Universidade de Lisboa

Faculdade de Farmácia

DEVELOPMENT OF FACIAL SERUM FOR BEARD GROWTH USING PHYTOSOMES WITH *ALLIUM CEPA* SKIN EXTRACT

Iolanda dos Ramos Hilário Tomás

Dissertação orientada pela Doutora Sandra Simões, Investigadora Principal com Agregação e coorientado pela Doutora Manuela Carvalheiro, Investigadora Auxiliar.

Mestrado em Cosmetologia Avançada

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Abstract

The cosmetics industry has traditionally focused on female-oriented formulations, often overlooking the structural and physiological characteristics of male skin and challenges associated with beard grooming. The growing interest in natural ingredients has driven research into plant-based bioactives, such as onion extracts (*Allium cepa*), rich in phenolic compounds like quercetin and fisetin, which possess antioxidant and hair growth-promoting properties.

In this study, onion skins, an agri-food by-product, were evaluated as a source of bioactive extracts for use in a beard growth serum. Red onion skin extract (ROSE) was selected due to its high flavonoid content and antioxidant activity. It was shown to be well-tolerated in keratinocyte cytotoxicity assays.

To enhance stability and delivery, ROSE was incorporated into phytosome nanocarriers. Lecithin-based phytosomes demonstrated high encapsulation efficiency and favourable physicochemical properties, outperforming cholesterol-based systems. These phytosomes were used to develop a novel facial serum for beard growth. The optimized formulation, containing ROSE, lecithin, caffeine, and arginine exhibited stable nanoscale vesicles, appropriate rheology, good spreadability, and minimal occlusion. *In vitro* release and permeation studies indicated sustained delivery of fisetin. A sensory evaluation test conducted in voluntary males confirmed the good acceptance of the developed product, intended to be brought into contact with the skin.

Overall, these results highlight the potential of red onion skin extract, a sustainable by-product source, in combination with phytosome nanocarriers, to create beard growth serum with promising antioxidant and hair-growth–related bioactivities.

Keywords: Cosmetics; Nanotechnology; Beard growth; *Allium cepa* extract; Phytosomes

Resumo

Ao longo do tempo, a indústria cosmética tem priorizado formulações direcionadas ao público feminino, ignorando as particularidades estruturais e fisiológicas da pele masculina, bem como os desafios específicos associados à barba. O interesse crescente por ingredientes naturais tem impulsionado a investigação de bioativos vegetais, como os extratos de cebola (*Allium cepa*), ricos em compostos fenólicos como quercetina e fisetina, conhecidos pelas suas propriedades antioxidantes e potenciadoras do crescimento capilar.

Neste estudo, foram avaliadas cascas de cebola, um subproduto agroalimentar, como fonte de extratos bioativos para incorporação num sérum destinado a estimular o crescimento da barba. Foi escolhido o extrato obtido a partir de cascas de cebola roxa (ROSE) pelo seu elevado teor de flavonoides e pela sua atividade antioxidante. Este extrato apresentou boa tolerância em ensaios de citotoxicidade em queratinócitos.

De forma a otimizar a estabilidade e entrega dos compostos ativos, o extrato foi incorporado em fitossomas. Os fitossomas à base de lecitina mostraram elevada eficiência de encapsulação e características físico-químicas apropriadas, tendo sido escolhidos para a formulação do sérum. A formulação otimizada, composta por extrato, lecitina, cafeína e arginina, apresentou vesículas estáveis em escala nanométrica, propriedades reológicas adequadas, boa espalhabilidade e efeito oclusivo mínimo. Estudos *in vitro* confirmaram a cedência e permeação sustentada da fisetina. O teste de avaliação sensorial realizado em voluntários do sexo masculino confirmou a boa aceitação do produto desenvolvido, destinado ao contato com a pele.

Estes resultados evidenciam o potencial do extrato de casca de cebola roxa, um subproduto sustentável, em combinação com fitossomas, para a formulação de um sérum facial com promissoras propriedades antioxidantes e de estimulação do crescimento da barba.

Palavras-chave: Cosméticos; Nanotecnologia; Crescimento de barba; Extrato de cebola; Fitossomas

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List of Abbreviations

ADME/T: Absorption, distribution, metabolism, excretion, and toxicity of a compound

DHT: Dihydrotestosterone

DLS: Dynamic light scattering

DNA: Deoxyribonucleic acid

EE: Encapsulation efficiency

ELS: Electrophoretic light scattering

F: Occlusion factor

GA: Gallic acid

GAE: Galic acid equivalents

HPLC: High-performance liquid chromatography

IC50: Inhibitory concentration

IVPT: *In vitro* permeation test

IVRT: *In vitro* release test

NO: Nitric oxide

NP: Nanoparticles

PBS: Phosphate-buffered saline

PDI: Polydispersity index

ROS: Reactive oxygen species

ROSE: Red onion skin extract

SC: Stratum corneum

TE: Trolox equivalents

TEWL: Transepidermal water loss

TPC: Total phenolic content

UV: Ultra-violet radiation

UVA: Ultra-violet radiation A

UVB: Ultra-violet radiation B

1. Introduction

1.1 Male skincare: historical trends and current needs

Throughout history, the cosmetics industry has predominantly focused on women, emphasizing beauty enhancement, health maintenance, and anti-ageing, while neglecting male skincare needs. However, with the rise of online visibility through social media and videoconferencing, especially during and after the COVID-19 pandemic, men have become more conscious of their appearance. Despite this growing awareness, many still avoid skincare due to its perceived feminization (1,2). Male and female skin differ in key aspects, yet most products are not designed with these differences in mind (3–9).

Studies indicate that male skin is in a more chronically inflamed state, exacerbated by infrequent skincare use and grooming practices, highlighting the urgent need for targeted products that address men's specific dermatological concerns (1,10). Hair thinning or growth concerns, which are common among men, are often treated with prescription medications such as finasteride, corticoids and minoxidil (11–13). Although these options can be effective, they may not be suitable for all individuals due to potential side effects and accessibility constraints. Currently, the cosmetic market still lacks safe and effective alternatives designed specifically to promote hair growth, particularly in areas such as the beard, despite the rising demand for easily accessible cosmetic alternatives. This need highlights the importance of exploring new bioactive ingredients capable of addressing hair growth in a safer and sustainable way.

1.2 Key structural differences between male and female skin

Male and female skin exhibit a fundamentally similar structure and function, with comparable biochemical pathways, cellular mechanisms, and sensory responses (10). Certain structural differences make men less prone to showing signs of ageing like thicker skin, high-density collagen, and increased sebum production. This is mostly due to hormones' significant effect on the skin, with estrogen affecting females and androgens, such as testosterone, affecting males (1,14). Figure 1 shows the histological variances between male and female skin. Male skin typically exhibits a thicker epidermal layer compared to female skin. A higher presence of connective tissue results in increased collagen bundles in male skin in contrast to female skin.

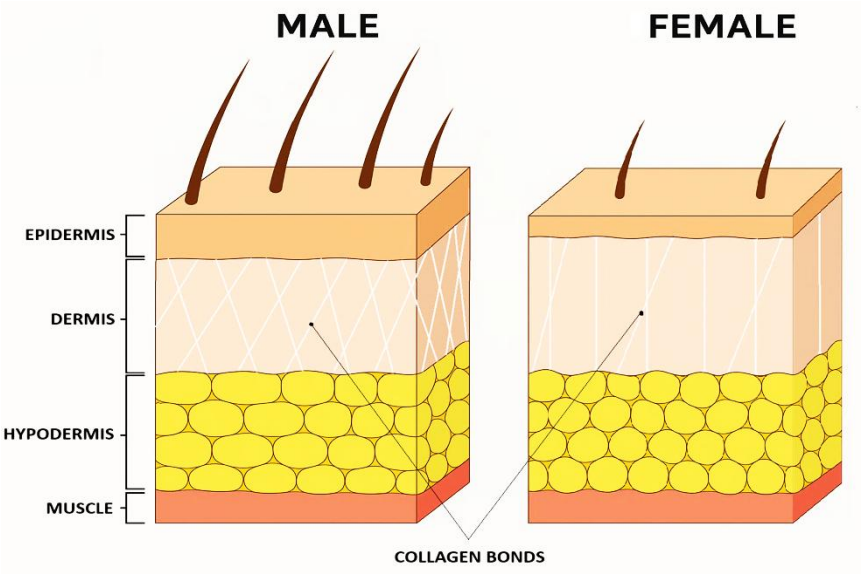

Figure 1: Different architecture of female and male skin (15).

Androgens promote collagen and elastin production, leading to increased epidermal and dermal thickness, making male skin approximately 25% thicker than female skin (1,3,9). Additionally, higher skeletal muscle mass and lower subcutaneous fat contribute to more pronounced deep expression wrinkles in men as they age, in comparison to women (1,9,16) The androgens are also responsible for more sebum secretion in male skin which has more and larger sebaceous glands, especially on the face linked to beard hair (5,16). Other physiological differences can be seen in Table 1.

Table 1: Summary of differences between male and female skin (3–9).

