research, n.d.)**, and increased investment in research and development of new products. Moreover, the flourishing of the e-commerce sector is anticipated to boost market growth further **(Grand View Research, 2022)**. A market analysis detected a shift in customer opinion between 2017 and 2021 about the necessity of not using environmentally damaging products. This trend has grown in European countries, most notably in France, Germany, Spain, and the United Kingdom, which are Europe's major markets. According to the report, the utilization of naturally produced components has been more essential to consumers globally over the last five years. For example, in 2017, 23% of French customers indicated it was a reason to buy face and body products, rising to 32% in 2021. As a result, natural and vegan products are becoming increasingly popular as people throughout the world become more aware of the ingredients and sustainability of the products they purchase **(CBI, 2023)**.

3. Grape by-products and their environmental impact

Grapevine (*Vitis vinifera L.*) is a perennial woody fruit crop used for the production of wine, juice, dried fruit, and distilled liquor, and for fresh consumption (table grapes) **(Goufo *et al.*, 2020)**. Grapes are one of the world’s biggest fruit crops, being one of the most popular fruits consumed worldwide, with approximately 80% of its production being destined for winemaking **(Martin *et***

***al.*, 2020; Nunes *et al.*, 2017)**. High levels of organic waste are produced during the winemaking process, which primarily entails pressing and macerating grapes. Those wastes are primarily made up of grape pomaces (62%), lees (14%), stalks (12%), and dewatered sludge **(Salem *et al.*, 2022)**, reaching approximately 14.5 million tons per year in Europe **(Chouchouli *et al.*, 2013)**. In line with this, a significant amount of these by-products (**Figure 1**) is produced in a short amount of time, posing ecological issues, since 35.9 million tons of grapes are pressed worldwide each year to make wine. Grape pomace is composed of 25% seeds, 25% stalks and 50% skins **(Hoss *et al.*, 2021)**. These by-products are either composted, fed to animals, or dumped in landfills. As an agro-industrial by-product, grape seeds may be responsible for environmental problems, when improperly managed **(Ferreira and Santos, 2022; Salem *et al.*, 2022)**, due to the low pH and high concentration of phenolic compounds, which can resist biological degradation **(Salem *et al.*, 2022)**, having the potential to unbalance the ecosystems, by modifying organic compound pathways and nutrients cycle **(Hoss *et al.*, 2021)**. In addition, the high organic carbon content of pomace, between 31 and 54%, can cause water pollution and bad odours.

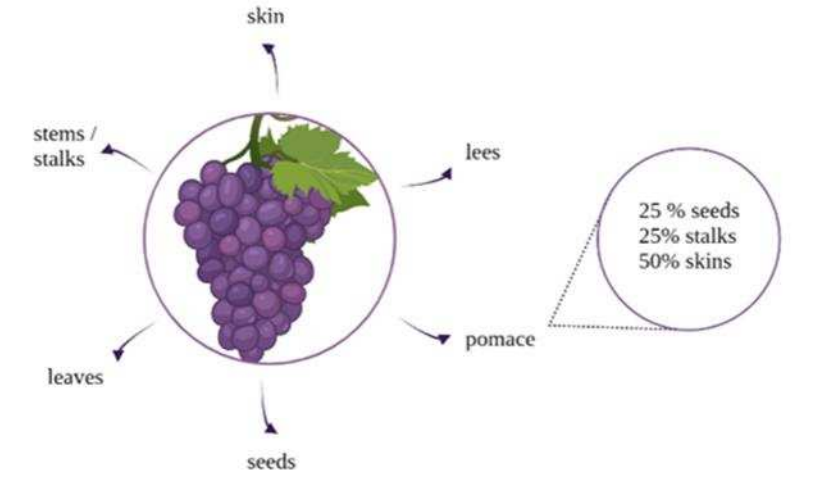

Figure 1 – Grape by-products.

Notwithstanding, it is possible to effectively valorise these wastes by means of extraction of their bioactive compounds, especially antioxidant phenolic molecules, but also vitamin E and fatty acids, using a safe, green, and environmentally friendly extractive medium (i.e., water-glycerol solution) **(Ferreira and Santos, 2022; Salem *et al.*, 2022)**.

Phenolic compounds are secondary plant metabolites that can be used as adjuvant treatment for a number of illnesses, including diabetes, diseases associated with the aging of the brain, and hypertension **(Brezoiu *et al.*, 2020)**. Structurally, they have an aromatic ring with one or more hydroxyl groups, and are mainly found in fruits, vegetables, nuts, seeds, and cereals. In particular, the phenolics produced by grapevine range from structural cell wall compounds, such as lignin and tannins, to specialized ones such as phenolic acids and flavonoids, and exhibit several properties such as antioxidant, anti-inflammatory, antibacterial, anticarcinogenic, antidiabetic, and cardioprotective effects, having great potential for several industries, in particular the nutraceutical and cosmetic ones **(Brezoiu *et al.*, 2020; Carvalho *et al.*, 2021; Goufo *et al.*, 2020)**.

4. Grapes and their by-products for cosmetic and skincare applications

4.1. Identified skincare biological activities

As mentioned before, there are several types of grape by-products exhibiting potential health benefits. Since these ingredients are biodegradable and have therapeutic and functional qualities that are of value, they have an advantage over synthetic compounds **(Ferreira and Santos, 2022)**. In **Table 1** it can be seen a list of studies regarding these by-products and their skincare biological effects. In most of the cases presented, the grapevine variety used is *Vitis vinifera L.*, and the explored by-products comprise extracts from seed and oil, pomace and oil, grape skins, shoot, lees stalk and stem, and seed paste. Nonetheless, other studies tested the biological activities of other grapevine varieties, such as *Vitis labrusca L.* **(Maluf *et al.*, 2018)**. Moreover, several other cultivars, such as *Mamaia, Carignano, Tempranillo, Touriga Nacional, Touriga Francesa*, and *Tinta-roriz*, have been explored **(Brezoiu *et al.*, 2020; Ferreira and Santos, 2022; Matos *et al.*, 2019; Perra *et al.*, 2021)**.

Since many ingredients in the formulation of cosmetics are prone to oxidation, the presence of antioxidant agents, as the ones present in the grape by-products, is essential for their functionality and maintenance, extending their quality and shelf life. Given that the bioactive compounds extracted from grape by-products present more than one functionally, as showed below, they may be used in cosmetic formulations as multifunction agents, minimizing the use of extra or even synthetic compounds.

Table 1 – Grape by-products and their potential skincare biological effect.

Grapevine variety	Grape by-product	Type of study	Biological effect	Reference source
Na	Seed extract	Human Dermal Fibroblasts	Increased cell viability, UVA protection	Yarovaya *et al.* (2020)
Vitis vinifera L.	Seed extract	*In vitro*	UVA photoprotection, antioxidant activity	Yarovaya and Khunkitti (2019)
Vitis vinifera L.	Seed extract	*In vitro*	Antioxidant activity	Salem *et al.* (2022)
Vitis labrusca L.	Pomace	*In vitro*		Maluf *et al.* (2018)
Mamaia	Pomace extract	*In vitro*		Brezoiu *et al.* (2020)
Carignano	Skins extract	*In vitro*		Perra *et al.* (2021)
Na	Seed extract	*In vitro*	Antioxidant activity, sun protection factor (SPF) booster	Limsuwan and Amnuikit (2017)
Touriga Nacional, *Touriga Francesa*, and *Tinta-roriz*	Pomace/seed extract and oil	*In vitro*	Antioxidant and antimicrobial effects	Ferreira and Santos (2022)
Vitis vinifera	Shoot extract	*In vitro, in vivo*	Antioxidant and anti-aging effects	Cornacchione *et al*. (2007)
Tempranillo	Lees extract	*In vitro*, keratinocyte and fibroblast cell cultures	Antioxidant, anti-tyrosinase, -elastase, and -collagenase activities; cellular protective effect against oxidative damage	Matos *et al.* (2019)
Red grape	Seeds and stalks extract	*In vitro*, keratinocyte and fibroblast cell cultures	Antioxidant activity, cellular protective effect against oxidative damage	Manca *et al.* (2019)

Vitis vinifera L.	Stems extract	*In vitro*	Antioxidant, anti-elastase and -tyrosinase activities, antimicrobial activity against *Staphylococcus aureus*, anti-inflammatory, and anti-aging effects	Leal *et al.* (2020)
Vitis vinifera L.	Seed extract	*In vivo* (Guinea pigs skin)	Tyrosinase inhibition (whitening effect)	Yamakoshi *et al.* (2003)
Vitis vinifera L.	Seed paste	*In vitro* enzymatic assay	Anti-tyrosinase, -elastase, and -collagenase activities	Michailidis *et al.* (2021)
Vitis vinifera	Seed extract	*In vivo*	Skin whitening, anti-aging, and anti-acne effects	Sharif *et al.* (2015)
Vitis vinifera L.	Pomace extract	*In vitro* enzymatic assays, human embryonic kidney HEK293 cells ATCC	Tyrosinase and elastase inhibition, anti-inflammatory activity	Ferri *et al.* (2017)
Vitis vinifera	Pomace	*In vivo*	Anti-inflammatory activity	Nishiumi *et al.* (2012)
Na	Seed extract	Raw 264.7 macrophages		Terra *et al.* (2007)

Na – not available

4.1.1. Grape seeds: extract, oil, powder, and paste

Several studies report important biological effects coming from grape seeds. For instance, grape seed extract is reported to increase cell viability, to protect against UVA damage, and to exert antioxidant activity **(Limsuwan and Amnuikit, 2017; Manca *et al.,* 2019; Salem *et al.*, 2022; Yarovaya *et al.*, 2020; Yarovaya and Khunkitti, 2019)**. Additionally, it is reported to have anti-tyrosinase, anti-aging, anti-acne, and anti-inflammatory activities **(Sharif *et al.*, 2015; Terra *et al.*, 2007; Yamakoshi *et al.*, 2003)**. Grape seed extract is also reported to have anticaries, antidandruff, antifungal, antibacterial and antioxidant properties, and to have light stabilizing and sunscreen applications. In addition, grape seed powder is reported to function as an abrasive and exfoliant by-product **(Belsito *et al.*, 2012; Fiume *et al.*, 2014)**. In the case of grape seed oil, it has been associated with antioxidant and antimicrobial activities **(Ferreira and Santos, 2022)**. Moreover, grape seed paste was described as exhibiting anti-tyrosinase, -elastase, and -collagenase activities **(Michailidis *et al.*, 2021)**.

4.1.2. Pomace: extract and oil

As discussed before, grape pomace consists of a mixture containing seeds, stalks, and skins (**Figure 2**), and it has been described as a source of antioxidant and anti-inflammatory compounds (**Brezoiu *et al.*, 2020; Ferri *et al.*, 2017; Maluf *et al.*, 2018; Nishiumi *et al.*, 2012**) and also as a tyrosinase and elastase inhibitor **(Ferri *et al.*, 2017)**. Moreover, both the grape pomace extract and the oil present antimicrobial activity against gram-positive bacteria (*S. aureus* and *S. epidermidis*), being the latter equally associated with antioxidative protection **(Ferreira and Santos, 2022)**.

4.1.3. Grapevine shoot and grape stems/stalks

Besides grape pomace, also vine shoot, which results from the pruning of vineyards during winter **(Baroi *et al.*, 2022)**, has been studied for its biological effects. **Cornacchione *et al.* (2007)** describes vine shoot as having antioxidant and antimicrobial properties. Regarding grape stems or stalks, which are the skeletons of grape clusters or bunches **(Atatoprak *et al.*, 2022; Blackford *et al.*, 2021)**, they have been reported to exert antioxidant, anti -elastase and -tyrosinase activities, antimicrobial action against *S. aureus*, anti-inflammatory, and anti-aging effects **(Leal *et al.*, 2020; Manca *et al.,* 2019)**.

4.1.4. Others

Furthermore, there are other examples of grape by-products, which have been explored for its biological potentialities. For instance, wine lees are reported by **Matos *et al.* (2019)** to have antioxidant, anti -tyrosinase, -elastase, and -collagenase activities, and to protect the cells against oxidative damage. This by-product is defined according to the European regulation EEC No. 337/979 as “the sediment/residue formed at the base of the deposit or barrel containing wine after fermentation, during storage or after performing authorized treatments to the product, as well as residues obtained from the filtration or centrifugation of said product” **(Fia, 2016; Sancho-Galán *et al.*, 2020)**. It consists of microorganisms (especially yeasts), tartaric acid, colloids, phenolic compounds, ethanol and inorganic matter **(Sancho-Galán *et al.*, 2020; Troilo *et al.*, 2021)**.

Another example are grape skins, whose extract has been associated with antioxidant activity **(Perra *et al.*, 2021)**.

4.2. Market expression

According to Cosmetics Europe – The Personal Care Association, the vast majority of Europe's 500 million consumers use, in a daily basis, a wide variety of cosmetic products such as soaps, shampoos, deodorants, perfumes, skincare products, make-up, among others **(Cosmetics Europe - The Personal Care Association, 2023)**. As it is one of the basic features in this field, over the last 20 years, the innovation in the cosmetic industry has been enormous, resulting in a wide range of different products. Also, the consumers are getting more concerned about their appearance, trying to accept the new social paradigms and with great enthusiasm and interest for natural trends and eco-friendly products. These tendencies have been increasing the use of bio-based extracts as active ingredients, leading to the development of new *green* compounds, which tend to be obtained considering sustainable principles **(Nunes *et al.*, 2017; Rodrigues *et al.*, 2016; Rodrigues *et al.*, 2018)**.