Attribute	Female	Male
Skin Colour	Lighter	Darker
Red Skin Tones	Lower	Higher
Yellow Skin Tones	Higher	Lower
Skin thickness	Thinner	Thicker
Amount of collagen	Less	More
Rate of collagen loss	Same	Same
Subcutaneous fat	More	Less

Skeletal muscle	Lower	Higher
Sebum secretion	Lower	Higher
Sweat secretion (Eccrine and apocrine secretions)	Lower	Higher
Stratum corneum hydration	Lower	Higher
Skin temperature	Colder	Warmer
Transepidermal Water Loss (TEWL)	Higher	Lower
pH	Not consensual	
Appearance of aging	Faster	Slower

Androgens also play a crucial role in hair development in males. In the cells of the hair follicle, androgen receptors bind testosterone and the more potent metabolite dihydrotestosterone (DHT). DHT results from the transformation reaction of testosterone when catalysed by the enzyme 5α-reductase(17) . During male puberty, a significant rise in serum androgen levels occurs, playing a crucial role in the conversion of vellus hair (usually referred to as “peach fuzz”) into terminal hair, which is longer, thicker, and more pigmented, in areas such as the pubic, axillary, chest, and facial regions (17). However, it is important to note that increased production of dihydrotestosterone, elevated cellular androgen receptor levels, and higher 5α-reductase activity have been linked to cases of alopecia (3,17,18).

On average, a male face has 500 hair follicles per centimetre, each as richly innervated as the surrounding skin. Facial hair can be beneficial as it protects against harmful ultra-violet (UV) rays when compared to skin otherwise exposed (4,19). However, shaving, which is a daily practice for most men, can induce mild abrasion of the outer skin layers, eliciting a cascade of physiological responses. These responses involve the stimulation of cutaneous nerve endings and subsequent activation of cell signalling pathways, ultimately leading to the release of cytokines. These cytokines are implicated in the manifestation of typical shaving-induced irritation, characterized by sensations of burning and itching, accompanied by increased skin erythema due to heightened blood flow (20). Compromised skin barrier integrity, combined with the occurrence of ingrown hairs, can predispose individuals to developing the inflammatory disorder known as pseudofolliculitis barbae (9). The neck region is the most susceptible to this occurrence due to rougher skin topography and a lower hair emersion angle (10). The structural properties of the hair shaft significantly affect shaving as larger fibres require more

force to cut the hair. The force needed to cut beard hair is almost three times higher than that needed to cut scalp or leg hair (4). As male skin is usually more deeply pigmented than female skin, the probability of post-inflammatory hyperpigmentation caused by trauma due to poor shaving techniques is higher (4).

1.3 Nanotechnology in cosmetic formulation development

Nanotechnology is a promising and transformative field with wide-ranging applications spanning cosmetics, dermatology, and biological sciences, among others. It has the potential to enhance existing products and unlock new delivery systems, offering opportunities for researchers, industries, and consumers alike (21). The use of nanotechnology in cosmetics dates to 1986, when Lancôme and Dior introduced their first nanotechnology-based products (22). Now, it is widely integrated into skincare formulations due to its ability to improve performance, bioavailability, and stability. This technology also enabled the design of biomimetic particles capable of delivering compounds to targeted cells, optimizing transcutaneous penetration through epidermal and dermal layers, and ultimately improving efficacy (22).

1.3.1 Types of nanomaterials

The European Regulation nº 1223/2009 of the European Parliament and of the Council, Article 2, Section K, defines nanomaterials as insoluble or bio-persistent and intentionally manufactured materials with one or more external dimensions, or an internal structure, on a scale from 1 to 100 nm (23).

Nanomaterials can be used for various cosmetic purposes as active ingredients, rheology modifiers, and carriers for bioactive molecules, among other roles. The most popular nanomaterials used in cosmetics, and their possible advantages and disadvantages are reviewed in Table 2.

Table 2: Summary of different nanomaterials used in cosmetics and their possible advantages and disadvantages (21,24–26).

Types of nanomaterials				Advantages/ Disadvantages
Organic Nanoparticles				
Lipid and surfactant derived nanoparticles	**Vesicular**	**Liposome**	Vesicles composed of a bilayer of phospholipids	**Advantages:** Biodegradable, biocompatible, amphiphilic (ability to compartmentalize and solubilize both hydrophilic and lipophilic materials), and higher skin penetration **Disadvantages:** Lower medication stacking, lower reproducibility, and lower chemical stability. May trigger an immune response
		Ethosome	Liposome formulation with inclusion of a very high concentration of ethanol	**Advantages:** Higher efficiency and penetration of cosmetic delivery into the skin **Disadvantages:** Poor yield problems, stability, and possibility of coalescence
		Transferosome	Liposome formulation with inclusion of edge activators (i.e. softening surfactants)	**Advantages:** Superior penetration through skin barrier
		Niosome	Vesicles composed of non-ionic surfactants	**Advantages:** Cheaper raw materials, higher efficiency, penetration, bioavailability, and prolonged stability of drugs (especially when compared to phospholipid-based vesicles) **Disadvantages:** Higher cost of production, physical and chemical instability, leakage of the drug, time-consuming production
	Non-vesicular	**Solid Lipid Nanoparticles**	Inner lipid core (solid at body temperature) formed only by solid lipids prepared by microemulsion and high-pressure homogenization	**Advantages:** Potential carriers of chemically labile molecules, occlusive properties that promote skin hydration and penetration of bioactive agents, higher duration of action, ease of large-scale production, higher bioavailability and biodegradability **Disadvantages:** Lower shelf life, and decreased drug encapsulation

Types of nanomaterials				Advantages/ Disadvantages
		Nano-structured Lipid Carrier	Inner lipid core (solid at body temperature) formed by solid and liquid lipids prepared by microemulsion and high-pressure homogenization	**Advantages:** Potential carriers of chemically labile molecules, occlusive properties that promote skin hydration and penetration of bioactive agents, the inclusion of oils in lipid matrix decreases crystallinity improving loading capacity, long-term encapsulation and stability of the system leading to higher shelf life, ease of large-scale production. **Disadvantages:** Lower duration of action
		Nano-emulsion	Biphasic systems composed of oil-phase and liquid-phase and one or more emulsifying agents	**Advantages:** If non-ionic surfactants are used results in more stability against agglomeration and precipitation, and the large interfacial area displayed by their droplets makes the material transfer faster enhancing delivery performance, fluid and transparent appearance, and amphiphilic. **Disadvantages:** Less thermodynamic stability when compared to microemulsion, more difficult to prepare, acid sensitive, lower duration of action
Polymeric Nanoparticles	**Nano-sphere**	Polymeric matrix in which numerous active ingredients can be entrapped or into which they can be adsorbed		**Advantages:** Improved performance of chemically labile, poorly water-soluble, or volatile molecules, sustained release of the loaded active molecules prolonging their beneficial effects.
	Nano-capsule	Liquid core surrounded by polymeric shell – the inner core holds the bioactive agent while the polymeric shell controls its release		**Advantages:** Improved performance of chemically labile, poorly water-soluble, or volatile molecules) protection of ingredients), masking of undesirable odours, sustained release of the loaded active molecules, resolution of incompatibility issues between formulation components, sustained release formulation. **Disadvantages:** An additional purification step is required after nanocapsule formation

Types of nanomaterials		Advantages/ Disadvantages
Nanocrystals	Clusters made up of thousands of molecules joined together in a fixed pattern to form a group with sizes ranging from 10 to 400 nm stabilised by surfactant/polymeric coating.	**Advantages:** Possible solution to poorly water-soluble agents, large surface area and poor crystallinity lead to higher drug solubility, particle distribution, adhesiveness, and dissolution rate. 100% drug loading ability. **Disadvantages:** Possibility of aggregation, not appropriate for aqueous APIs, only stable to a certain extent
Dendrimers	Three-dimensional nanostructured macromolecules that are extensively branched	**Advantages:** Higher solubility of lipophilic drugs, controlled-release drug formulation, maintenance of the stability of the drug in cosmetic formulations, and higher shelf life of formulation **Disadvantages:** Poor suitability for hydrophilic drugs, potential cellular toxicity, and higher manufacturing costs
Inorganic Nanoparticles		
Titanium Dioxide and Zinc Oxide	Inorganic UV radiation filters ZnO: more effective for UVA TiO_2: more effective for UVB	**Advantages:** TiO_2 has a higher sun protection factor at the nanoscale, which makes it more effective and results in a superior restorative effect due to its transparency, in contrast with its original colour due to the large surface-area-to-volume ratio **Disadvantages:** Inhalation of a large amount of these NPs is harmful
Gold and Silver	Gold NPs play a substantial role in fixing skin damage and improving skin surface, grace, and flexibility. Silver nanoparticles can be utilized as successful inhibitors of various microorganisms.	**Advantages:** Stability, biocompatibility, antifungal, antibacterial, and anti-ageing benefits **Disadvantages:** The safety of colloidal silver in nanostructures concerning its use in oral and dermal cosmetic items is ambiguous