In this section, emphasis will be given on commercial products having grape related ingredients in their composition. In this line, as observed in **Table 2**, the products available commercially contain by-products such as crushed grape seeds, seed oil, seed extract, and powder, grape water and juice, and resveratrol. Among them, resveratrol, which is a natural polyphenol synthesized by plants, such as grapevine, in response to various stressful conditions (e.g., UV radiation exposure), has been one of the most explored bioactive ingredients in cosmetic industry. This compound has several biological effects, including antioxidant and anti-inflammatory properties **(Crăciun and Gutt, 2023; Santos *et al.*, 2023)**. It is found in both *cis* and *trans* versions, with the *trans* thought to be the active form **(Augustin *et al.*, 2013)**, being more thermo- and photostable than the *cis* form, which is very unstable **(Soleymani *et al.*, 2019)**. The biological effects on skin of some of these by-products were described in the previous section, and, in most cases, they are considered as key ingredients in the respective topical formulations. Some of the companies commercializing the products are TheraVine™, CAUDALIE®, DVINE Skin, Vinoble Cosmetics, BIOLAVEN and KORRES©, and the claims made include removal of dead skin surface cells, skin smoothing, turning the skin more receptive to other active ingredients, skin texture uniformization, among others. The commercial unit price, with variable unit volume, range from 7.60 to 199 €, depending on the ingredient composition and target claims. **Table 2** summarizes the different products commercially available, employing grape-derived ingredients, along with its target claims and potentialities.

Table 2 – Commercialized cosmetic products containing grape-derived ingredients.

Type of cosmetic	Application site	Company / Brand	Designation	Ingredients	Claims	Price	Reference source
Scrubs	Face	TheraVine™	Grape seed Facial Exfoliator	Crushed grape seed beads*, grape seed oil*	Removes dead cells; more radiant, refined and youthful skin	Na	TheraVine™ (n.d.)
		CAUDALIE®	VINOCLEAN Gentle Buffing Cream	Grape seed oil*	Removes dead cells; cleans and purifies the face; clean, smooth, and radiant skin	21 € (75 mL)	CAUDALIE® (2020)
		DVINE Skin	Light Harvest Facial Exfoliator	Grape seed powder*, grape seed extract*	Removes dead cells l; cellular renovation improvement; uniform skin texture	22.40 € (125 mL)	DVINE (2022)
		Vinoble Cosmetics	Cleansing scrub	Grape seed oil*	Removes dirt, sebum, and excess skin cells; skin protection; cooling effect	129 € (200 mL)	VINOBLE Cosmetics GmbH (2022)
			Enzyme scrub	Grape seed extract*	Frees the skin from dander; makes the skin more receptive to the following active ingredients	56 € (50 mL)	
	Body	TheraVine™	Grape seed Body Exfoliator	Crushed Pinotage grape seeds*	Lift impurities and dead surface skin cells	Na	TheraVine™ (n.d.)
		Vinoble Cosmetics	Salt & grape seed scrub	Grape seed extract*	Loosens dead skin cells and smooths the skin; detoxifying and purifying	22 € (100 mL)	VINOBLE Cosmetics GmbH (2022)

		The Body Shop®	Spa of the World™ French grape seed scrub	Grape seed oil, grape seed powder	Helps to invigorate, exfoliate, and refine the skin; smoother and softer skin	15.88 € (100 mL)	The Body Shop International Limited (2020)
		CAUDALIE®	Crushed Cabernet Scrub Vinosculpt	Grape seed oil*, grape seed powder	Gently eliminates dead cells; reduces skin irregularities; smoother skin	28.90 € (225 g)	CAUDALIE® (2020)
Masks	Face	CAUDALIE®	Glycolic peeling mask Vinoperfect	Grape seed oil, grapevine shoot extract	Anti-tyrosinase, anti-blemishes	25.50 € (75 mL)	CAUDALIE® (n.d.)
			Hydrating cream mask Vinosource-Hydra	Grape water*, grape seed oil*, grape seed extract, grape juice	Hydrates, soothes, antioxidant effect	23.90 € (75 mL)	
		Vinoble Cosmetics	Regenerating & detoxifying mask	Grape seed oil*, grape flower cell extract, vine extract	Regenerates and purifies	199 € (50 mL)	VINOBLE Cosmetics GmbH (2022)
		TheraVine™	HydraVine™ Chardonnay Grape tissue Mask	Grape seed Extract*	Soothes, desensitizes and hydrates	Na	TheraVine™ (n.d.)
Creams	Face	dieNikolai	Grapeseed Oil Darling	Grape seed oil*, grape skin extract	Moistures and strengthens skin	69 € (50 mL)	dieNikolai (n.d.)
		BIOLAVEN	BIOLAVEN Day Face Cream	Grape seed oil*	Hydrates and softens	7.60 € (50 mL)	BIOLAVEN (n.d.)

		Schaf Skincare©	Restore	Grape seed oil	Balances skin's moisture barrier	95.52 € (30 mL)	Schaf Skincare© (2023)
Sun protection	Hair	KORRES©	Red Vine Hair sun protection	Grape seed extract	Water-resistant UV filters	15.50 € (150 mL)	KORRES© (n. d.)
Eye care	Eye	Patchology©	SERVE CHILLED™ ROSÉ EYE GELS	Resveratrol*	Calms inflammation	33.76 € (15 units)	Patchology© (2023)

Na – not available; * key-ingredient

5. Encapsulation trends

In the last decades, the delivery of grape by-products-derived compounds in micro or nanocarriers has been suggested as an innovative and effective way to enhance their stability, bioavailability, and biological activity at the target areas **(Perra *et al.*, 2022)**. Nanomaterial-based cosmetics have distinct advantages over micro-scale cosmetics, since they have a wider contact surface, allowing for more effective and long-lasting effects **(Ferreira *et al.*, 2023)**. According to the European Commission, "*Nanomaterials consist of differently shaped small particles no larger than one hundred nanometres*" **(European Commission, 2022)**. Despite the growing interest in nanocarriers due to their unique features, there are also some concerns related to incomplete knowledge of its interactions with biological systems and consequent implications upon consumer's health **(Ferraris *et al.*, 2021)**. Materials at this scale can penetrate biological barriers, including cell membranes and subcellular structures, to a much greater extent compared to larger particles. This heightened ability to breach cellular and sub-cellular boundaries raises concerns about potential undesired and uncharacterized effects on human health **(Joseph *et al.*, 2023; Sahu, 2023)**. These problems must be addressed, and lead to the need of regulation over the incorporation of nanomaterials/nanotechnologies in cosmetic products. However, this raises another problem, as each country or world region follows its own legislation **(Ferraris *et al.*, 2021)**. For example, in the European Union (EU), the Regulation (EC) nº 1223/2009 establishes the legal basis for all manufactured or sold cosmetic products **(Ferreira *et al.*, 2023)**. According to this document, each nanomaterial must be properly identified in the list of ingredients, using the word "nano" (in brackets) as a suffix to the material's name **(Ferraris *et al.*, 2021; Gupta *et al.*, 2022)**, which, due to the misinformation of the general public, may cause some reluctance to purchase products incorporating such materials. A good example of that was the distrust of a significant part of the world population towards the COVID-19 vaccines, which was partly due to the alleged incorporation of nanotechnologies in their formulations. On opposite, micro scaled encapsulation does not involve such issues, which means that besides low regulation and/or less risk to health, larger particle sizes have less permeability on the skin, allow greater texturing or better appearance of the product to the touch, allow the encapsulation of high molecular weight compounds, may present greater stability over time or less ability to aggregate, sometimes having more automated, simple and fast manufacturing processes (since size is not a restrictive criterion) **(Ngwuluka *et al.*, 2021)**. Moreover, according to clinical studies, resveratrol and grape seed extract are very effective on a variety of skin conditions such as aging, wounds, acne vulgaris, redness, among others. In line with this, well-designed clinical research, particularly with micro and nanoformulations, appear to be very promising and are needed **(Soleymani *et al.*, 2019; Taofiq *et al.*, 2019; Zhang *et al.*, 2020)**. Among the several carriers designed for different activities, particularly tailored for skin delivery, liposomes have been the favoured option since they protect the encapsulated compound, prevent its degradation and are able to transport and release it more effectively on the targeted layers of the skin. On their own, liposomes are also

beneficial since they repair the skin's protective barrier **(Perra *et al.*, 2021; INdermal, 2022)**. A schematic representation of different encapsulation techniques is presented in **Figure 2**.

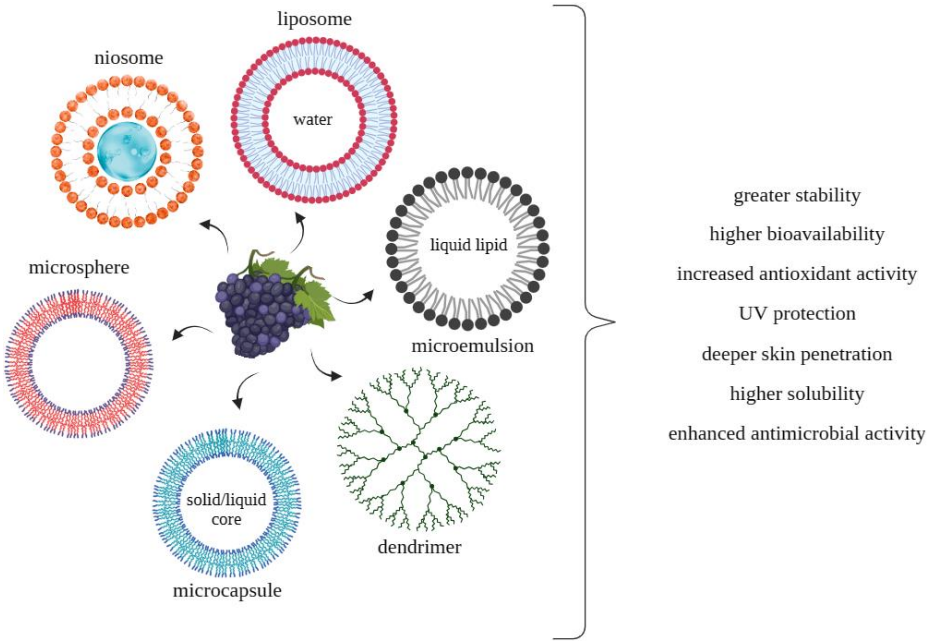

Figure 2 – Encapsulation techniques and their respective advantages.

5.1. Liposomes

A liposome, **Figure 2**, consists of a spherical vesicle composed primarily of phospholipids that form a bilayer structure when dispersed in water **(INdermal, 2022)**. **Perra *et al.* (2021)** developed liposomes and phospholipid-based nanovesicles, modified with glycerol, or Montanov 82®, or a combination of the two, as carriers for grape pomace skin extract. The antioxidant activity of the extract was slightly boosted when placed into the vesicles, which were biocompatible and capable of protecting fibroblasts against oxidative stress. In a study conducted by **Jøraholmen *et al.* (2015)**, chitosan-based liposomes successfully loaded resveratrol (0.1 and 0.2% in distilled water ($w.v^{-1}$)) with an encapsulation efficiency of ca. 75% for topical treatment of vaginal inflammation and infections. **Park *et al.* (2014)** also reported the development of chitosan-coated liposomes for transdermal delivery of resveratrol (0.1%), allowing to increase the proportions of resveratrol that permeate the skin from 96.85 $\mu g.cm^{-2}$ with non-coated to 126.93 $\mu g.cm^{-2}$ with coated liposomes, which may help delay the skin aging process. In another study, PEG-resveratrol liposomes were shown to be more stable than simple resveratrol liposomes. In the PEG-based liposomes, no significant physical alterations (e.g., mean particle size) and no significant vesicle migration and aggregation were observed, and there was a constancy of the PI (polydispersity index) **(Caddeo *et al.*, 2018)**. Moreover, **Manconi *et al.* (2016)** combined a grape pomace extract (4 or 5%) in small sized sodium alginate (SA)- or arabic gum (AG)-based liposomes with good encapsulation efficiency, potentiating its applicability in the nutraceutical industry. Depending on the used extract concentration, the encapsulation efficiencies were 91 and 53% when using SA, and 84 and 52% when using AG, for 4 and 5% of grape pomace extract, respectively. **Caddeo *et***

***al.* (2016)** evaluated an integrated resveratrol- and quercetin-loaded liposomal formulation, made from Lipoid S75, oleic acid and ATX Tris buffer, in an animal model of cancer-mediated skin tissue injury, leading to the enhancement of human dermal fibroblasts viability (from 20 to 60% at 400 μM), presumably via reducing intracellular reactive oxygen species (ROS) levels. **Caddeo *et al.* (2008)**, developed resveratrol-loaded liposomes, made from enriched soy phosphatidylcholine (P90G), diacetyl phosphate (DCP), cholesterol or lecithin alone. This system showed to be stable for 60 days, without significative variation in particle size, zeta potential, and loaded resveratrol quantity, or signs of trans/cis-isomerization, thus increasing the efficacy of the treatment and prevention of skin disorders caused by excessive UV radiation exposure. **Castangia (2017)** created an *in vitro* formulation of propylene glycol-liposomes and simple liposomes, both stabilized with grape-silver nanoparticles and loaded with grape pomace extract. The mixture was shown to be effective in removing several pathogens (*S. aureus* and *Pseudomonas aeruginosa*) and free radicals, as well as in protecting fibroblasts and keratinocytes against oxidative stress, suggesting a suitable formulation for topical treatment of skin lesions. In agreement with the last study, **Vitonyte *et al.* (2017)** showed that resveratrol and gallic acid co-loaded (0.5% ($w.v^{-1}$) each) into phospholipid liposomes were able to protect fibroblasts and keratinocytes from oxidative stress and from pathogenicity (antimicrobial activity boost). Another research, focused on ultra-deformable liposomes (UDL), composed of 3ß-[N-(N', N'-dimethylaminoethane)-carbamoyl] cholesterol hydrochloride (DC-Chol), cholesterol and sodium deoxy cholate (SDC), and loaded with resveratrol and psoralen (from 2.5% to 10% ($w.w^{-1}$), with respect to the lipids weight), highlighted their potential for vitiligo treatment. The procedure consisted in the administration of two doses of the liposomes, significantly increasing tyrosinase activity by ca. 1.70-fold for both doses, and melanin concentration by 1.69 and 2.12 for first and second drug-delivery moments, respectively. Interestingly, liposomes loaded only with resveratrol did not show significant improvements for these parameters **(Doppalapudi *et al.*, 2017)**.

Liposomes were the approach chosen for the development of this research. Nonetheless, there are other delivery systems and during the next sections a few examples of each are given.

5.2. Niosomes

Niosomes, **Figure 2**, are non-ionic surface-active compounds with a bilayer structure composed of surfactants and cholesterol **(Bhardwaj *et al.*, 2020)**. A study by **Pando *et al.* (2013)** focused on resveratrol-loaded niosomes made from Span 80 or a Span 60-cholesterol mixture. In this work, Span 80 niosomes were very stable but showed low entrapment efficiency (13.66% in the best-case scenario). Span 60-cholesterol niosomes had the opposite results (better entrapment efficiency, 40.96%, but lower stability), releasing resveratrol at a slower rate than the Span 80 niosomes. In other study, **Pando *et al.* (2015)** showed that smaller niosomes for topical use, made from Gelot 64 and either oleic or linoleic acid, exhibiting a size range of 299-402 nm, were more effective in maintaining the stability of resveratrol and in enhancing its entrapment efficiency. With nutraceutical purposes, **Schlich *et al.* (2020)** prepared resveratrol-loaded niosomes, made of Tween 20/Span 60 (surfactants) and cholesterol, which exhibited minimal cytotoxicity for intestinal

cells, and safety for potential oral intake. A niosome-based hydrogel made from Cholesterol and Span 80 was developed by **Negi *et al.* (2017)** for delivery of resveratrol. These delivery systems were able to increase the resveratrol biological half-life, as well as decrease its T_{max} (time until maximum cutaneous concentration is obtained).

5.3. Microparticles

Microparticles, are micrometer-sized (usually between 1 and 1000 μm) drug delivery systems made from synthetic or natural polymers. They can be employed as effective carriers for delivering bioactive compounds to several body sites **(Chew *et al*., 2017; Kumar *et al*., 2017; Mishra and Singh, 2020)**, and comprise, for example, microcapsules and microspheres **(da Silva *et al*., 2023)**, **Figure 2**. For instance, **Böger *et al*. (2018)** developed microspheres from gum arabic and a mixture of gum arabic and maltodextrin 10DE (1:1 ratio) for grape seed oil encapsulation. Comparing to free grape seed oil, the loaded microspheres presented a lower total phenolic content, with only 47 and 60% being preserved for gum arabic and gum arabic/maltodextrin microspheres, respectively. On the other hand, the loaded microspheres allowed a higher ferric reducing power. In another study, **Surini *et al*. (2018)** produced grape seed oil-ethylcellulose (1:4 ratio)-based microcapsules, with a mean diameter of 202.74 μm, which allowed an entrapment efficiency of 93.87%. Also, these microcapsules were combined with a gel, and the authors concluded that this solution would be interesting for cosmetic application as a moisturizer.

5.4. Microemulsions

Microemulsions, **Figure 2**, are transparent, thermodynamically stable, isotropic liquid combinations of oil, water, and surfactant, frequently in conjunction with a cosurfactant. A microemulsion is distinguished by its thermodynamic stability rather than its size **(Murthy and Shivakumar, 2010; Simonazzi *et al*., 2018)**. **Juškaitė *et al*. (2017)** produced microemulsions, with mean droplet size of 112 nm, using PEG-8 caprylic/capric glycerides as surfactant (57%) and polyglyceryl-6 isostearate as cosurfactant (38%). Resveratrol was incorporated as the oil phase (5%). The incorporation of resveratrol into the formulation enhanced the photostability of active compounds and delayed its photodegradation. *In vitro* testing revealed that up to 60% of resveratrol was released within 6 hours under a constant rate. Also, resveratrol's capacity to protect cells from oxidative stress and promote cell viability was demonstrated *in vivo*. Other study, by **Alibade *et al*.(2022)** showed that red grape pomace-loaded microemulsions (3 – 15% ($w.w^{-1}$)), made from a Tween 80 and Span 20 mixture as surfactant mixture and isopropyl-myristate as oil phase, were able to remain stable at 25 and 37 ºC for 30 days, and that radical scavenging activity of encapsulated pomace improved up to 13% when compared to the free extract. **Arunprasert *et al*. (2023)** studied the impact of several surfactants (polysorbate 80 and polysorbate 20) and co-surfactants (ethanol, isopropyl alcohol, Cetiol® HE, and glycerol) on the efficacy of grape seed oil-loaded microemulsions. The tested grape seed oil concentration ranged from 5-15% ($w.w^{-1}$). All formulations had good thermodynamic stability, with no apparent variations after 30 days. Microemulsions made from polysorbate 80, and Cetiol® HE at 1:2 ratio revealed

the largest microemulsion area, had a particle size > 300 nm, and the results showed that this mixture could be used with relevant antioxidant activity.

5.5. Dendrimers

A dendrimer, **Figure 2**, is a hyperbranched spherical polymer composed of a hydrophobic central core, branched monomer and functional peripheral groups **(Mu *et al.*, 2020)**. A research by **Pentek *et al.* (2017)** showed that the application of polyamidoamide dendrimer enhanced resveratrol solubility and stability in water and semisolid dosage forms. Also, the dendrimer allowed higher efficiency in resveratrol loading and skin penetration. In another study **Shi *et al.* (2020)** used glucan-based dendrimers to encapsulate resveratrol, showing that its bioactivity and bioavailability was significantly improved. For instance, its solubility increased about 9.1 times when compared to the raw resveratrol solution. Also, the antioxidant activity of encapsulated resveratrol was significantly higher (from about 30% of DPPH radical scavenging activity with raw resveratrol to 94.1% when encapsulated), its apparent permeability coefficient (P_{app}, $cm.s^{-1}$) was slightly higher (2.12×10^{-6} $cm.s^{-1}$) than those of common permeable compounds (1×10^{-6} $cm.s^{-1}$), while the resveratrol uptake by cells was markedly enhanced, raising from 3.47 nmol with raw resveratrol to almost 6 nmol with the dendrimer encapsulation technique.

Table 3 summarizes the different reported studies related to delivery systems incorporating grape-related bioactive compounds with applications in cosmetics.

Table 3 – Delivery systems used for the delivery of compounds from grape by-products in cosmetics and their advantages/applications.

Delivery System	Material	Loaded Compound	Advantages / Application	Reference source
Liposomes	Glycerol, Montanov 82®, or a mix	Grape pomace skin extract	Boosted antioxidant activity	Perra *et al*. (2021)
	Chitosan	Resveratrol	Ca. 75% encapsulation efficiency; Topical treatment of vaginal inflammation and infections	Jøraholmen *et al.* (2015)
	Chitosan		Increased amount of resveratrol that permeates skin	Park *et al*. (2014)
	PEG		Improved liposome stability	Caddeo *et al.* (2018)
	Sodium alginate or arabic gum	Grape pomace extract	Good encapsulation efficiency; Applicability in nutraceutical industry	Manconi *et al*. (2016)
	Lipoid S75, oleic acid and ATX Tris buffer	Resveratrol and quercetin	Enhanced human dermal fibroblasts viability	Caddeo *et al.* (2016)
	P90G, DCP, cholesterol or lecithin alone	Resveratrol	Good liposome and resveratrol stability; Increased efficacy of the treatment of UV-caused skin disorders	Caddeo *et al.* (2008)
	Propylene glycol; Grape-silver nanoparticles for stabilizing the liposomes	Grape pomace extract	Antimicrobial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*; Antioxidative stress effect	Castangia (2017)
	Phospholipid	Resveratrol and gallic acid	Antimicrobial activity boost; Antioxidative stress effect	Vitonyte *et al.* (2017)

	DC-Chol, cholesterol and SDC	Resveratrol and psoralen	Increased tyrosinase activity and melanin production; Potential for vitiligo treatment	Doppalapudi *et al.* (2017)
Niosomes	Span 80 or Span 60-cholesterol mix	Resveratrol	Na	Pando *et al.* (2013)
	Gelot 64 and oleic acid/linoleic acid		Enhanced stability and entrapment efficiency	Pando *et al*. (2015)
	Tween 20/Span 60 (surfactants) and cholesterol		Minimal cytotoxicity	Schlich *et al.* (2020)
	Span 80 and cholesterol		Increased biological half-life; Decreased time until maximum cutaneous concentration	Negi *et al.* (2017)
Microparticles	Microspheres: gum arabic or gum arabic/maltodextrin (coat)	Grape seed oil	Improved ferric reducing power	Böger *et al*. (2018)
	Microcapsules: ethylcellulose (coat)		High entrapment efficiency (93.87%); Potential as a moisturizer agent	Surini *et al*. (2018)
Microemulsions	PEG-8 caprylic/capric glycerides (surfactant); polyglyceryl-6 isostearate (cosurfactant)	Resveratrol (oil phase)	Increased photostability; 60% release within 6 h; Oxidative stress protection and improved cell viability	Juškaitė *et al*. (2017)
	Isopropyl-myristate (oil phase); Tween 80 and Span 20 (surfactants)	Red grape pomace	High stability over 30 days, at 25 and 37 ºC; Improved radical scavenging activity up to 13%	Alibade *et al*. (2022)
	Cetiol® HE (co-surfactant); Polysorbate 80 (surfactant)	Grape seed oil (oil phase)	Good thermodynamic stability over 30 days; antioxidant activity	Arunprasert *et al*. (2023)

Dendrimers	Polyamidoamide	Resveratrol	Enhanced solubility and stability; More efficiency in loading and skin penetration	Pentek *et al.* (2017)
	Glucan		Improved bioactivity, bioavailability, and solubility; Higher antioxidant activity and cellular uptake	Shi *et al.* (2020)

Na – not applicable

Work Outline

1. Aim of the thesis

The main goal of this master's thesis was to obtain, develop and characterize a grape seed extract for potential skincare and cosmetic application. In addition, we aimed to employ an encapsulation method in order to improve the extract's stability and delivery. Moreover this research proposes a sustainable approach to a common by-product, promoting a circular economy. This thesis already generated a review paper, regarding the use of grape by-products in sustainable cosmetics, which is the state-of-the-art of this work. Data of this thesis is being used to create a research paper that will be submitted soon for publication.

2. Thesis organization

This dissertation is divided into several sections. The first section, **Introduction** comprises a theoretical background indispensable to the understanding of the work. Topics like the anatomy of the skin and its pathologies, the relevancy of the use of skincare and cosmetic products, the environmental impact of grape by-products, their identified biological properties and market expression, as well as the advantages and trends of the encapsulation of such products are explored. The second section, **Work Outline** provides a description of the work organization to further improve the understanding of this thesis, working as a guide. The following section, **Materials and Methods** depicts all materials and methodologies used to develop this research, comprising the preparation and biological and physicochemical characterization of the grape seed extract (GSE), as well as the liposomes. The fourth section, **Results and Discussion** portray all the obtained results and their respective interpretation. The final sections, **Conclusions** and **Future Work** provide a closure to the work and at the same time the possibility to further explore and optimize the developed research.

3. Outputs

Castro, M. L., Ferreira, J. P., Pintado, M., Ramos, O. L., Borges, S., Baptista-Silva, S. 2023. Grape By-Products in Sustainable Cosmetics: Nanoencapsulation and Market Trends. *Applied Sciences* **13**: 9168. doi:10.3390/app13169168

Apolinário, E., Castro M. L., Pintado, M., Ferreira, J. P., Baptista-Silva, S., Borges, S. 2024. Endowed polyphenols for vaginal infections in advanced delivery systems. *Microorganisms*. (Accepted for publication).

Materials and Methods

1. Biomass

The seeds of red grapes were grown in the Alto Douro region of Portugal and were provided by Symington Family Estates, a wine company and Port wine house in Portugal. In this study, two types of red grape seeds were used: (i) from one wine grape variety, commonly known as wine for regular consumption (GSE-Ov), and (ii) from five selected grape varieties, commonly known as port wine (GSE-Sv). The 100% hydro-ethanolic solvent used throughout the work was purchased to Carlo Erba Reagents S.A.S. (France). All the remaining reagents are analytical grade or higher as discriminated bellow as used.

2. Preparation of Grape Seed Extracts (GSEs)

The GSE was obtained as described by **Bucić-Kojić *et al*. (2013)**, with some modifications. Briefly, milled grape seed was subject to a solid-liquid extraction process using a hydro-ethanolic solvent (1:1) at a 1:40 (w/v) ratio. The process was carried out in a shaking water bath (Shaking Bath UNITRONIC VAIVÉN, JP SELECTA, Barcelona, Spain), for 3 h at 80 ºC. After extraction, the mixture was decanted for solid-liquid separation, and the supernatant's ethanol was evaporated using an R-210 Rotavapor System (Rotary Evaporator BÜCHI R-210, Switzerland). Finally, the resultant supernatant was frozen at -80 ºC, and then lyophilized/freeze-dried (GAMMA 2–16 LSC plus, Christ, Germany) for further testing.

3. Antioxidant activity

The antioxidant activity of both GSEs (i.e. GSE-Ov and GSE-Sv) was assessed using three different methods: 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay and Folin-Ciocalteau method. For that, freeze-dried GSEs were weighed and solubilized in methanol.