Types of nanomaterials		Advantages/ Disadvantages
Silica	Mainly composed of amorphous silica nanodispersions with a size range of 5 to 100 nm. Can deliver both hydrophilic and lipophilic entities to their respective targets by encapsulation	**Advantages:** Hydrophilic surfaces favour extended distribution and low manufacturing costs, improve the adequacy, surface, and shelf life of cosmetics **Disadvantages:** Concerns about their safety. Size and surface changes are factors that ought to be considered while surveying its toxicity
Carbon Black	Colorant - CI77266	**Advantages:** Micron-sized NPs have a lower propensity of causing cytotoxicity, aggravation, and changes in phagocytosis in human monocytes when compared to NPs **Disadvantages:** Can be used in cosmetic items when there is no danger of being breathed in
Tris-Biphenyl Triazine	Novel, powerful, and photostable filter	**Advantages:** Broad-spectrum UV protectant **Disadvantages:** Not dangerous if applied to solid, unbroken skin. Concerns related to possible harmful impacts with the potential to bioaccumulate in selected tissues
Bucky Balls (Buckminsterfullerene/C60)	Carbon fullerene is a three-dimensional spherical compound that comprises a carbon ring with an odd number of atoms	**Advantages:** Antioxidative properties (potent scavenging ability of free radical oxygen species (ROS)). **Disadvantages:** Fullerenes alone have limited applications due to their hydrophobic nature, but the use of surface-active agents in a suitable concentration has improved their aqueous solubility

1.3.2 Advantages of nanotechnology in cosmetic applications

Cosmetic formulations are designed to remain on the outer layers of the skin without ever reaching systemic circulation, yet they must penetrate the skin to a certain degree to achieve their desired effects. During pre-formulation studies, various molecules and extracts of interest, known for their beneficial effects, face challenges related to poor solubility and

penetration which can hinder their absorption. Additionally, some of these compounds may negatively impact the homogeneity or organoleptic properties of the final product due to strong colours or odours. Nanotechnology offers a promising solution to these challenges (22,27).

Nanoscale delivery systems or nanomaterials can overcome the limitations of traditional systems by improving the penetration of ingredients through the skin and facilitating direct interaction with the skin outmost layer, the *stratum corneum*, and skin appendages. This approach increases the contact area between the ingredients and corneocytes, protects molecules from physicochemical and enzymatic degradation enhancing their stability without compromising bioavailability, controls their release, and enhances both dermal penetration and the duration of their effect in the skin (27,28).

Nanonization, the process of reducing substances to nanoparticles (NP) to sufficiently penetrate the skin, while maintaining their chemical properties in a way that does not compromise their efficacy serves as a strategy to address these issues, despite comprising a significant challenge (22,27).

The use of nanotechnology can most definitely offer several advantages over traditional formulations by enhancing the penetration, absorption, organoleptic characteristics, texture, spreadability, and adherence of the formulations to the skin, while prolonging the action of ingredients through controlled delivery, site-specific targeting, and increased ingredient-loading capacity. Nanomaterials, with their distinct characteristics, can also improve ingredient stability resulting in a longer shelf life for the final product, and may even act as active agents themselves (22,25).

1.2.3 Health and environmental hazards of nanotechnology

Although nanotechnology provides significant technical and economic advantages in cosmetic formulations, increasing concerns regarding its potential health and environmental risks arise (21). Due to their nanoscale dimensions, large surface area, and often positively charged surfaces, certain nanomaterials exhibit bio-persistent properties, raising concerns about their accumulation on the skin or within the upper layers of the *stratum corneum*. Although most nanoparticles are designed to remain on the skin's surface, studies suggest that under specific conditions, such as prolonged exposure, formulation-specific properties, or compromised skin integrity, they may penetrate deeper and potentially reach systemic circulation, raising toxicity concerns (22).

Their unique physicochemical properties, which allow them to traverse biological membranes and infiltrate cells, tissues, and even organs, enhance their bioavailability but

simultaneously present risks related to cellular interference. By interacting with intracellular components, they can disrupt normal cellular processes, induce oxidative stress, trigger inflammatory responses, and, in severe cases, lead to apoptosis or necrosis, posing a significant health hazard. Substances that are typically considered non-hazardous at the macroscale may exhibit altered physicochemical properties when reduced to the nanoscale, leading to heightened chemical reactivity and enhanced biological activity (24).

One of the primary toxicological concerns associated with nanoparticles is their ability to generate excessive ROS, a mechanism central to many adverse biological responses. Elevated ROS levels can compromise cellular homeostasis, leading to oxidative stress, inflammation, and widespread molecular damage. This oxidative imbalance can deteriorate cellular membranes, denature proteins, and induce DNA mutations, which have been linked to carcinogenesis and genotoxic effects (24). Additionally, interactions between nanosize delivery systems and biological targets remain only partially understood, further complicating safety assessments and reinforcing the need for continued research (27).

In cosmetic applications, the most relevant route for nanoparticles to enter the body is through dermal absorption. Special consideration is needed for cosmetics formulated as sprays, powders or aerosols, as inhalation of nanoparticles poses additional health risks (25). On the other hand, nanomaterials exhibit dose-dependent toxicity across various routes of administration. It is well established that the bioavailability of an active ingredient is primarily influenced by its dosage rather than its intrinsic physicochemical properties. In cosmetic formulations, a major concern is that nano formulations may enhance systemic absorption of active ingredients, potentially increasing their concentration in the bloodstream and exacerbating toxic effects (24).

Experimental studies further reinforce these concerns. Research by Jessica P. Ryman-Rasmussen *et al*. (26) demonstrated that specific nanomaterials could penetrate deeper layers of pig skin within just 24 hours of exposure (24). Additionally, scientific evidence has shown that exposure to silver nanoparticles can significantly decrease cell viability and metabolic activity, while also impairing cell migration and proliferation(25). Prolonged exposure has been associated with caspase 3/7 activation and DNA damage, resulting in both genotoxic and cytotoxic effects (25).

Beyond human health, nanotechnology also presents potential environmental challenges. While it has been recognized for its role in reducing waste, mitigating greenhouse gas emissions, and limiting hazardous chemical discharge, its environmental impact remains insufficiently understood. Due to their unique physical and chemical properties, nanoparticles

can disrupt biochemical processes in ecosystems. During manufacturing and product use, nanomaterials may be released into water, air, and soil, posing significant ecological risks. Their environmental behaviour is influenced by factors such as their mobility, dispersion mechanisms, solution chemistry, redox potential, and overall stability under different environmental conditions. Additionally, exposure levels throughout the product lifecycle, including transportation, use, and disposal, further determine their ecological footprint (24). Given these uncertainties, further research is essential to fully assess the long-term environmental consequences of nanoparticle exposure from cosmetic applications.

1.4 *Allium cepa* properties

Allium cepa (onion) has long been used in traditional medicine for its anti-inflammatory, antimicrobial, antioxidant, wound healing, and hair growth properties. It is commonly associated with maintaining healthy skin and hair, and in Thai folklore it has been used to treat skin infections, tinea capitis and hair loss (7). Beyond traditional knowledge, *in silico* studies also suggest that onion extract may exert DHT-blocking effects (40). *In silico* analyses of its active compounds against 5α-reductase reported favourable docking scores and ADME/T properties, supporting its potential (40).

The main bioactive compounds in onions are phenolic compounds such as quercetin and fisetin, which are recognized for their anti-inflammatory and antioxidant potential (7,8,41). Inflammation in hair follicles is often triggered by oxidative stress and androgens. Quercetin has demonstrated both anti-inflammatory and antiandrogenic activity through inhibition of steroid 5α-reductases and downregulation of androgen receptors (7). Similarly, Kubo *et al.* showed that fisetin promotes hair growth by stimulating growth factors such as IGF-1 and KGF, both essential for hair follicle development and proliferation (9). Ruksiriwanich *et al*. studies show that quercetin enhances the expression of the CTNNB1 gene that encodes β-catenin. β-catenin plays a critical role in initiating the telogen to anagen transition and hair cycle progression. Disruption of this gene had been linked to abnormal hair growth in animal studies. Thereby increased β-catenin levels are thought to prolong the anagen phase and promote hair regrowth (7,42). Fisetin also activated β-catenin and induced hTERT expression in epidermal cells, leading to stem cell activation and new follicle formation, thereby reinforcing its role in the β-catenin signalling pathway and hair regeneration (9).

Nitric Oxide (NO) is also a mediator of inflammatory responses in hair follicles that increases in response to DHT levels (43). Ruksiriwanich *et al.* describe that onion extract reduces NO production and secretion, which in turn contributes to anti-inflammatory effects,

suppression of androgen-related gene expression, and activation of hair growth-related genes, suggesting a dual role in inhibiting inflammatory and androgen pathways (7).

The traditional uses of *Allium cepa* and accumulating experimental evidence highlights its potential for promoting hair growth through multiple mechanisms, making it a promising candidate for the development of topical formulations for beard hair loss and stimulation of beard hair growth.

2. Objectives

The present study aimed to produce an extract from onion skins and incorporate it in phytosome nanocarriers to improve stability and performance. The ultimate objective was the development of a novel facial serum for beard growth, combining consumer demand for effective cosmetic alternatives while contributing to sustainable product development.

Firstly, the phenolic compounds were extracted from onion skins varieties and then characterized for its phenolic content, specific bioactive composition (quercetin and fisetin), structural features, antioxidant activity and cellular viability. Using a phytosome system, chosen based on encapsulation efficiency and physiochemical properties (particle size, polydispersity index (PDI), surface charge), a facial serum will be formulated. The final formulation was characterized for its phenolic content, encapsulation efficiency, physicochemical stability (particle size, PDI, surface charge and pH), stability, spreadability, rheological behaviour, occlusion properties, quercetin and fisetin quantification, *in vitro* release and permeation, and voluntary sensory evaluation.

This work highlights the potential of *Allium cepa* skin extract, rich in phenolic compounds with antioxidant and anti-inflammatory properties that have been associated with hair growth and hair loss reduction. The transformation of onion skins, often discarded as waste in the agri-food industry, into a high value ingredient, not only offers a sustainable solution to waste management but also opens the possibility of innovative cosmetic applications. This study further evaluates phytosome systems to optimize encapsulation and stability and demonstrates their ability to produce a stable facial serum formulation. This addresses a gap in male skincare and provides a sustainable, scientifically grounded approach to cosmetic products development.

3. Materials and Methods

3.1 Materials

The plant materials used in this study included red onion (*Allium cepa*, code 83345, size: hybrid 50-70 mm), obtained from grower, packhouse and supplier José João Ribeiro Zina, Lda, Torres Vedras, Portugal; sweet onion (*Allium cepa*, code 83376, size: 75-100 mm), obtained from grower, packhouse and supplier Fuencampo XXI SL, Zaragoza, Spain; white onion (*Allium cepa*, code 83125, size: 65-85 mm), obtained from grower, packhouse and supplier Hortobatista, Alto Estanqueiro, Portugal; and shallot (*Allium cepa var. aggregatum*, code 8048283, size: 20-40 mm), obtained from grower, packhouse and supplier Scamark Iberia, Oliveira do Bairro, Portugal. Quercetin, Gallic acid, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and Folin-Ciocalteu's reagents were obtained from Sigma-Aldrich (Madrid, Spain). Fisetin was purchased from MedChemExpress (Princeton, USA) and Geogard Ultra™ was purchased from Lonza (Basel, Switzerland). Lecithin (food grade) was obtained from Santiveri, Casa Santiveri SL, Spain. L-Arginine was purchased from TCI Chemicals (Tokyo, Japan), and caffeine and Triton X-100 were obtained from Merck (Darmstadt, Germany). Acetonitrile, methanol and ethanol were of high-performance liquid chromatography (HPLC) grade and were purchased from Sigma-Aldrich (Madrid, Spain). Ultrapure water was provided by reverse osmosis in a MILLI-Q System Elix® 3 from Millipore® (Massachusetts, USA). All other reagents were of analytical grade and were used without further purification.

3.2 Methods

3.2.1 Extraction process

The protocol for the extraction process was adapted from a previously established protocol (29). The onion skins were dried in a convection oven with forced air circulation for 1h at 60 °C. After drying, the skins were grinded with a kitchen blender (Kunft, Worten, Portugal) for 3 min, followed by mixing them with 70% ethanol (in H_2O Milli-Q, v/v) in a proportion of 1:45 (w/ v) and heated in a water bath (Julabo U3) at 60 °C for 3 h with manual agitation every 30 min. The extracted solution was then subjected to a simple filtration using filter paper. The resulting solution was placed in a rotary evaporator (RE111, Büchi, Switzerland) coupled to an ultra-thermostatic bath (B-461, Büchi, Switzerland) at 60 °C and 90 rpm of agitation until the solvent completely evaporated. The dry residue was stored in an ultrafreezer at -63 °C overnight and then freeze-dried (Christ Alpha 1-4, BBraun Biotech International, Melsungen, Germany). The freeze-dried extract was stored in a hermetically sealed bottle and in a freezer

at -17 ± 3°C. The resulting dry residue was subsequently reconstructed with H_2O Milli-Q in the ratio of 1:50 (w/v). The extraction solution was subjected to centrifugation at 2000 rpm for 10 min at room temperature (Beckman GPR, USA; rotor GH-3.8; Beckman, Brea, CA, USA) to eliminate the precipitated material.

3.2.2 Characterization of the extract

3.2.2.1 Total phenolic content

Total phenolic content (TPC) of the extract was determined by the Folin-Ciocalteu method adapted from a previously described protocol with modifications (30) using gallic acid (GA) as a standard. A calibration curve of a standard solution of GA was prepared starting from a stock solution of 1000 mg/mL and subsequently diluted to six different concentrations ranging from 20 to 500 µg/mL. The assay was done in triplicate. An aliquot of 100 µL of the extract, previously diluted 50-fold, was transferred to the test tubes. To each experimental test tube, 200 µL of 10% Folin-Ciocalteu reagent solution was added, and the tubes were subsequently kept in a dark place at room temperature for 5 min. Then, 1 mL of 15% Na_2CO_3 and 2 mL of Triton 2% were added to each test tube, and the mixture was kept in a dark place at room temperature for 60 min. Following the incubation, the absorbance of each sample was analysed using UV-visible spectrophotometer (U-2000 spectrophotometer, Hitachi, Tokyo, Japan) at a wavelength of 765 nm.

3.2.2.2 Quantification of quercetin and fisetin by high-performance liquid chromatography

Quercetin and fisetin in the red onion skin extract (ROSE) were quantified in a HPLC system with a 126 pump direct control, 168 diode array detector (DAD) and a Midas type 830 auto-sampler (Beckman Instruments, Brea, CA, USA) following a previously described protocol (31,32). The mobile phase consisted of methanol and acidified water (70:30, v/v), and the analysis was performed using an Inertsustain C18 column (5 µm, 4.6 x 250 MM, GL Sciences) with a flow rate of 1 mg/mL, a 20 µL injection loop, and detection at 360 nm. Calibration curves were prepared using standard concentrations of both fisetin and quercetin ranging from 3 to 250 µg/mL. A stock solution of fisetin and quercetin (1 mg/mL of each in methanol) was prepared and then diluted to obtain seven different concentrations.

3.2.2.3 Fourier-transform infrared spectroscopy (FTIR) analysis

The dried extract was analysed in a FTIR spectrometer, model Nicolet TM iS 5 from ThermoFisher Scientific® (Waltham, USA). The measurement conditions comprise Attenuated

Total Reflectance mode, a Diamond ATR Crystal Plate detector, a resolution of 2 cm^{-1}, and a spectral range of 500-4000 cm^{-1}. Before the scan, the ATR crystal was cleaned with propranolol, and a background was collected. As extract is solid, it was necessary to use the punch to compress the sample against the crystal. The spectrum was obtained using MATLAB version R2021b (MathWorks, Natick, USA).

3.2.2.4 Antioxidant activity

The free radical scavenging capacity of the obtained extracts was evaluated using the ABTS decolorization assay, as described by Re *et al*. (1999). Trolox, a water-soluble vitamin E analogue, was used as a standard to express results in Trolox equivalents. Antioxidant activity was quantified by measuring the percentage of decolorization of the ABTS radical cation, with results expressed as mg of Trolox equivalents per mg of sample (33,34).

3.2.2.5 Cellular viability

The cytotoxic effect of ROSE was assessed in HaCaT keratinocytes cell line using the colorimetric MTS assay, following a previously established protocol (35). HaCaT cells were seeded in 96-well plate at a density of $2x10^4$ cells per well and incubated for 24 h to allow cell attachment and 60–70% confluence. Subsequently, cells were exposed to various concentrations of ROSE prepared in appropriate cell medium (8.88 mg/mL to 0.52 mg/mL) and incubated for 24 h at 37 °C in a humidified incubator with 5% CO_2 atmosphere. Untreated cells maintained in culture medium served as negative controls. At the end of the incubation, cells were washed with phosphate-buffered saline (PBS) and further incubated for 2 h with 100 µL incomplete medium and 20 µL CellTiter 96® Aqueous One solution at 37 °C. Since the extract had a pH of 3, HEPES buffer was used to stabilize the medium and maintain suitable experimental conditions. Cellular viability was determined by the absorbance at 490 and 630 nm with a Microplate Reader (BioTek® Instruments, USA). Results were expressed as the mean percentage ± SD of viable cells relative to untreated cells (considered as 100% viability). The ROSE concentration able of inhibiting 50% of cell growth (inhibitory concentration IC_{50}) was calculated.

3.2.3 ROSE-Lecithin phytosomes

The required amounts of ROSE and Lecithin (**Table 3**) were dissolved in H_2O Milli-Q and then homogenized (Kinematica PT 3000, Polytron, Germany) until no lecithin granules were visible.

Table 3: Formulation of lecithin phytosomes.