3.1. ABTS Radical Cation Decolorization Assay

The ABTS assay was performed according to **Gonçalves *et al*. (2009)**. The $ABTS^+$ stock solution was prepared by mixing two aqueous solutions (ultrapure water), one of ABTS with a concentration of 7 mM, and the other of potassium persulfate ($K_2O_8S_2$) with a concentration of 2.44 mM. The resulting solution was left stirring for approximately 16 h, covered in aluminium foil, and then stored at 4 ºC for a maximum of 1 month. The $ABTS^+$ working solution was made by filtering the prior with a 0.45 µm syringe filter and diluting it with water until the absorbance, at 734 nm, was 0.70 ± 0.02. Also, a trolox solution was prepared with a 1000 µM concentration and stored at -20 ºC. For this solution, trolox was solubilized in methanol and the volume was completed with a PBS solution (75 mM NaH_2PO_4), pH 7.4 adjusted with a strong monovalent base or acid. Briefly, for the procedure, in each well of a 96-well microplate, 20 µL of the sample (each dilution), trolox, or solvent (methanol) for the control, were pipetted in triplicate, followed by 180

µL of $ABTS^+$ working solution. After incubation for 6 min at 30 ºC, the reaction was measured at 734 nm using a microplate reader (Microplate Reader Biotek Synergy H1, USA). The samples' scavenging activity (SA) was determined using Equation (1) and compared to the trolox standard calibration curve (25 – 175 µM). The results were expressed as IC_{50} milligrams per gram of dry extract (mg.g^{-1} dry extract).

$$SA\ (\%) = \left[\frac{Abs_{CTL} - Abs_{SPL}}{Abs_{CTL}}\right] \times 100 \quad (1),$$

where, Abs_{CTL} is the absorbance of the control and Abs_{SPL} is the absorbance of the samples.

3.2. DPPH Radical Cation Decolorization Assay

The DPPH assay was performed according to **Brand-Williams *et al*. (1995)**. First, a DPPH stock solution was prepared with a 600 µM concentration and then stored protected from the light at -20 ºC. From the previous one, a DPPH working solution was prepared (by dilution with methanol) until its absorbance was 0.600 ± 0.100, at 515 nm. Also, a trolox solution was prepared in methanol with a 1000 µM concentration and stored at -20 ºC. Briefly, in each well of a 96-well microplate, 25 µL of sample (each dilution), trolox, or solvent (methanol) used as control, were pipetted in triplicate, followed by 175 µL of DPPH working solution. After incubation for 30 min at 25 ºC, the absorbance was measured at 515 nm using a microplate reader. The samples' scavenging activity (SA) was also determined using Equation (1) and compared to the trolox standard calibration curve (25 – 175 µM). The results were expressed as IC_{50} milligrams per gram of dry extract (mg.g^{-1} dry extract).

3.3. Folin-Ciocalteau colorimetric method

The total phenolic content of GSE were determined by the Folin-Ciocalteau method with some modifications **(Coscueta *et al*., 2018)**. First, a 7.4% (w.v^{-1}) Na_2CO_3 solution was prepared in deionized water (dH_2O). Also, a Folin-Ciocalteau working solution was prepared by diluting 2 mL of Folin-Ciocalteau reagent in the final 10 mL with dH_2O. This solution was prepared daily, when needed. Furthermore, a gallic acid standard solution with a concentration of 1 mg.mL^{-1} was prepared in methanol and stored at -20 ºC. Briefly, for the procedure, in each well of a 96-well microplate, 30 µL of sample (each dilution), gallic acid, or solvent (methanol) for blank were pipetted in triplicate, followed by 100 µL of Folin-Ciocalteau working solution, and 100 µL of 7.4% Na_2CO_3, in this order. After incubation for 30 min at 25 ºC, the absorbance was measured at 765 nm using a microplate reader. The samples' total phenolics content was determined by interpolation with a gallic acid calibration curve (0.025 – 0.200 mg.mL^{-1}) and expressed as milligrams of gallic acid equivalent per gram of dry extract (mg GAE.g^{-1} dry extract).

4. Antimicrobial activity

For the evaluation of the GSEs (i.e. GSE-Ov and GSE-Sv) antimicrobial activity, several microbial relevant strains usually present in skin microbiota were used: *S. epidermidis*, methicillin-susceptible *S. aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), and *C. acnes* (obtained

from the collection culture of CBQF, Portuguese Catholic University). The method was performed as described by **Pinela *et al*. (2022)**, with modifications. Colonies of the microbial strains were transferred and cultured overnight in Mueller Hinton (MH) agar (Biokar, France) plates at 37 ºC for 24 h. *C. acnes* was grown in Tryptic Soy (TS) agar (Biokar, France) supplemented with 5% sheep blood (Thermo Fisher Scientific, Massachusetts, USA), with an anaerobic atmosphere controlled at 37 ºC for 48 h. After incubation, the inoculum suspensions were adjusted with the turbidity of 0.5 McFarland (equal to 1.5×10^8 colony-forming units (CFU) mL^{-1})) in sterile saline solution. For antimicrobial activity, 100 µL GSEs (100 $mg.mL^{-1}$) was mixed with 100 µL media and serially diluted in the same media. After, 100 µL of inoculant was added to each GSE dilution and incubated for ca. 24 h at 37 ºC. After incubation, all samples were plated using the drop technique **(Miles and Misra, 1938)** in MH agar or TS agar supplemented with blood and then incubated for ca. 24 h or 48 h at 37 ºC, for MBC (minimum bactericidal concentration) determination. Culture media and culture media with inoculum were used as controls.

5. Fourier-Transform Infrared (FTIR) Analysis

A FTIR analysis of the GSEs was carried out using a Spectrum 100 FTIR spectrometer with a horizontal attenuated total reflectance samples accessory (PIKE Technologies, USA), and a diamond/ZnSe crystal. The Horizon MBTM FTIR software was used. All spectra were collected with 16 scans and a 4 cm^{-1} resolution, in the 4500 – 500 cm^{-1} region. In addition, baseline, point adjustment, and spectra normalization were performed. All samples were analyzed in triplicate.

6. GSEs composition analysis by High Performance Liquid Chromatography (HPLC)

The GSEs were precisely weighed, dissolved in hydro-ethanolic solvent (1:1) at a final concentration of 10 $mg.mL^{-1}$, and then filtered into vials using 0.45 µm syringe filters. For the extracts' composition analysis, samples were analysed on a Waters Alliance e2695 Separate Module HPLC coupled to a C_{18} Phenomenex column (250 x 4.6 mm x 5 µm, Kromasil) and to a Diode Array Detector (DAD). Detection was achieved at wavelengths of 280 (derivates of hydroxybenzoic acids), 320 (derivatives of hydroxycinnamic acids), 360 (flavonols), and 528 nm (anthocyanins). The mobile phase was composed of (A) acetonitrile/water (5:95 $v.v^{-1}$) 0.1% Trifluoroacetic acid (TFA), and (B) acetonitrile 0.1% TFA. The flow rate was set at 1.0 $mL.min^{-1}$, and the injection volume was 20 µL.

7. GSE-Ov encapsulation with liposomes

Liposomes were prepared by lipid film hydration method, as described by **Machado *et al*. (2014)**. Briefly, lecithin (Alfa Aesar, Massachusetts, USA) was solubilized in absolute ethanol at a 1:20 (w/v) ratio, and the mixture was stirred for 1 min. Then, a R-210 Rotavapor System (Rotary Evaporator BÜCHI R-210, Switzerland) was used to evaporate the ethanol until a lipid film was observed in the flask walls. After, 0.2 g of GSE-Ov and 20 mL of dH_2O (for loaded liposomes) or just 20 mL of dH_2O (for empty liposomes) were added to the flask, and the mixture was left in a

shaking water bath at 60 ºC for 90 min. Finally, 1% (w.v^{-1}) pectin aqueous solution was mixed with the liposomal mixture in a 1:1 (v.v^{-1}) ratio, in order to enhance liposomes stability (adjust the electrical charge).

7.1. Liposomes characterization

7.1.1. Zeta potential

For liposomes characterization, zeta potential measurements were performed at room temperature (25 ºC) in a folded capillary cell using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK) with a He-Ne laser-wavelength of 633 nm and a detection angle of 173º. The analysis was performed in triplicate, and the results were expressed as average ± standard deviation (mV).

7.1.2. Size distribution

For particle size distribution assessment, it was used a Malvern Mastersizer 3000 - Laser Diffraction (Malvern Instruments Ltd.) with a refractive index of 1.457 and absorption index of 0.01 parameters selected. dH_2O was used as a dispersant. According to the laser diffraction through the particles of material, a scattering pattern was generated and used to calculate the particle size via Mie theory. The sample was analyzed six times, and the presented data was the mean of all measurements.

7.1.3. Morphology

Liposome morphology was assessed using a Pro Scanning Electron Microscope (Thermo Scientific™, MA, USA). Before the procedure, the liposome samples (empty liposomes) were freeze-dried and were analysed in splinters and powder forms. For sample preparation, they were placed onto metal plates and coated with a thin gold layer using a Sputter Coater (Polaron, Bad Schwalbach, Germany) under vacuum. SEM was used with the magnifications of 500, 1000, 2000, and 4000 x, and with an electron beam of 10 kV. The images presented are representative of the morphology of the sample.

7.1.4. FTIR analysis

A FTIR analysis of encapsulated GSE-Ov and encapsulant was carried out as previously described in **section 5 (Material and Methods)**.

7.1.5. Encapsulation efficiency

The liposomes encapsulation efficiency (EE) was assessed by an indirect method, as follows. After GSE encapsulation by the liposomes, the supernatant was recovered and analysed by the Folin-Ciocalteau method for total phenolics content determination, as described in **section 3.3 (Material and Methods)**. The EE was calculated by the difference between the total phenolic content of the extract and the total phenolic content of the supernatant after encapsulation, according to Equation (2).

$$EE\ (\%) = \left[\frac{TPC_{GSE} - TPC_{SN}}{TPC_{GSE}}\right] \times 100 \quad (2),$$

where, TPC_{GSE} is the total phenolic content of the GSE, and TPC_{SN} is the total phenolic content of the supernatant after encapsulation. This analysis was performed in triplicate.

7.1.6. *In vitro* polyphenols release assay

The release profile of polyphenols by the liposomes was assessed as follows. After GSE encapsulation by the liposomes, the mixture was centrifuged (5 000 rpm, 9 min; Hettich Zentrifugen, Tuttlingen, Germany), and a sample was retrieved for initial total phenolics content determination (0 h). After, the supernatant was removed and replaced with 5 mL PBS at pH 5.4, in order to mimic skin's physiological conditions. Then, 500 µL samples were taken at the following times: 1, 2, 4, 6 and 24 h. Immediately after collecting the samples the retrieved volume was replaced with PBS for volume maintenance. Finally, all samples were centrifuged (13 220 rpm, 5 min; Hettich Zentrifugen) and analysed using the Folin-Ciocalteau method as previously described in **section 3.3 (Material and Methods)**. During the experiment the mixture was kept stirring at 100 rpm and 37 ºC, and the analysis was performed in triplicate.

8. Cell culture

Immortalized human keratinocytes (HaCaT) and Human dermal fibroblast (HDF) were obtained from ATCC. The cells were cultured at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2, as monolayers using Dulbecco's Modified Eagle Medium (DMEM, Gibco, Thermo Fisher Scientific), supplemented with 10% ($v.v^{-1}$) Fetal Bovine Serum (FBS, Gibco, Thermo Fisher Scientific) and 1% ($v.v^{-1}$) antibiotic/antimycotic. HaCaT were used between passages 52 and 56, and HDF between passages 4 and 6.

9. Cytotoxicity

Cytotoxicity of GSE-Ov, encapsulated GSE-Ov and encapsulant was assessed through PrestoBlue™ Cell Viability test, as specified by the manufacturer. HaCaT and HDF cells were seeded at 1.0 x 10^5 $cells.mL^{-1}$ in 96 well microplates and were left to adhere in incubation overnight. After, the cells were exposed to GSE-Ov, encapsulated GSE-Ov or encapsulant for 24 h, at 37 ºC with 5% CO_2 in a humidified environment, using DMEM. The concentrations studied were 0.0187 – 10 $mg.mL^{-1}$ (1:2 dilutions), 0.3 – 10 $mg.mL^{-1}$ (1:2 dilutions) and 10 $mg.mL^{-1}$, respectively. In two separate studies, each sample was evaluated in quadruplicate. Concisely, 90 µL of the sample, culture medium for positive control or 10% dimethyl sulfoxide (DMSO, Sigma Aldrich, Missouri, USA) for negative control, were added to each well.

After incubation, 10 µL of PrestoBlue™ Cell Viability Reagent (Thermo Fisher Scientific) was added to each well of the plate and left to incubate, protected from the light, for approximately 2 h in a humidified atmosphere at 37 ºC and 5% CO_2. Lastly, the fluorescence was measured using a microplate reader. The results are presented in percentage of metabolic inhibition in comparison to the positive control, according to the ISO 10993-5 standard, where an inhibition of more than 30% is deemed cytotoxic.

10. Quantification of IL-6 by ELISA

HaCaT cells were seeded at 2.5 x 10^5 $cells.mL^{-1}$ in 12 well (1 mL per well) microplates for 24 h. After, the cells were exposed to GSE-Ov, encapsulated GSE-Ov or encapsulant in the following

concentrations: 0.1 and 0.025 mg.mL^{-1}, 2 and 0.5 mg.mL^{-1} and 10 mg.mL^{-1}, respectively. The microplate was left to incubate for 24 h in the conditions mentioned above. Briefly, culture medium was used as control, betamethasone at 20 μM was used as an anti-inflammatory standard and SRM 1648 Urban Particulate Matter obtained from the NIST (Gaithersburg, MD) was used as an inflammatory stimulus. Each sample was evaluated in duplicate, in two independent studies.