Ingredients	Composition (g)
Lecithin, food grade	9
ROSE	9
Water	q.s. 50 mL

3.2.4 Phytosomes physicochemical characterization

The particle size and polydispersity index (PDI) were determined by dynamic light scattering (Malvern Zetasizer NanoS, Malvern, UK), while zeta potential (surface charge) was evaluated by laser doppler anemometry (Malvern Zetasizer Nano Z, Malvern, UK), at 25°C. Phytosome samples were diluted with H_2O Milli-Q to suitable concentration. All measurements were performed in triplicate.

3.2.5 Encapsulation efficiency

Encapsulation efficiency (EE%) of the ROSE-SPC phytosomes was determined by comparing the TPC present in the extract with the amount quantified in the supernatant after ultracentrifugation (Beckman Coulter Optima NL-90, rotor 70Ti; Beckman, Brea, USA) at 180,000 x*g*, 2 h, 15 °C. The TPC was measured as described in **3.2.2.1**. The TPC measured in the supernatant was considered the non-encapsulated fraction, and the encapsulated fraction was determined by subtracting this value from the extract TPC. EE% was expressed as a percentage according to the following Equation 1:

$$EE(\%) = \frac{Encapsulated\ TPC}{Extract\ TPC}\ x\ 100 \quad (1)$$

3.2.6 Serum formulations

To develop a facial serum formulation for beard hair growth incorporating the ROSE-Lecithin phytosomes, a series of formulations were developed and tested (**Table 4**). To optimize the serum's effect on beard hair growth, caffeine and arginine were chosen as additional ingredients, together with the ROSE-lecithin phytosomes and a preservative.

Geogard Ultra was selected due to its ECOCERT certification as the preservative for the assays, and the percentage used was according to manufacturer indication. Caffeine and arginine were specifically included to support hair growth.

Table 4: Various serum formulations produced and tested.

Ingredients	F0	F1	F2	F3	F4	F5	F6	F7	F8
ROSE (g)	0	9	9	9	9	9	9	9	9
Lecithin (g)	9	9	9	9	9	9	9	9	9
Caffeine (g)	0	0	4	7.5	0	7.5	0.5	1.5	0.5
Arginine (g)	0	0	1	1	1	0	0	0	1
Preservative (g)	1	1	1	1	1	1	1	1	1
Water (g)	q.s. 50								

3.2.7 Characterization of serum formulations

3.2.7.1 Centrifugation test

To evaluate physical stability under stress, 10 g of each formulation was transferred into centrifuge tubes and subjected to centrifugation at 3000 rpm for 30 min at room temperature (Beckman GPR, USA; rotor GH-3.8; Beckman, Brea, USA). Following centrifugation, the samples were visually inspected for evidence of phase separation or other macroscopic changes (30,36).

3.2.7.2 Spreadability

The spreadability of the formulations was evaluated by placing 0.5 g of the sample within a pre-marked circle (2 cm diameter) on a glass plate. A second glass was carefully placed on top, and a 500 g weight was applied for 5 min, following a previously described protocol (37). After removal of the weight, the final diameter of the spread area was measured to determine spreadability. Each measurement was performed in triplicate, and the mean values with standard deviation were calculated.

3.2.7.3 Rheology

The flow behaviour of the formulations was first evaluated using a Brookfield Rotation Viscometer DV-E (Brookfield Engineering Laboratories, Middleborough, USA) at room temperature (ISO 7884-2). Spindles 63 and 64 were selected according to sample consistency, and the formulations were subjected to an ascending and descending shear sweep with rotational speeds of 1, 2,5, 5, 10, 20, 50 and 100 rpm. This provided an initial screening of viscosity and flow behaviour across multiple formulations.

Subsequently, the rheological behaviour of the formulations was assessed using a rotational rheometer equipped with a CP1/50 cone-plate geometry at 25°C. Viscosity measurements were performed as function of a shear rate (0.01-10 s^{-1}) to evaluate flow behaviour. Thixotropic behaviour was assessed by applying a shear rate of 0.01-10 s^{-1} for 5 min to observe structural recovery after shearing. Frequency sweep tests were performed at a shear strain of 0.10% over a frequency range of 0.1-10 Hz to determine storage (G') and loss (G'') moduli and evaluate viscoelastic properties. Ten replicates were analysed for each formulation to ensure reproducibility.

3.2.7.4 Occlusion test

The occlusive properties of the formulation were determined following the method described by Wissing and Müller with adaptations (38). Beakers of 50 mL were filled with 15 mL of water and covered with standard filter paper. Formulations were applied evenly on the filters at a dose of 13.3 mg/cm^2. The beakers were stored at 32 °C and 50-55% relative humidity for 48 h. Water loss from the beakers was recorded at 6 h, 24 h, and 48 h. The occlusion factor (F) was calculated according to Equation 2, where A represents the water loss from the reference beaker without sample and B is the water loss from the beaker covered with the formulation. Each experiment was performed in triplicate. Vaseline was included as a positive control to provide a reference for maximum occlusive effect.

$$F = 100\ x\ \frac{A-B}{A} \quad (2)$$

3.2.7.5 *In vitro* release and permeation studies

Formulation F8 was evaluated using a Franz diffusion cell system for both release and permeation studies (31,32,39). The receptor compartment (~4 mL) was filled with PBS containing 2% Tween 80 to maintain sink conditions, and the donor compartment was loaded with 300 µL of the formulation. The effective diffusion area between the donor and receptor

compartments was 1 cm². The receptor medium was continuously stirred and maintained at 32 °C.

For release experiments (IVRT), cellulose nitrate membranes 0.45 µm (Sartorius, Göttingen, Germany) were employed as the diffusion barrier. Samples of 500 µL were withdrawn from the receptor phase at 1, 2, 4, 6, and 8 h, and immediately replaced with fresh preheated buffer.

For permeation experiments (IVPT), Strat-M membranes (Merck Millipore, Billerica, USA) were used. Sampling was performed at 1, 2, 4, 6, 8, and 24 h, and immediately replaced with fresh preheated buffer.

All experiments were performed in duplicate. The cumulative amount of active compounds was quantified by HPLC as described in **3.2.2.2**.

3.2.7.6 Cell growth

HaCaT cells (1.0 × 10⁴ cells/well) were seeded onto a 96-well plate and incubated overnight. Cells were then treated daily with fisetin (10 µM) or ROSE extract diluted to 10 µM fisetin. Cell proliferation was monitored using the MTT assay, as described by Kubo *et al*. (40).

3.2.7.7 Voluntary sensory evaluation

A voluntary sensory evaluation of the formulation was conducted following approval by the Faculty of Pharmacy, Universidade de Lisboa ethics committee ("Avaliação da cosmeticidade de um sérum para aplicação na pele do rosto masculino"). Participants applied a single drop (**Figure 2**) of the formulation on the left forearm and spread it using their right hand. After application, volunteers completed a questionnaire (Google Form) to access the sensory attributes of the product. Five parameters (colour, odour, spreadability, oiliness and freshness) were evaluated using a 5-point scale, where 1 represented "very pleasant" and 5 "unpleasant". Participants also indicated whether any residue was visible after application (yes/no). Finally, they rated their likelihood of using the product as a facial serum on a scale from 1 "most likely" to 5 "never". Copy of the study design, the subject inclusion criteria, the written informed consent form and other written information provided to subjects are in **Annex II**.

Figure 2: Amber glass dropper bottle with glass pipette used for serum evaluation. The label provided a QR code to assess the Google Form with the related questionnaire.

4. Results and discussion

4.1 Red Onion Skin Extract (ROSE) characterization

The average yield of the extraction was approximately 24% (w/w), calculated as the mass of freeze-dried extract divided by 100 g of dry onion skin mass. This result compares favourably with reported values for hydroalcoholic extraction of onion skins (42). The extracts, with an average concentration of 20 mg/mL, showed a mean value of 0.247 ± 0.012 mg GAE/mL, corresponding to 10 mg GAE/g extract. While this value is lower than some reported TPC values for red onion skin extracts (147-289 mg GAE/g) (43,44), several methodological factors explain this result. The extended extraction time of 3 hours at 60°C, though intended to maximize efficiency, may have contributed to thermal degradation of phenolic compounds, as both quercetin and fisetin exhibit significant degradation at elevated temperatures (45). The reconstitution of freeze-dried extract in pure water (Milli-Q H_2O, 1:50 w/v) followed by centrifugation likely resulted in significant quercetin loss due to its extremely poor water solubility (approximately 1-7 µg/mL at room temperature), causing precipitation that was removed during centrifugation (46–48).

Quercetin and fisetin were quantified by HPLC as fully described in **4.2.1.** The extract presented quercetin and fisetin contents of 4 µg/g extract and 6.5 µg/g extract, respectively. Despite quercetin being the most common flavonoid in onion skins, fisetin content exceeded quercetin in the aqueous extract (49,50). This is explained by fisetin's superior stability compared to quercetin under the extraction conditions used, as fisetin demonstrates lower degradation rate constants than quercetin (51).

Despite these challenges, the extract successfully retained both flavonoids, and the 24% yield represents a promising recovery. However, the extraction process requires further optimisation to minimise active molecules degradation and loss.