After 24 h, the supernatants of each well were collected in order to quantify the level of proinflammatory cytokine IL-6 using the ELISA MAX™ Deluxe Set Human IL-6 kit (Biolegend, CA, USA), according to manufacturer's instructions. In short, 100 μL of diluted Capture Antibody solution were added into each well and left to incubate overnight (2 to 8 ºC). The day after, the microplate was washed 4 times, 200 μL of 1X Assay Diluent A were added and it was left to incubate at room temperature for 1 h on a plate shaker. In this order, the microplate was washed 4 times, 100 μL of standard or supernatant were added, and it was left to incubate for 2 h at room temperature with shaking. Again, the microplate was washed 4 times and 100 μL of diluted Detection Antibody solution were added to each well and it was left to incubate for 1 h at room temperature with shaking. After 1h, the plate was washed 4 times, 100 μL of diluted Avidin-HRP were added and the microplate was incubated in the same conditions, this time for 30 min. Then and for the final time, the microplate was washed 5 times in 30 s to 1 min intervals. 100 μL of TMB Substrate Solution were added and the plate was then left incubating for 15 minutes, at room temperature, in the dark with no agitation. Lastly, 100 μL of Stop Solution was added and the absorbance was measured at the wavelength of 450 and 570 nm using a microplate reader (Microplate Reader Biotek Synergy H1, USA).

After the cells were lysed with 300 μL of deionised water, total protein was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific), as recommended by the manufacturer. Briefly, the assay kit requires the preparation of a working reagent that consists of the combination of reagent A with B (50:1, Reagent A, B). In this order, 25 μL of standard or supernatant and 200 μL of the working reagent were added to each microplate well. Posteriorly, the microplate was stirred for 30 sec, covered with aluminium foil and incubated at 37 ºC for 30 min. Finally, the absorbance was measured at the wavelength of 562 nm using a microplate reader (Microplate Reader Biotek Synergy H1, USA). The final results were presented in pg of cytokine.mg^{-1}of total protein.

11. Quantification of IL-1α by ELISA

HaCaT cells were seeded at 2.5 x 10^5 cells.mL^{-1} in 12 well (1 mL per well) microplates for 24 h. Thereafter the cells were exposed to GSE-Ov, encapsulated GSE-Ov or encapsulant in the following concentrations 0.1 and 0.025 mg.mL^{-1}, 2 and 0.5 mg.mL^{-1} and 10 mg.mL^{-1}, respectively. In the conditions mentioned above, the microplate was incubated for 24 h. The controls and the inflammatory stimulus were identical to the ones used in **section 10**. In two separate studies, each sample was evaluated in duplicate.

The supernatants from each well were collected after 24 h to determine the level of proinflammatory cytokine IL-1α. For that, ELISA MAX™ Deluxe Set Human IL-1α kit (Biolegend,

CA, USA), as recommended by the manufacturer. Concisely, 100 µL of diluted Capture Antibody solution were added into each well and left to incubate overnight (2 to 8 ºC). After 24 h, the microplate was washed 4 times, 200 µL of 1X Assay Diluent A were added and it was left to incubate at room temperature for 1 h on a plate shaker. In this order, the microplate was washed 4 times, 50 µL of Assay Buffer D was added to the standard and supernatant wells, 50 µL of standard or supernatant were added the wells, and the microplate was left to incubate for 2 h at room temperature with shaking. Again, the microplate was washed 4 times and 100 µL of diluted Detection Antibody solution were added to each well and it was left to incubate for 1 h at room temperature with shaking. Afterwards, the plate was washed 4 times, 100 µL of diluted Avidin-HRP were added and the microplate was incubated in the same conditions, this time for 30 min. Then and for the last time, the microplate was washed 5 times in 30 s to 1 min intervals. 100 µL of Substrate Solution F were added and the microplate was then left incubating for 15 minutes, at room temperature, in the dark with no agitation. Finally, 100 µL of Stop Solution were added and the absorbance was measured at the wavelength of 450 and 570 nm using a microplate reader. Cells were lysed with 300 µL of deionised water and the BCA method was used to quantify total protein, as previously described in **section 10 (Materials and Methods)**. The results were expressed in pg of cytokine.mg^{-1} of total protein.

12. Human pro-Collagen I α1 quantification

HDF cells were seeded at 3 x 10^5 cells.mL^{-1} in 12 well (1 mL per well) microplates for 24 h. After that the cells were exposed to GSE-Ov, encapsulated GSE-Ov or encapsulant in the following concentrations 0.1 and 0.025 mg.mL^{-1}, 2 and 0.5 mg.mL^{-1} and 10 mg.mL^{-1}, respectively. Culture medium was used as control. In two separate studies, each sample was evaluated in duplicate. Total protein quantification was measured as indicated in **section 10 (Materials and Methods)**. The quantification of collagen I α1 was performed using the Human Pro-Collagen 1 alpha 1 CatchPoint® SimpleStep ELISA® Kit (ABCAM, ab229389), as recommended by the manufacturer. The total protein used was 100 ng. Briefly in this order, 50 µL of sample or standard and 50 µL of the cocktail solution were added into the wells, followed by an incubation period of 1 h in a plate shaker (400 rpm), at room temperature. After, the wells were washed 3 times with the washing buffer PT and the microplate was firmly tapped into paper to remove excess liquid. Lastly, 100 µL of the prepared CatchPoint HRP Development Solution were added and the microplate was incubated for 10 min in the dark on a plate shaker (400 rpm). The fluorescence was then measured at Ex/Cutoff/Em 530/570/590 nm using a microplate reader. The final results were presented in pg of collagen.µg^{-1}of total protein.

13. Statistical analysis

The IBM® SPSS® Statistics 26 program was utilized for statistical analysis. The data was first checked for normality (Shapiro-Wilk test, n < 50, or Kolmogorov-Smirnov test, n > 50). To assess differences between more than two groups, a one-way ANOVA test (normal distribution) with Tukey's HSD post hoc test or a Kruskal-Wallis test (non-normal distribution) were used. A

student's t-test (normal distribution) or a Mann-Whitney test (non-normal distribution) was used to compare two groups. The significance level was set at 0.05.

Results and Discussion

1. Initial screening of GSEs potential and characterization

1.1. Antioxidant activity

The antioxidant activity of both GSEs was evaluated using ABTS and DPPH assays, and the obtained results are presented in **Table 4**. For both methods, the GSE-Ov presented higher antioxidant activity (i.e., lower IC_{50} – half-maximal inhibitory concentration – values). For this extract, the obtained values were 0.138 and 0.079 $mg.mL^{-1}$ for ABTS and DPPH, respectively, whereas for GSE-Sv the obtained values were 0.163 and 0.088 $mg.mL^{-1}$, respectively. Grape species, individual cultivars, cultivation conditions, maturation stage and seasonal fluctuations, according to **Mandić *et al*. (2009)**, may impact phenolic biosynthesis and antioxidant capacity of grape seeds, which can be a possible explanation for the obtained differences between GSEs. In fact, GSE-Ov presented a higher total phenolics content (258.96 $\pm$ 30.94 mg GA $eq.g^{-1}$ dry extract) than the GSE-Sv (203.72 $\pm$ 42.64 mg GA $eq.g^{-1}$ dry extract), which is in accordance with the antioxidant activity results. Comparing both methods, DPPH assay allowed to obtain higher antioxidant activity for both GSEs. This is due to the nature of the assays themselves, once the samples were prepared in methanol and therefore only in a methanol-like solvent (like the one used for DPPH) they can present proper antioxidant activity **(Duarte *et al*., 2023; Tournour, 2016)**. Besides that, GSEs were obtained with an organic solvent, hence the extracted compounds should be more soluble with this kind of solvents (e.g. methanol, ethanol, etc).

Comparing the obtained results with the antioxidant activity of two benchmarks, ascorbic acid and butylated hydroxytoluene (BHT), which present IC_{50} values within a 0.04 – 0.28 $mg.mL^{-1}$ range **(Duarte *et al*., 2023)**, we can conclude that grape seeds are a relevant source of antioxidant compounds since all the obtained values are within the indicated range. Impressively, the GSEs, which are a complex mixture of different compounds, were able to present similar antioxidant capacity to the tested benchmarks, which are pure compounds. Indeed, previous studies have reported the potential of natural plant-derived compounds, including grape, as antioxidant agents for cosmetic/skincare application **(Hoang *et al*., 2021; Michalak, 2022)**.

Table 4 – Total phenolics content and antioxidant activity values (mean $\pm$ SD) determined by Folin-Ciocalteau, ABTS and DPPH assays for both GSEs and for the two antioxidant benchmarks (ascorbic acid and butylated hydroxytoluene (BHT)).

Analysis	GSE-Ov	GSE-Sv	Ascorbic acid	BHT
Total phenolics (mg GA $eq.g^{-1}$ dry extract)	258.96 $\pm$ 30.94 [a]	203.72 $\pm$ 42.64	---	---
ABTS IC_{50} ($mg.mL^{-1}$)	0.138 $\pm$ 0.034 [a]	0.163 $\pm$ 0.015	0.05 *	0.13 *
DPPH IC_{50} ($mg.mL^{-1}$)	0.079 $\pm$ 0.003 [a, b]	0.088 $\pm$ 0.004 [b]	0.04 *	0.28 *

[a] significantly different from GSE-Sv ($p < 0.05$) within a given row; [b] significantly different from ABTS ($p < 0.05$) within a given column; * values retrieved from Duarte *et al*. (2023)

1.2. Antimicrobial activity

Both GSEs were also tested for their antimicrobial potential against skin microbiota commensal bacteria such as *S. epidermidis*, Methicillin Sensitive *S. aureus* (MSSA), Methicillin Resistant *S. aureus* (MRSA), and *C. acnes*. The extracts were tested at the following concentrations: 50, 25, 12.5, 6.25, 3.13, and 1.56 $mg.mL^{-1}$ for MBC determination. Minimum inhibitory concentrations (MICs) were not achieved since the extracts precipitated, making it impossible to assess the microbial growth-caused turbidity of the medium. Therefore, only the MBC results were evaluated and presented in **Table 5**. From the tested microorganisms, only *C. acnes* was not inhibited by any of the extracts at the tested concentrations. Moreover, *S. epidermidis*, commonly found on human skin as it is considered a normal part of the skin microbiota, was only inhibited by the highest tested concentration for both GSEs. However, this concentration is quite high and, therefore, this concentration is not considered for application. In fact, the non-inhibition of these bacteria can be seen as a positive outcome since, in balance, these bacteria are important for skin homeostasis maintenance, host defence, and innate immune response **(Fournière *et al*., 2020)**. Besides that, *S. epidermidis* is capable of inhibiting the adhesion of virulent *S. aureus* strains **(Christensen and Brüggemann, 2014; Fournière *et al*., 2020)**. This is relevant since, as discussed before, *S. aureus* has been associated with exacerbating skin diseases like atopic dermatitis. Having that said, the obtained results for *S. aureus* inhibition are quite promising because they may lead the way for a possible transfer of these samples' antimicrobial potential towards the treatment and/or prevention of such skin conditions. Comparing both extracts, the results were similar, except for MSSA, where GSE-Ov showed higher antimicrobial capacity.

Table 5 – GSEs antimicrobial activity against *Staphylococcus epidermidis*, Methicillin Sensitive *Staphylococcus aureus* (MSSA), Methicillin Resistant *Staphylococcus aures* (MRSA), and *Cutibacterium acnes*.

Microorganism	**GSE-Ov**	**GSE-Sv**
	MBC $mg.mL^{-1}$	
Staphylococcus epidermidis	50	50
Methicillin Sensitive *Staphylococcus aureus* (MSSA)	3.125	6.25
Methicillin Resistant *Staphylococcus aureus* (MRSA)	6.25	6.25
Cutibacterium acnes	> 50	> 50

1.3. Individual polyphenols identification by HPLC

As discussed earlier, phenolic compounds are secondary plant metabolites (e.g., derived from grapevine) which exhibit several biological properties of relevance, such as antimicrobial, antioxidant and anti-inflammatory activities, and therefore, have great potential for cosmetic applications. In this line, both GSEs were analysed by HPLC for individual polyphenols identification. In total, four different compounds were identified from the chromatograms (**Figure 3**).

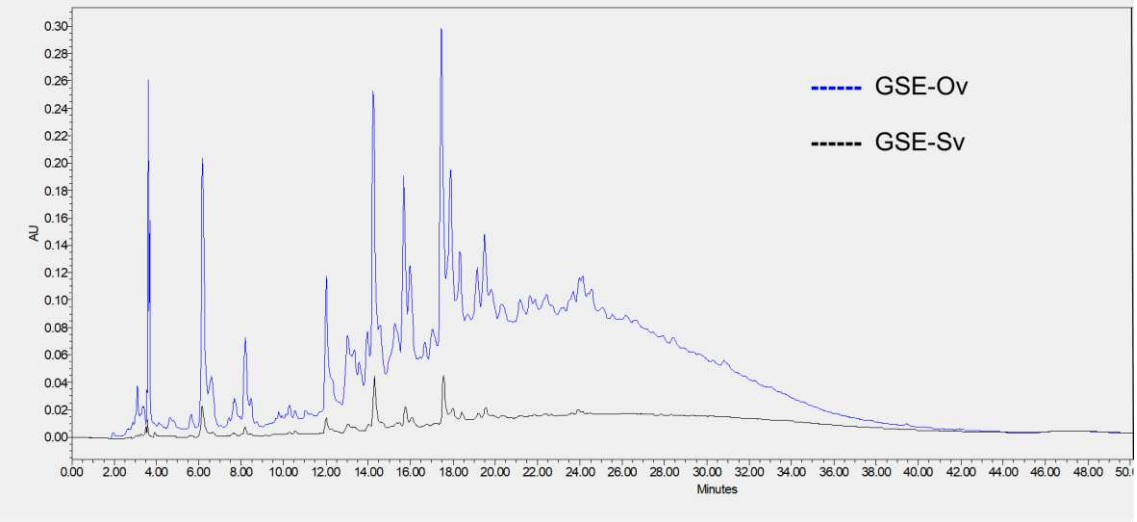

Figure 3 - Chromatogram of the HPLC analysis of the GSEs and respective identified polyphenols.

The identified polyphenols were gallic acid, catechin, procyanidin B1, and procyanidin B2. The latter was only found in GSE-Sv, and the results are presented in **Table 6**. All the identified polyphenols are normally found in grape seed extracts (e.g., Pinot Noir, Cabernet Sauvignon, Marselan, Tamyanka) **(Krasteva *et al*., 2023)**. Regarding the first three identified polyphenols, all were found in higher quantities in GSE-Ov, as it is possible to see by the peak area (**Table 6**). This way, and despite the procyanidin B2 case, these findings are consistent with the antioxidant activity results, where GSE-Ov showed higher total phenolics content and higher radical scavenging activity. The area results are comparable since both extracts were analysed at the same concentration. Besides their known antioxidant potential, these compounds are also reported to be beneficial for skincare applications. For example, gallic acid has been suggested as a candidate for the prevention of UVB-induced premature skin aging, by decreasing skin dryness, thickness, and wrinkle formation. Also, IL-6 production was supressed (anti-inflammation) and type-I procollagen was stimulated **(Hwang *et al*., 2014)**. Another study showed that gallic acid inhibited melanin production by down-regulation of tyrosinase activity, being capable of acting as a skin whitening agent **(Kumar *et al*., 2013)**. Furthermore, (+)-catechin has been found to inhibit the inflammatory mediator prostaglandin E_2, tumor necrosis factor-alpha (TNF-α), and proinflammatory cytokines IL-1β and IL-6 **(Monga *et al*., 2014)**. Regarding procyanidins, procyanidin B2 anti-inflammatory activity has also been reported, through the inhibition of NF-kB, IL-6 and pro-IL-1 β **(Martinez-Micaelo *et al*., 2014)**. Lastly, both procyanidins (B1 and B2) have shown moderate inhibitory effect over collagenase and elastase activities,

although in a dose-dependent manner **(Wittenauer *et al*., 2015)**. By analyzing the chromatogram, corroborating the activities described above, it can be concluded that there is a more pronounced profile of GSE-Ov polyphenols and therefore a greater biological activity.

Table 6 – Identified polyphenols in both GSEs; A - area.

Polyphenol	Retention Time (min)	GSE-Ov (A1)	GSE-Sv (A2)	A1 / A2
Gallic acid	6.422	2130502	52335	40.7
Catechin	14.274	2613543	331686	7.9
Procyanidin B1	13.193	951038	26698	35.6
Procyanidin B2	17.569	---	386677	---

1.4. Fourier Transform Infrared analysis

Fourier Transform Infrared (FTIR) spectroscopy has advanced significantly in the last decade, offering a faster and more objective way to investigate the interactions between matter and infrared radiation, resulting in a spectrum. Each substance has a unique spectrum fingerprint that differentiates it from other compounds **(Fadlelmoula *et al*., 2022)**. The results of GSEs FTIR analysis are presented in **Figure 4**.

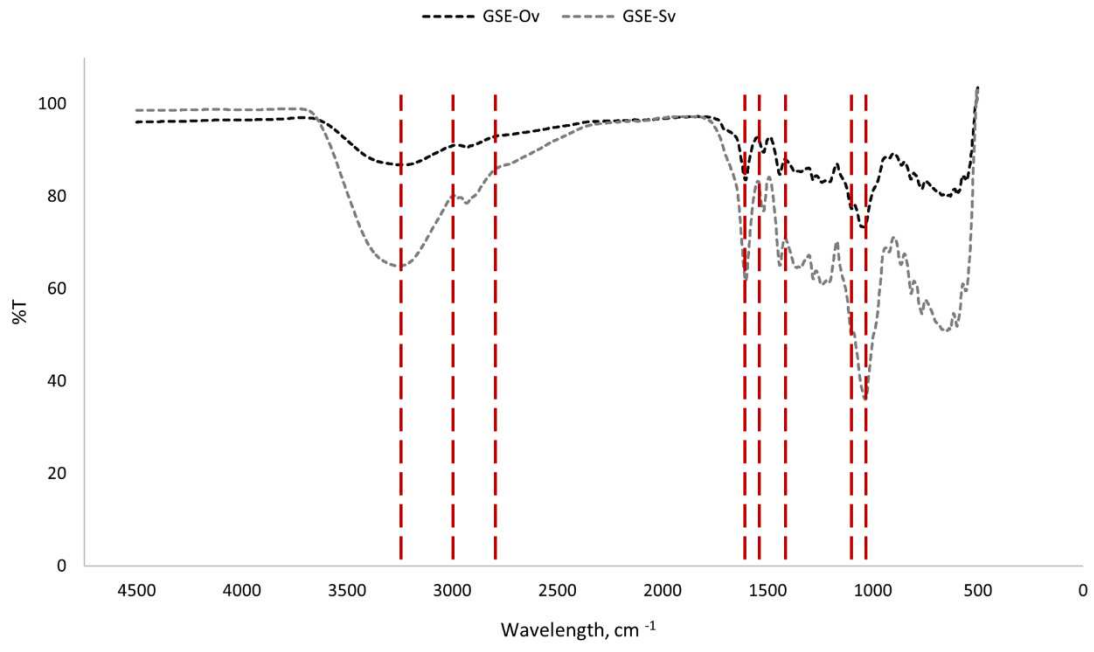

Figure 4 - FTIR spectra of the GSEs.

Analysing the mentioned figure, the first conclusion we can take is that both GSE have identical spectra, and, therefore, the main functional groups. So, from the spectra it was possible to distinguish several peaks/bands which represent functional groups and modes of vibration, and also the components to which they are related to. The broad band peaking at approximately 3250 cm^{-1} represents a stretching O-H bond, which may be due to the presence of polysaccharides

and lignins. The zone between around 2800 and 3000 cm^{-1}, which seems to be composed of two unresolved peaks, corresponds to asymmetric and symmetric stretching bonds of CH_2 groups, derived from lignins and lipids. The peak around 1600 cm^{-1} is relative to COO^- groups and aromatic C=C bonds, found in pectins and phenolics. The two peaks around 1500 cm^{-1} correspond to aromatic stretching C-C bonds from phenolic compounds. The peak around 1150 cm^{-1} is due to aromatic stretching C-H bonds from phenolics, and around 1000 cm^{-1} there is a peak relative to stretching C-O and C-C bonds from polysaccharides and pectins. This spectra analysis was made according to **Gunter and Popeyko (2022)**, **Nogales-Bueno *et al*. (2017)**, and **Lucarini *et al*. (2019)**, and also demonstrated that the extraction process was successful and that the main functional groups of both extracts were preserved.

2. GSE-Ov encapsulation – Liposomes

As discussed before, advanced delivery systems, at the nano and/or micro scales, offer several advantages for the administration of specific compounds. These personalized systems protect the encapsulated compound, preventing its degradation, and can transport and release it more effectively on the targeted layers of the skin. Herein, liposomes are the chosen system due to their lipid character and to the perfect interaction with the skin and its ceramides, which are one of the main lipids of the skin and play a vital role in its barrier function **(Vovesná *et al*., 2021)**. For these reasons, we aimed to develop a liposome capable of efficiently entrapping and deliver the GSE-Ov to the skin, since it was the one that demonstrated the best polyphenol profile and biological properties, as mentioned above. The optimization of the liposomes production process and the physicochemical characterization of the final formulation will be discussed in the following sections.

2.1. Optimization of the encapsulation process

To optimize the liposome production process, several tests were carried out considering the Zeta potential (ζ) and Polydispersity Index (PDI) as fundamental parameters for the development of the system (**Table 7**). In relation to these two measurements, ζ is better the higher its absolute value, which represents a greater charge, and therefore a greater repulsion between the particles and consequent stability over time. A lower PDI value represents greater liposome size homogeneity. The lower the PDI, the lower the index of particle subpopulations **(Mudalige *et al*., 2019; Samimi *et al*., 2019)**. For this purpose, two different liposome production methods were tested: (I) Lipid Film Hydration (LFH) and (II) Reverse Phase Evaporation (RPE). LFH allowed better ζ and PDI results and, therefore, was the method chosen to carry out the investigation. In fact, this method (LFH) is often preferred in laboratory settings as it is simple and does not require specialized equipment **(Machado *et al*., 2014)**.

Once chosen the method, the attempt III consisted of adding the extract to the liposomes, which resulted in a relatively unstable and prone to agglomerate formation mixture (decreased ζ value). In order to solve this issue, for attempt IV chitosan was added as a liposome coat **(Kumar *et al*., 2020; Lee *et al*., 2019)**, leading to the intended increase of ζ value from -5.1 to -9.3 mV, although

not that significant. However, the PDI value increased from 0.405 to 1.000, which was not intended. Moreover, chitosan precipitated which was also not desired. So, for attempt V, chitosan was replaced by pectin **(Kumar *et al*., 2020)** and, indeed, the ζ improved significantly to -20.3 mV and the uniformity index (similar to PDI) was 0.637. Pectin was the biopolymer chosen, as it is found in grape pomace **(Spinei and Oroian, 2023)**, and in future studies, the pectin itself can be extracted and used from the same pomace. No sonication step was needed because the measurement equipment already provided ultrasounds of its own. In this line, the final formulation consisted of LFH method for GSE-Ov-loaded liposomes coated with pectin.

Table 7 – Optimization of liposomes production until the final formulation. Values of ζ and PDI / Uniformity (mean ± SD) for each attempt.

Attempt #	Process description	ζ (mV)	PDI / Uniformity
I	LFH + 1 min sonication (70% amplitude)	- 29.7 ± 2.9	0.407 ± 0.008
II	RPE (Reverse Phase Evaporation) method	- 14.4 ± 1.3	0.432 ± 0.031
III	Attempt #I + GSE-Ov	- 5.1 ± 1.1	0.405 ± 0.014
IV	Attempt #III + chitosan	- 9.3 ± 0.7	1.000 ± 0.000
V (final)	LFH + GSE-Ov + pectin	- 20.3 ± 2.4	0.637 *

***** Uniformity was measured instead of PDI.

2.2. Final Liposome characterization

2.2.1. Zeta Potential, size distribution and morphology

After the optimization of the production process, the final liposomes were characterized concerning some features. The first evaluated feature was zeta potential (ζ), and the obtained value was -20.3 mV. The results allowed us to conclude that the developed system is stable, with no tendency to agglomerate over time, since its charge demonstrated to be close to that described in the literature (ζ ~ ± 30 mV) as ideal for long-term stability **(Németh *et al*., 2022)**. The negative electrical surface charge is due to the presence of lecithin **(Assis *et al*., 2014; Machado *et al*., 2019)**, and pectin was used to increase the charge stability.

Another assessed characteristic was the size distribution. This variable is of major importance since it directly impacts the liposomes cellular uptake, transportation, and accumulation behaviour **(Choi *et al*., 2023)**. The results are presented in **Figure 5**. Liposomes had a mean diameter of 13.6 μm, which meats the micro-scale range as desired. This average size found for liposomes tend to be used for surface applications such as in skin **(INdermal, 2022)**. In fact, micro-scaled liposomes have some advantages such as higher encapsulation efficiency with size **(Nsairat *et al*., 2022)**, namely relative to large biomolecules, and the fact that it allows to avoid nano-bioaccumulation and nano-related legislation issues (labelling, etc.) **(Castro *et al.,* 2023)**. Furthermore, a uniformity size value (similar to PDI) of 0.637 was obtained, which indicates some heterogeneity of the liposomes based on their size, yet acceptable **(Mudalige *et al*., 2019)**. However, this value reveals a rather narrow size range. Since PDI is close to 0.5, it indicates a

prevalence of size or a majority population, in this case, close to 15 μm. Higher size values may be associated with possible agglomerates in the process liposomes development.

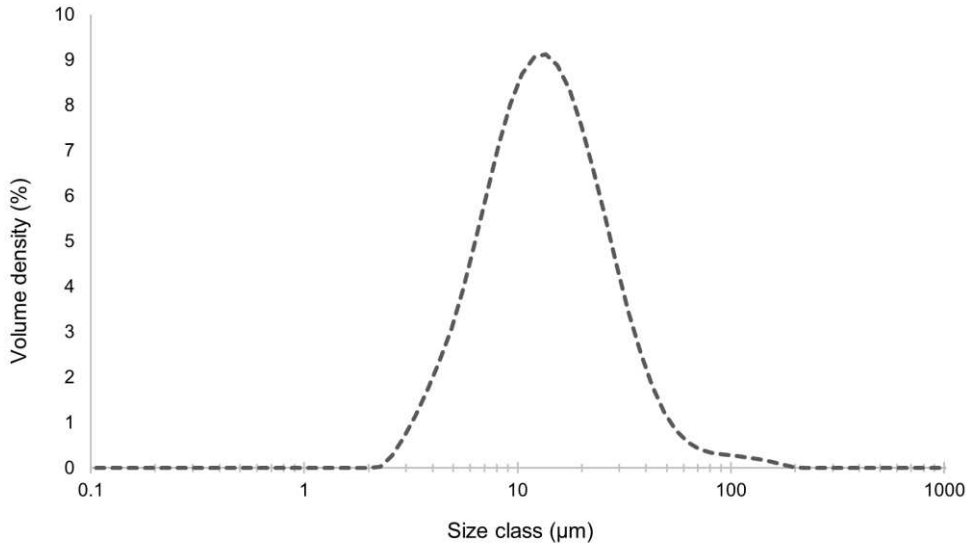

Figure 5 - Size distribution of GSE-Ov-loaded liposomes.