FTIR spectrum of ROSE was measured in the 500-4000 cm^{-1} spectral region (**Figure 4**) and reveals distinct peaks due to the presence of bioorganic molecules. The broad band at 3287 cm^{-1} is assignable to OH stretching vibration and indicates the presence of free OH from flavonoids and phenols (52). The width of this band is due to intermolecular hydrogen bonds between the organic molecules. The weak bands at 2850 and 2950 cm^{-1} are assignable to the stretching of the aliphatic and aromatic C–H bond, respectively. The N–H (amine) bends and the ring C–C stretch of phenyl corresponds to bands at 1680 cm^{-1} and 1600 cm^{-1}, respectively. The functional groups from carbohydrates are what cause the wide peak at

around 1000 cm^{-1}. These FTIR spectral features are consistent with previously reported onion peel extracts (53,54).

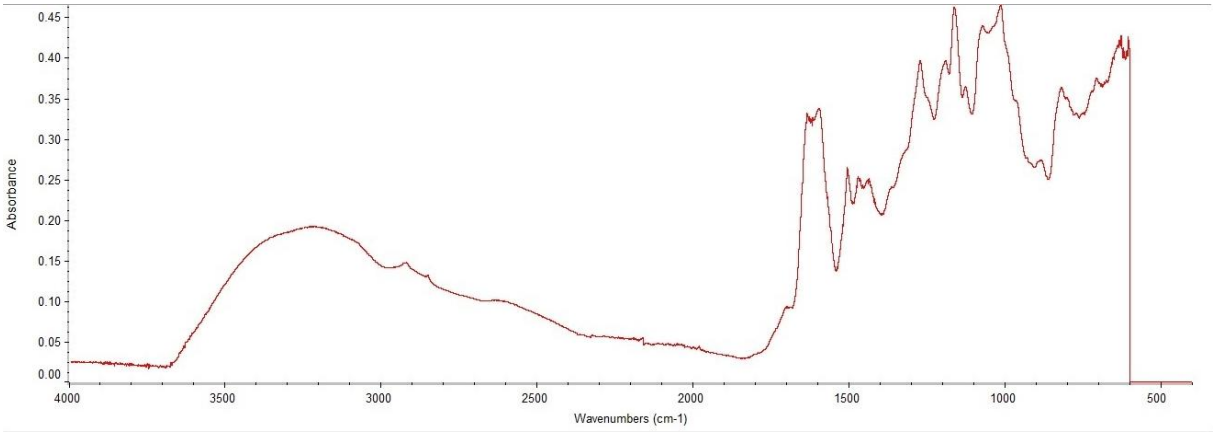

Figure 3: FTIR spectrum of ROSE.

The extract obtained, at a concentration of 20 mg/mL, presented substantial free radical scavenging activity, resulting in 56.87% decolorization using the ABTS decolorization assay. This corresponds to an antioxidant activity of 0.28 mg Trolox equivalents (TE) per mg of extract (mg TE/ mg extract). These results demonstrate promising antioxidant potential, consistent with the presence of quercetin and fisetin in the extract, both flavonoids with well-documented free radical scavenging properties, comparable to previously reported values and even surpassing those of 0.011 mg TE/mg extract for similar 70% ethanol red onion skin extracts (55), thereby indicating successful retention of bioactive phenolic compounds (56,57).

4.1.1 Quantification of quercetin and fisetin by high-performance liquid chromatography

Calibration curves were constructed for both quercetin and fisetin using standard solutions at concentrations of 3.90, 7.81, 15.62, 31.25, 62.5, 125 and 250 µg/mL. The calibration curves showed excellent linearity within this range, with the equation for quercetin being y = 153332x + 721470 (R^2 = 0.9967), and for fisetin y = 228398x + 71574 (R^2 = 0.9999). The retention time was 13.5 min for fisetin and 22.2 min for quercetin (**Figure 5**). Using this quantification method, it was possible to assess both molecules concentration in the analysed samples with one single chromatographic run.

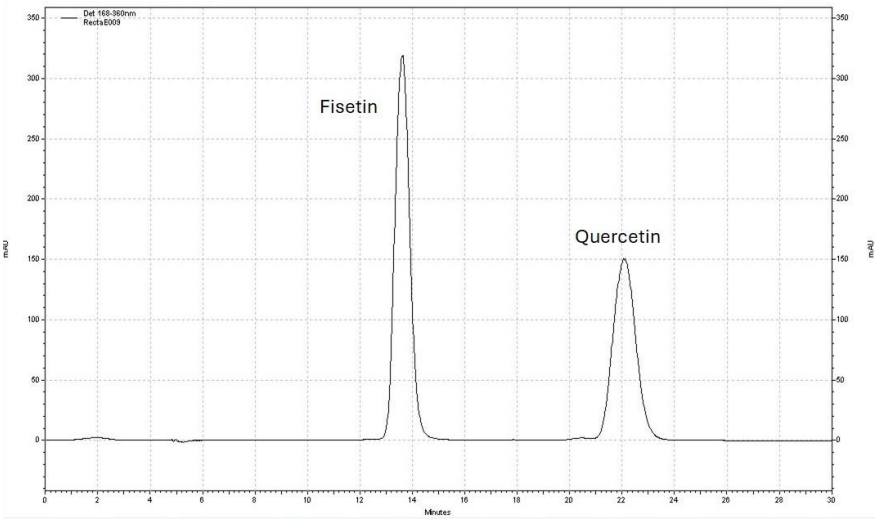

Figure 4: Chromatogram of the calibration curve standard containing 125 µg/mL fisetin and 75 µg/mL quercetin, showing peaks corresponding to fisetin and quercetin eluted during the same chromatographic run.

4.1.2 Cellular viability

The cytotoxic potential of the ROSE was evaluated in HaCaT keratinocytes using the MTS assay. Cells were treated with extract concentrations ranging from 8.88 mg/mL to 0.52 mg/mL for 24 h. Cell viability remained relatively high at lower extract concentrations, suggesting no cytotoxicity. A pronounced decrease in viability was observed at higher concentrations as seen in **Figure 6**.

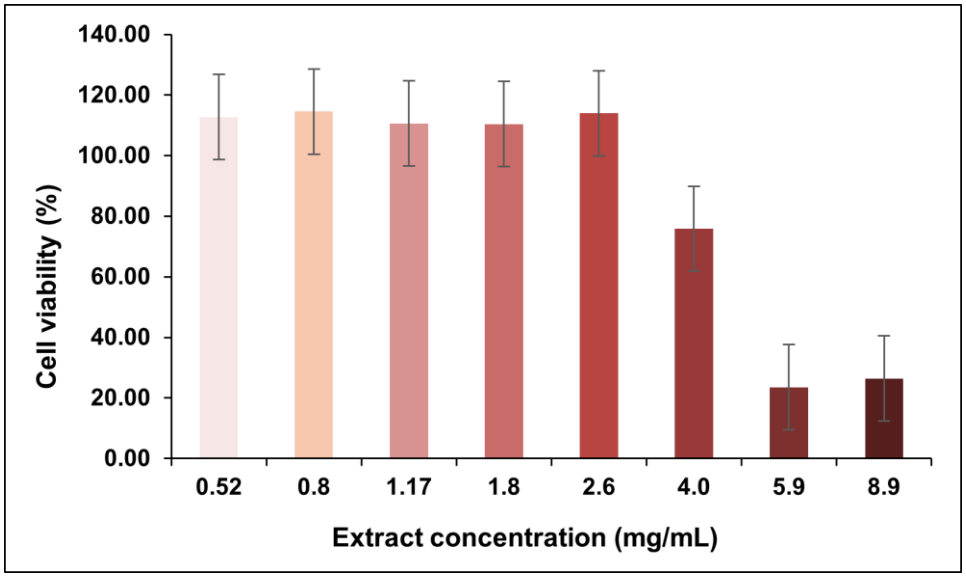

Figure 5: Cell viability (%) of HaCaT cells exposed to different concentrations of ROSE.

The dose-response data reveal that the cytotoxic effect of ROSE is concentration-dependent with an IC_{50} value of 4.82 mg/mL (log IC_{50} = 0.68). These results indicate that ROSE

exhibits cytotoxic effects only at concentrations toward the higher end of the tested range, while concentrations at the lower end appear to be well tolerated by HaCaT cells. These results are consistent with literature reports showing red onion extracts are well-tolerated at lower concentrations (58). It should be noted that this cytotoxicity assay was performed on monolayer HaCaT keratinocytes, which are directly exposed to the extract without the protective barriers present in intact skin. *In vivo*, the SC provides additional protection reducing the impact of ROSE on keratinocyte viability.