For liposome morphology, a scanning electron microscopy (SEM) analysis was performed, and the images are presented in **Figure 6**. In all pictures, somewhat spherical-shaped and regular liposomes can be seen, in addition to some agglomerates. Also, some size heterogeneity between liposomes can be observed, associated to liposome process development. In fact, the agglomerates seem to be formed out of larger liposomes. These findings are consistent with the above-mentioned zeta potential and uniformity values. Also, it has been reported that sample preparation for SEM analysis may be also responsible for the formation of some agglomerates

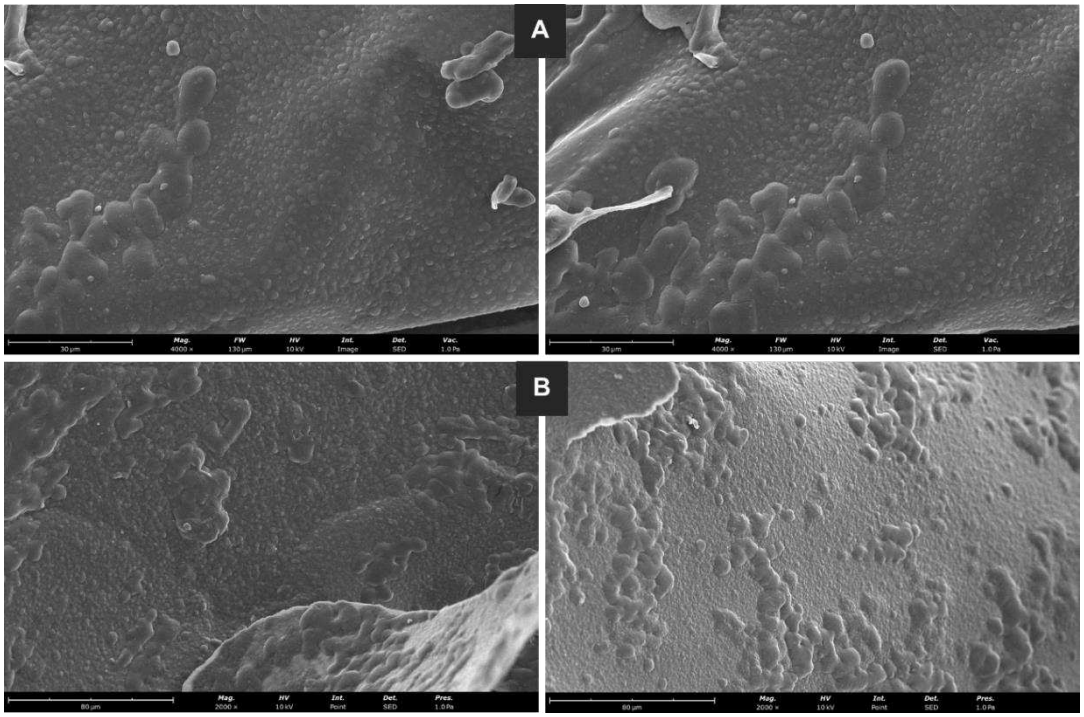

Figure 6 - SEM images of powdered (A; magnification of 4000 x) and splintered (B; magnification of 2000 x) lyophilized GSE-Ov loaded liposomes.

(Baldino and Reverchon, 2023). The results were similar for powdered (**Figure 6A**) and splintered (**Figure 6B**) lyophilized GSE-Ov-loaded liposomes.

2.2.2. FTIR analysis

A FTIR analysis was performed for empty and GSE-Ov-loaded liposomes in addition to the one made for both GSEs. The resultant spectra are presented in **Figure 7**.

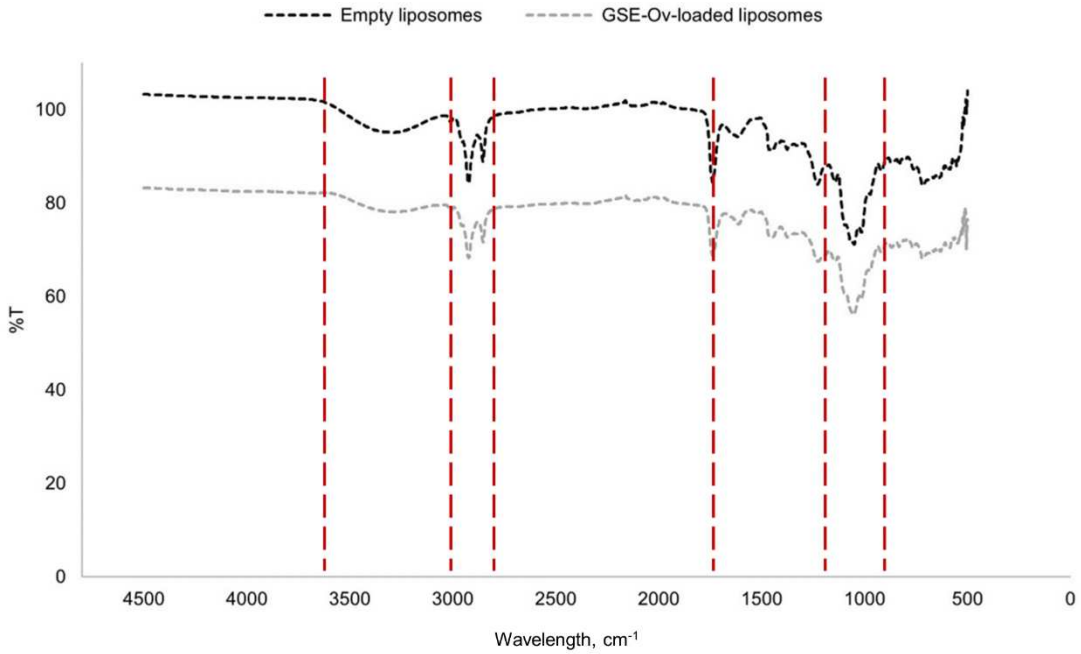

Figure 7 - FTIR spectra of empty and GSE-OV-loaded liposomes.

Analyzing the spectra, the first conclusion that can be made is that both empty and loaded liposomes presented a similar fingerprint, and, therefore, the analysis will be made for both. Importantly, the lack of shifts in the identified bands reveals that the encapsulation process neither alters the GSE structure nor promotes new bonds formation. Since the main component of the liposomes is soy lecithin, there is a group bands/peaks that are characteristic of this substance. For instance, the region between 2800 and 3000 cm^{-1}, composed of two peaks, corresponds to asymmetric and symmetric stretching bonds of CH_2 groups. The peak around 1750 cm^{-1} corresponds to stretching C=O bonds from aliphatic ester groups. The peaks in the zone approximately between 950 and 1200 cm^{-1} refer to stretching vibrations of P=O, P-O-C and C-O-C groups. Besides lecithin, pectin was used for liposome-coating, being responsible for the broad band approximately between 3000 and 3600 cm^{-1}, which corresponds to stretching O-H groups from intra- and intermolecular hydrogen bonds related to galacturonic acid. This spectra analysis was made according to **Merino *et al*. (2016)**, **Santos *et al*. (2020)**, **Whittinghill *et al*. (2020)**, and **Pezeshky *et al*. (2015)**.

2.3. Encapsulation efficiency and polyphenol release profile

The final developed liposomes presented an encapsulation efficiency of 88.8%, which, in comparison with the literature (ranging from 52 to 91%, reviewed in **section 5.1. (Introduction)**), is considered a very promising result. Besides encapsulation efficiency, the polyphenols release profile by the liposomes was also assessed, and the results are presented in **Table 8**. The assay was carried out for 24 h and the maximum percentage of released polyphenols was reached at $T_{24\ h}$, with 59.4% being released. Within the first 6 h, the polyphenols release profile was nearly linear, with almost 50% of the content being discharged at this time, allowing to conclude that from then forward the polyphenols release begins to stabilize. Interestingly, a study by **Lu *et al.* (2011)** reported similar values of release profile of polyphenols from GSE, at this time. Moreover, in another study, **Gibis *et al.* (2016)** obtained 55.5% of release within 24 h, which also resembles the presented result.

Table 8 – Polyphenols release profile by the liposomes.

Time stamps	Percentage of released GSE (mean ± SD)
$T_{1\ h}$	12.67 ± 0.46%
$T_{2\ h}$	24.95 ± 0.69%
$T_{4\ h}$	37.84 ± 0.40%
$T_{6\ h}$	48.18 ± 0.75%
$T_{24\ h}$	59.42 ± 0.41%

Hereupon, It can be concluded that the systems demonstrated excellent encapsulation efficiency, close to 90%, and they revealed a prolonged release over time. However, in this *in vitro* release study, realistic parameters, such as topical placement, mechanical abrasion, and/or enzymatic action were not considered **(Chen *et al*., 2021; Jeong *et al*., 2021)**. In this way, it is expected that *in vivo* release will be much higher and faster in time. However, so far as allowing us to simulate or estimate, the results appear promising for a topical compound delivery system.

3. Safety and skincare potential of encapsulated GSE-Ov

After physicochemical characterization, the GSE-Ov, GSE-Ov-loaded liposomes, and empty liposomes were tested for their skincare potential, including a safety assay and the assessment of their anti-inflammatory activity and capacity for pro-collagen I α1 production stimulation.

3.1. Cytotoxicity

Regarding their safety, these samples were tested on HaCaT and HDF cell lines, and the results are presented in **Figure 8**. GSE-Ov was safe for concentrations below ~0.15 $mg.mL^{-1}$ for HaCaT and HDF cell lines cell lines, reaching metabolic inhibitions of 27.86 and 32.49%, respectively. Concerning GSE-Ov-loaded liposomes, they showed to be safe approximately up to

concentrations of 5 mg.mL^{-1} for HaCaT and 2.5 mg.mL^{-1} for HDF cells, with metabolic inhibitions of 25.36 and 29.75%, respectively. According to this, liposomal content seems to be released in a safe way, highlighting the importance of the GSE-Ov encapsulation, since the extract alone showed to be significantly more cytotoxic. Even so, the antioxidant results are compatible with these, since the obtained IC_{50} values are within the safe range for both cells. Finally, for both cell lines, empty liposomes showed to be non-cytotoxic in the maximum tested concentration (10 mg.mL^{-1}), with metabolic inhibitions of -29.43 and 11.64% for HaCaT and HDF, respectively. In all cases, HDF cells showed to be more sensitive to the tested extracts. Similar results had been reported by **Duarte *et al*. (2023)**. In addition, negative inhibition values may have resulted from an increase in cellular proliferation **(Teixeira *et al*., 2021)**.

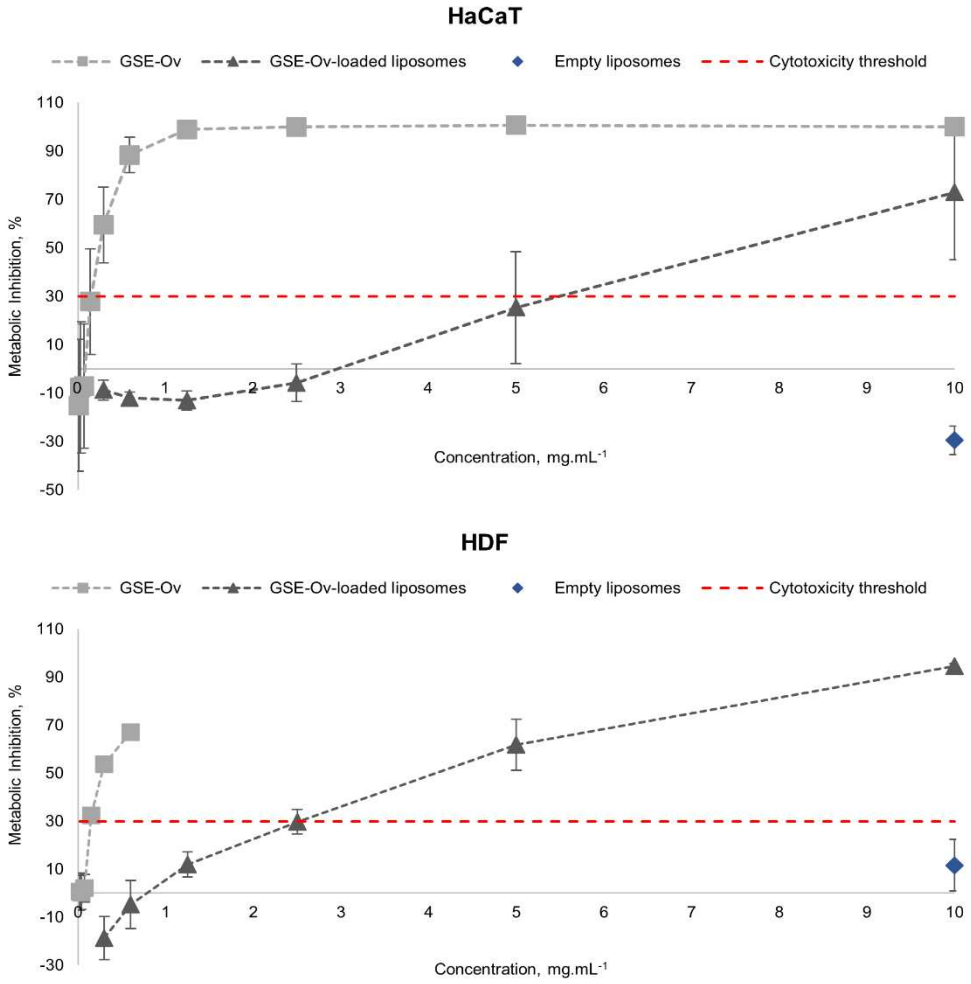

Figure 8 - Cytotoxicity study of GSE-Ov, GSE-Ov-loaded liposomes, and empty liposomes on HaCaT and HDF cell lines.

3.2. Anti-inflammatory activity

As discussed before, inflammation plays a major role in some skin diseases, and, therefore, it is important to understand if the extracts have anti-inflammatory potential. For this purpose, the effect of GSE-Ov, GSE-Ov-loaded liposomes, and empty liposomes on the production of two pro-

inflammatory cytokines (IL-1α and IL-6) by keratinocytes are presented in **Figure 9**. All samples were tested accordingly to their safe concentrations range.