4.2 ROSE-Lecithin phytosomes

4.2.1 Determination of encapsulation efficiency

The EE of the ROSE in Lecithin phytosomes was determined after ultracentrifugation by quantifying the non-encapsulated phenolic compounds in the supernatant. The TPC of the supernatant from the control formulation (without extract) was 0.014 mg/mL, while the formulation containing ROSE showed a TPC of 0.067 mg/mL. Considering the theoretical TPC of 0.16 mg/mL in the formulation based on the TPC of the extract, the calculated EE was 66%. These results indicate that a substantial proportion of the phenolic content was successfully incorporated into the phytosomes vesicles confirming the efficiency of the encapsulation method. This result is consistent with literature reports of 60-90% encapsulation efficiency for polyphenol-rich plant extracts in lecithin-based phytosomal formulations, confirming the efficiency and effectiveness of the encapsulation method for retaining bioactive compounds in the lipid complex (59–61).

From the extract characterization, the theoretical concentration of fisetin in the formulation was calculated to be 0.023 mg/mL. The measured concentration of fisetin in the supernatant was 0.010 mg/mL, corresponding to an EE of 56%, suggesting more than half of the fisetin remained associated with the phytosomal system.

Using the same approach the theoretical concentration of quercetin in the formulation was calculated as 0.013 mg/mL. However, quercetin could not be reliably detected in the supernatant, as the measured signal fell below the quantification limit of the method. As result, quantification could not be calculated for quercetin. As this analysis is done in the supernatant, we consider that the quercetin is completely encapsulated.

These encapsulation efficiency results, with fisetin at 56% and quercetin showing complete encapsulation, are consistent with literature reports of 67-98% encapsulation efficiency for flavonoids in lecithin-based phytosomal systems, demonstrating successful phytosome formation with efficient flavonoid retention (62,63).

No detectable peaks were observed in the chromatogram of the control supernatant (F0), confirming the absence of interference from the lecithin.

4.2.2 Physicochemical characterization

ROSE-Lecithin phytosomes exhibited very promising physicochemical properties, with average particle size of 256.9 nm, the PDI was 0.339 and zeta potential was -44.9 mV, which are consistent with literature reports for optimized lecithin-based phytosomal systems (64). Phytosomes were further incorporated into the developed serum formulations, and the results are discussed in the next sections.

4.3 Serum formulation with ROSE-Lecithin phytosomes

The serum contains 9 g (9 mL) of ROSE in a concentration of 20 mg/mL in a total of 50 g (50 mL) of formulation. This results in a final concentration of 3.6 mg/mL of extract in the serum formulation. This concentration is below the IC_{50} value determined in the MTS assay (4.82 mg/mL), suggesting that the extract, as incorporated into the formulation, does not pose a significant cytotoxic risk to HaCaT cells *in vitro*.

Caffeine was included in the serum due to its multiple actions relevant to hair growth such as inhibition of steroid 5α-reductase (reducing DHT production), stimulation of follicular metabolism and cell proliferation, antioxidant and anti-inflammatory effects, wound-healing properties, and enhancement of microcirculatory blood flow (65–67). It also contributes to skin barrier integrity, with evidence showing reduced TEWL particularly in male skin, which is directly relevant to facial serum formulations (68). This ingredient is commonly used in topical hair formulations, typically at concentrations around 3% (w/w) (65). Evidence from clinical and experimental studies suggests that higher concentrations may exert stronger biological effects. For example, Lupi *et. al* observed improved microcirculation with a 7% caffeine solution, and topical preparations containing 10% green coffee extract demonstrated enhanced wound-healing and skin penetration *in vivo* (65,66,69). Based on this, we evaluated a range of concentrations of 1%, 3%, 8% and 15% w/w to optimize composition.

Arginine is an amino acid that serves as a precursor for nitric oxide (NO) synthesis in cells. NO contributes to hair growth by dilating blood vessels, which enhances circulation around hair follicles, and by modulating potassium channels involved in follicular stimulation (70). Souza et. al reported that topical L-arginine improved skin resistance to tensile force, indicating potential benefits for tissue integrity, which can be particularly relevant for the beard area in men (70). In topical cosmetic products, arginine is typically used at concentrations

ranging from 1-3% (w/w) (71). For this study, arginine was added at 2% (w/w) in the serum formulation.

4.3.1 Physicochemical characterization

Stability studies are important to monitor how a formulation maintains its characteristics and quality over time. Changes in particle size over time can reveal tendencies towards aggregation or sedimentation, while zeta potential gives an indication of colloidal stability, since particles with higher surface charge are less likely to aggregate.

Table 5: Screening of formulations according to physiochemical characterization over time.

PARAMETER	FORMULATION	DATE OF DATA COLLECTION							MEAN	SD
		22/01/2025	30/01/2025	06/02/2025	12/02/205	19/02/2025	28/02/2025	12/03/2025		
Ave. Particle Size	F0	228.2	224.0	-	216.1	216.9	-	-	**221.3**	**5.8**
	F1	256.9	241.5	-	242.9	244.1	-	-	**246.4**	**7.1**
	F2	-	160.1	-	166.5	-	-	-	**163.3**	**4.5**
	F3	-	151.1	-	154.7	-	-	-	**152.9**	**2.5**
	F4	-	319.9	-	231.7	-	-	-	**275.8**	**62.4**
	F5	-	201.1	-	189.3	-	-	-	**195.2**	**8.3**
	F8	-	-	-	-	191.1	181.1	197.5	**189.9**	**8.3**
PDI	F0	0.258	0.307	-	0.226	0.228	-	-	**0.255**	**0.038**
	F1	0.322	0.334	-	0.286	0.298	-	-	**0.310**	**0.022**
	F2	-	0.179	-	0.175	-	-	-	**0.177**	**0.003**
	F3	-	0.175	-	0.171	-	-	-	**0.173**	**0.003**
	F4	-	0.441	-	0.346	-	-	-	**0.394**	**0.067**
	F5	-	0.279	-	0.247	-	-	-	**0.263**	**0.023**
	F8	-	-	-	-	0.231	0.220	0.279	**0.243**	**0.031**
ZP	F0	-53.60	-44.8	-	-47.4	-50.70	-	-	**-49.1**	**3.8**
	F1	-44.90	-40.8	-	-47.1	-48.70	-	-	**-45.4**	**3.4**
	F2	-	-56.7	-	-40.5	-	-	-	**-48.6**	**11.5**
	F3	-	-50.9	-	-48.8	-	-	-	**-49.9**	**1.5**
	F4	-	-57.7	-	-54.3	-	-	-	**-56.0**	**2.4**
	F5	-	-44.1	-	-46.2	-	-	-	**-45.2**	**1.5**
	F8	-	-	-	-	-47.20	-51.60	-49.50	**-49.4**	**2.2**
pH	F0	-	4.6	4.6	4.5	4.46	-	-	**4.5**	**0.1**
	F1	-	4.4	4.4	4.3	4.40	-	-	**4.4**	**0.1**
	F2	-	6.2	-	6.2	-	-	-	**6.2**	**0.0**
	F3	-	6.0	-	6.1	-	-	-	**6.1**	**0.1**
	F4	-	6.9	-	6.9	-	-	-	**6.9**	**0.0**
	F5	-	4.5	-	4.5	-	-	-	**4.5**	**0.0**
	F8	-	-	-		6.87	6.82	7.06	**6.9**	**0.1**

Table 6: Physiochemical characterization of formulation F8, prepared in triplicate, over time, starting one week post preparation of the formulation.

PARAMETER	FORMULATION	DATE OF DATA COLLECTION			
		T7	T28	T42	T56
Ave. Particle Size	F8-1	222.8	230.9	257.3	215.9
	F8-2	201.9	201.3	198.5	206.6
	F8-3	195.7	228.0	271.1	200.6
	Mean	**206.8**	**220.1**	**242.3**	**207.7**
	SD	**14.2**	**16.3**	**38.6**	**7.7**
PDI	F8-1	0.332	0.356	0.403	0.310
	F8-2	0.256	0.245	0.251	0.248
	F8-3	0.238	0.390	0.426	0.271
	Mean	**0.275**	**0.330**	**0.360**	**0.276**
	SD	**0.050**	**0.076**	**0.095**	**0.031**
ZP	F8-1	-68.0	-68.2	-61.5	-55.5
	F8-2	-66.4	-67.5	-60.7	-54.9
	F8-3	-64.5	-63.3	-61.1	-52.2
	Mean	**-66.3**	**-66.3**	**-61.1**	**-54.2**
	SD	**1.8**	**2.7**	**0.4**	**1.8**
pH	F8-1	6.6	6.7	6.8	6.8
	F8-2	6.7	6.9	6.8	6.8
	F8-3	6.7	6.8	6.8	6.9
	Mean	**6.7**	**6.8**	**6.8**	**6.8**
	SD	**0.1**	**0.1**	**0.0**	**0.1**

Overall, the formulations demonstrated good physical stability under the tested conditions with no major changes observed in particle size, PDI, and zeta potential during the studied period, consistent with literature reports for lecithin-based phytosomal formulations that typically show stable physicochemical properties with minimal changes in particle size and zeta potential over weeks to months of storage (72).

4.3.2 Centrifugation testing

Centrifugation test revealed marked differences in the physical stability of the formulations (**Figure 7**). Formulations F2, F3, F5, and F7 showed visible phase separation after centrifugation indicating poor stability. In contrast, formulations without caffeine or containing lower concentrations of this ingredient (F0, F1, F4, F6 and F8) remained stable under these conditions. The presence of arginine, at the tested concentrations, did not negatively affect stability. These findings are consistent with literature showing that caffeine above 1-2% w/v can destabilize colloidal systems by affecting interfacial tension and particle aggregation, while arginine does not compromise phytosomal stability and can enhance formulation compatibility (73).