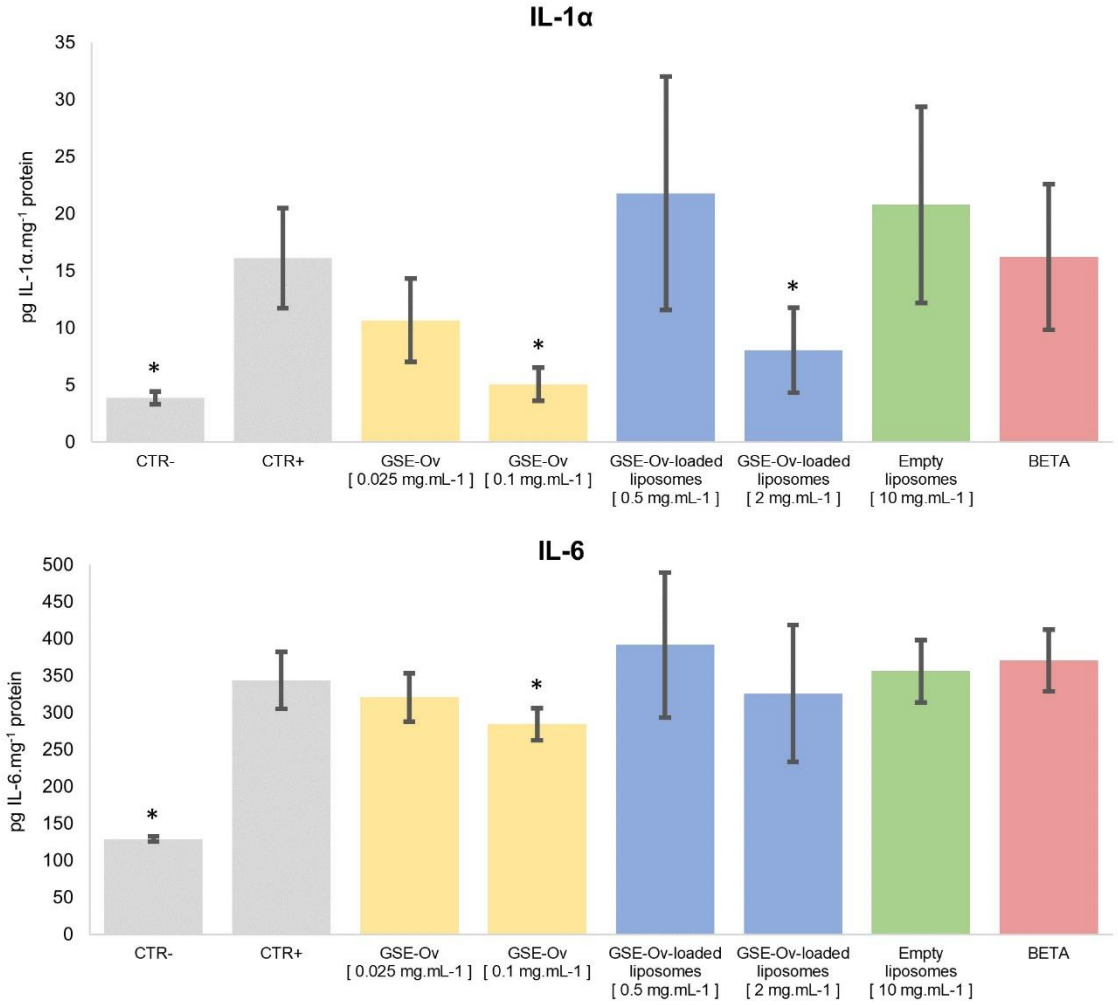

Figure 9 - Effect of GSE-Ov, GSE-Ov-loaded liposomes, empty liposomes, and betamethasone (BETA: anti-inflammatory standard) on keratinocytes (HaCaT) by assessing pro-inflammatory cytokines IL-1α and IL-6 levels under an inflammatory stimulus (pollution particles); Mean values (solid bars) are expressed as pg cytokine.mg^{-1} protein, and standard deviation is represented by bars; * significantly different from positive control (CTR+) ($p < 0.05$).

Regarding IL-1α, its overexpression has been associated to symptom exacerbation and disease progression in psoriasis, atopic dermatitis, and neutrophilic dermatoses (e.g., hidradenitis supurrativa). Nonetheless, it has important functions in the skin. For example, in psoriasis and atopic dermatitis lesions, keratinocytes extensively produce IL-1α, which has been suggested as a skin disease biomarker, also being capable of helping to maintain skin integrity and barrier **(Cavalli *et al.*, 2021; Galozzi *et al.*, 2021; Iznardo and Puig, 2022)**. Concerning the anti-inflammatory potential of the tested samples, the GSE-Ov was able to reduce IL-1α production for both concentrations tested, albeit in a concentration-dependent manner. In the case of 0.1 mg.mL^{-1} GSE-Ov, the amount of cytokine decreased from 16.1 to 5.1 pg IL-1α.mg^{-1} of protein ($p < 0.05$). For GSE-Ov-loaded liposomes, only the most concentrated samples (2 mg.mL^{-1}) was able to decrease the amount of cytokine (from 16.1 to 8.1 pg IL-1α.mg^{-1} of protein, $p < 0.05$). Such

effect could be due to the encapsulation of the GSE, leading to its higher protection, and therefore decreased immediate availability. For potentially better results, this study should be conducted over time. Finally, the empty liposomes did not exert any anti-inflammatory activity over IL-1α production.

On the other hand, IL-6 is produced by many different types of cells and is expressed under many states of cellular stress, including inflammation, infection, wound sites, and cancer **(Choy and Rose-John, 2017)**. In this case, none of the tested samples presented significant anti-inflammatory activity over IL-6 production, although a slight reduction in cytokine amount was observed for the GSE-Ov at 0.1 $mg.mL^{-1}$ ($p < 0.05$). Interestingly, a study by **Nallathambi *et al*. (2020)** reported that GSE was able to reduce IL-1α, and IL-6 expression. However, it was carried out for intestinal cellular models, and for IL-6, data showed a smaller expression than that found in our essay. Perhaps, in our work, the tested GSE-Ov concentration was not enough to induce such effects. Similarly to the GSE-Ov composition, catechin and procyanidin B1 were also detected in the mentioned extract. In this sense, more replicates should be done to better understand the effect on interleukins production. It is relevant to refer that betamethasone, as the anti-inflammatory standard, did not exhibited the expected activity.

3.3. Human pro-Collagen I α1 production

Collagen is the most abundant component of the extracellular matrix (ECM) and the skin's principal structural protein **(Alves *et al*., 2017; Sibilla *et al*., 2015)**. It is responsible for structure, stability, and strength, particularly within the dermal layers **(Kwatra, 2020)**, and it plays an important role in preventing skin aging. In fact, decreasing collagen density has been linked to the process of skin aging, particularly when related to sun exposure (photoaging), leading the skin to lose its integrity and elasticity **(Jhawar *et al*., 2020; Kwatra, 2020)**. For these reasons, the effect of GSE-Ov, GSE-Ov-loaded liposomes, and empty liposomes on Human pro-Collagen I α1 production by HDF was assessed and the results are presented in **Figure 10**. Empty liposomes, at 10 $mg.mL^{-1}$, inhibited collagen production, reducing from 773.32 to 191.46 pg $collagen.ug^{-1}$ protein ($p < 0.05$). Recalling the cytotoxicity study, this sample presented some metabolic inhibition, and, therefore, it may have stressed the HDF cells, leading to the misproduction of collagen. Interestingly, lecithin, the main component of liposomes formulation, has been reported to exert negative effects on collagen, like decreased accumulation (by stimulating collagenase activity) and gene expression **(Akit *et al*., 2016; Li *et al*., 1992)**. These findings could be an important landmark for future development and improvement of the GSE-Ov encapsulation process and to select the right concentration of this material for delivery system development. Moreover, GSE-Ov-loaded liposomes, at both concentrations, also inhibited some collagen with the most concentrated sample exerting a higher inhibition, which, as it is a concentration close to the limit of HDF viability (i.e. 2.5 $mg.mL^{-1}$), can follow the same compromise rationale of collagen misproduction. Concerning the GSE-Ov, at 0.025 $mg.mL^{-1}$, it stimulated the average production of collagen by 5.13%, although with no significant differences regarding the control ($p > 0.05$). This would be somehow expected, since there was no stress caused by this

concentration in HDF cells. In fact, GSE has been reported to promote collagen fiber deposition, strengthen collagen-based tissues, and to increase its synthesis **(Gupta *et al.*, 2020)**.

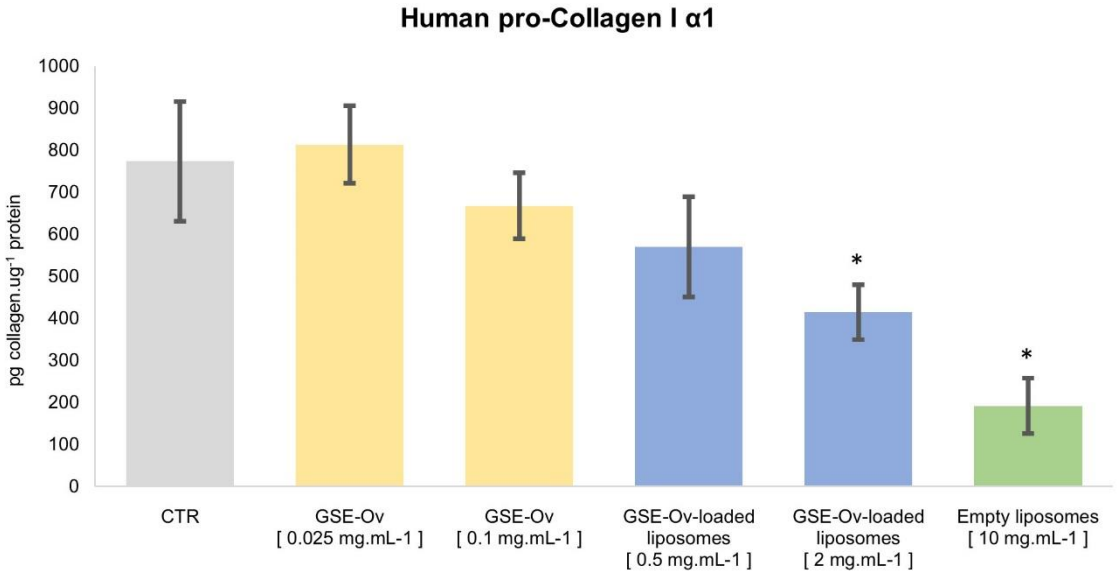

Figure 10 - Effect of GSE-OV, GSE-Ov-loaded liposomes, and empty liposomes on the production of collagen I α1 by HDF cells; Mean values (solid bars) are expressed as pg collagen.μg^{-1} protein, and standard deviation is represented by bars; * significantly different from control (CTR) ($p < 0.05$).

Conclusions

As previously mentioned, the primary goal of this thesis was the production, development and characterization of a GSE for potential skincare and cosmetic application. For that, two different GSEs were studied. It was possible to observe, through their antioxidant and antimicrobial activity, that the more promising extract was the GSE-Ov. The antioxidant result showed a huge radical scavenging potential, while the inhibition of *S. aureus* strains (MRSA and MSSA) together with the non-inhibition of *S. epidermidis* could indicate beneficial effects on several skin pathologies. Furthermore, we aimed to use an encapsulation technique as a way to potentiate the rather unstable extract delivery. Therefore, liposomes were used as delivery systems and several attempts were made to achieve the most stable and size appropriate delivery system. Amongst all attempts, soy lecithin liposome coated with pectin was the most suitable formulation. From this, it was concluded that the final liposome formulation was successful, presenting a very high encapsulation efficiency of 88.8%, and a controlled polyphenol release profile over time.

Regarding skin safety, GSE-Ov-loaded liposomes showed to be safe below 5 and 2.5 $mg.mL^{-1}$ for HaCaT and HDF, from which it can be concluded that the sample could be used as an antioxidant ingredient since the DPPH IC_{50} falls within the safe range. Furthermore, the extract exerted some potentialities as a skincare ingredient as it downregulated IL-1α production. Nevertheless, not all results were as intended, since IL-6 and pro-collagen I α1 production did not suffer significant variations.

Finally, it is believed that this new insight into GSE in skincare holds great potential since the GSE-Ov was successfully obtained and encapsulated and exercised antioxidant and anti-inflammatory properties. Besides that, the use of liposomes allowed for a safer, more controlled and more stable delivery of extract. Concluding, the aim of the work was achieved with success, and this dissertation also opens up an avenue of other possible applications considering these extracts and delivery systems.

Future work

Although very promising, the encapsulation process could undertake some improvements namely in the size uniformity, to improve stability over time and in the final product. In this sense, further testing could be done regarding the release profile and shelf life of the GSE-OV-loaded liposomes, e.g. stability test with a duration of 15 to 30 days. Afterwards, the hydration potential could be evaluated to verify if the encapsulated GSE has that potential. Also, the release *ex vivo* tests is vital since any *in vitro* tests lack several influencing factors, such as skin enzymes, and mechanical abrasion of application.

That being said, once passed the development stage of the liposome, the next step would be the incorporation of the GSE-OV-loaded liposomes in a cosmetic formulation, whether it is a cream, an exfoliant or a serum. A new range of tests of the new formulation should be conducted (e.g., cytotoxicity, anti-inflammation, collagen production, antimicrobial) to assure the safety and effectiveness of the product before being tested *in vivo*. The presence of colour in the GSE could pose a challenge for future commercialization in the skin cosmetics industry due to costumers' preference, hence an intermediate step of colour removal could be explored.

Following the product development step, an economic evaluation of the cost of production should be undertaken to see whether the end product is worth producing in comparison to other benchmark costs. The performance should also be weighed against other benchmarks when deciding whether to scale up.

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