Based on these findings, F8 was selected as the optimal formulation, as it combined the extract, lecithin, low caffeine content (1%) and arginine (2%), together with preservative, while maintaining stability.

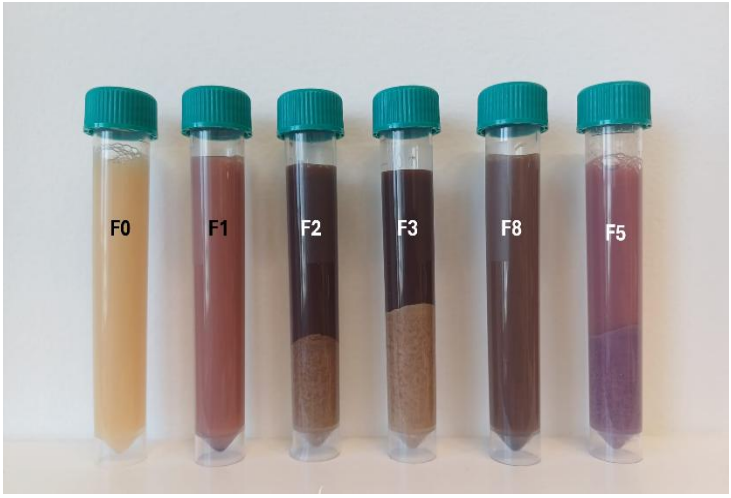

Figure 6: Formulations F0, F1, F2, F3, F8 and F5 after centrifugation. In F2, F3 and F5 phase separation can be observed very clearly.

4.3.3 Spreadability

Spreadability was assessed for formulations F0, F1 and F8 and the results are shown in **Table 5**. All formulations spread consistently under applied conditions, with F8 showing slightly greater spreadability compared to F0 and F1. The measured spreadability values fall within the optimal range of 5-7 cm for facial serums reported in literature (74,75). This property is advantageous for the topical application of a facial serum, as better spreadability can enhance ease use and uniform distribution of the product on the skin surface.

Table 7: Spreadability of formulations F0, F1 and F8.

FORMULATION	SPREADABILITY
F0	6.83 ± 0.29 cm
F1	6.43 ± 0.51 cm
F8	7.07 ± 0.42 cm

4.3.4 Rheology

Viscosity profiles obtained from ascending and descending sheer sweeps revealed all formulations displayed shear-thinning behaviour, with viscosity decreasing as the rotational speed increased.

Both F0 and F8 formulations were subjected to rheological characterization. F8 exhibited predominantly elastic behaviour, with storage modulus (G') exceeding loss modulus (G'') throughout the frequency range, indicating gel-like properties. Viscosity measurements revealed shear-thinning behaviour, with viscosity decreasing as shear rate increased. Thixotropy tests demonstrated recovery of the structure after shearing. The formulations displayed predominantly elastic behaviour, shear-thinning nature, and thixotropic recovery, which indicated that it is suitable for a facial serum (**Figure 8**).

This rheological profile is consistent with literature for optimal facial serum formulations, supporting easy dispensing through a dropper and ensuring uniform application on the skin, while maintaining structural integrity during handling and storage (76).

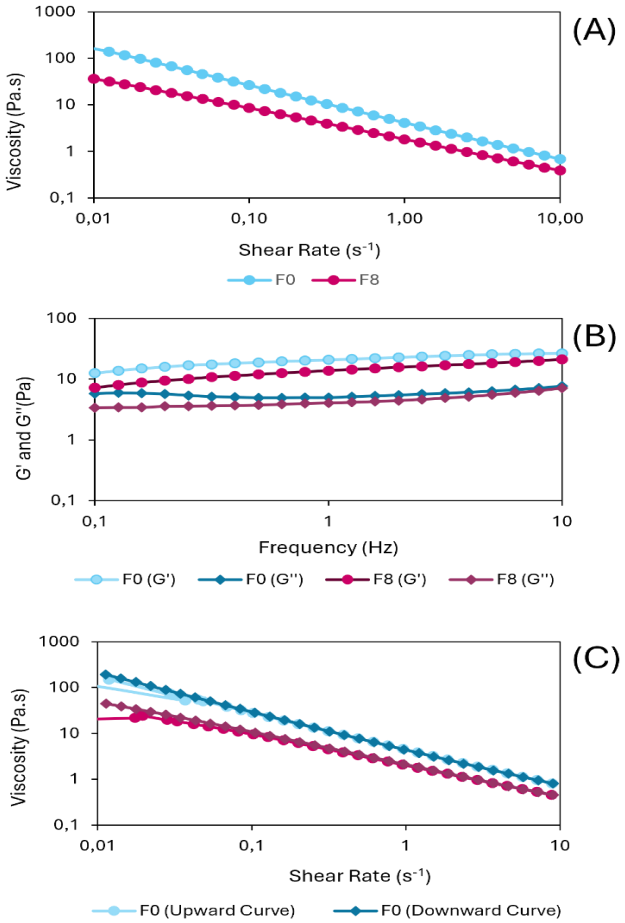

Figure 7: Rheological characterization of F0 and F8 formulations. (A): Viscosity as a function of shear rate. (B): Frequency sweep showing storage (G') and loss (G'') moduli. (C): Thixotropy test.

4.3.5 Occlusion

Occlusion studies revealed minimal occlusive effect for all tested formulations (F2, F4, F6 and F8), with values of F consistently lower than the positive control, Vaseline (**Figure 9**). Among the formulations, F8 exhibited the lowest occlusion, particularly noticeable at the 48h measurement. In most cases, F values were higher at the 24h time point compared to the 48h time point, suggesting a slight reduction in barrier effect over time, especially for F8.

The minimal occlusive effect observed for F8 (particularly at 48 h) is advantageous for a facial serum intended to avoid an overly heavy or greasy skin feel. This limited occlusion is consistent with literature showing that non-occlusive serums (occlusive effect < 30% that of Vaseline) provide comfortable skin feel and enhanced cosmetic acceptance while maintaining skin barrier function and allowing continued transepidermal water loss (77).

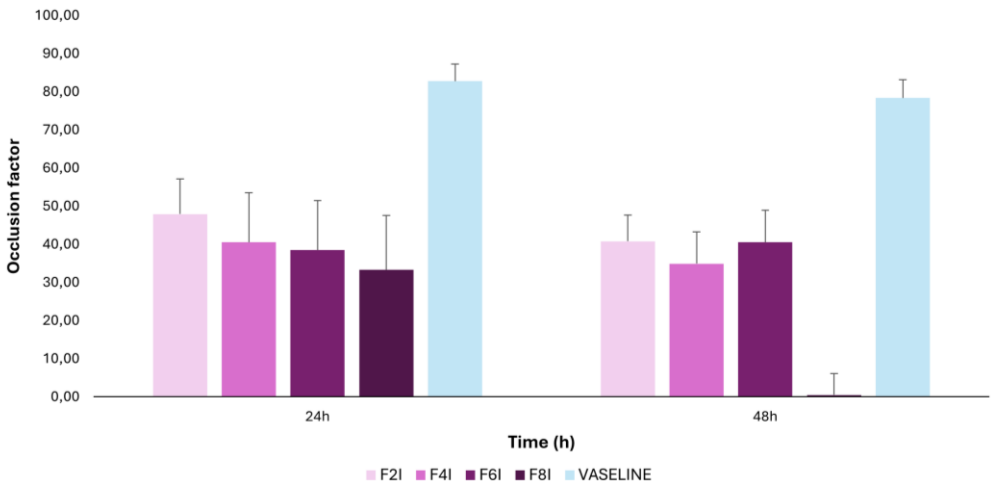

Figure 8: Occlusion factor of formulations F2, F4, F6 and F8 compared to Vaseline (positive control) over 24h and 48h.

4.3.6 *In vitro* release and permeation study

IVRT evaluated the release of quercetin and fisetin from the formulation F8 over 8h aiming to characterize their kinetic profiles. Quercetin was detected but the signal remained below the limit of quantification, preventing further kinetic analysis. In contrast, fisetin showed a rapid initial release within the first hour, after which the concentration remained relatively stable throughout the 8h period, with fluctuations as can be seen in **Figure 10A**. This profile suggests that fisetin undergoes a burst release followed by a slower, sustained release phase.

IVPT assessed the ability of quercetin and fisetin to diffuse from formulation F8 through a membrane over 24h. Quercetin was detected at 24h but the peak area was below the limit of quantification, preventing further quantitative analysis. Fisetin, however, displayed a continuous and time-dependent permeation profile, with cumulative amounts steadily increasing throughout the 24h period as seen in **Figure 10B**. The permeation curve shows an initial lag phase during the first hours, followed by a near linear increase, indicating that fisetin was able to diffuse across the membrane in a sustained manner.

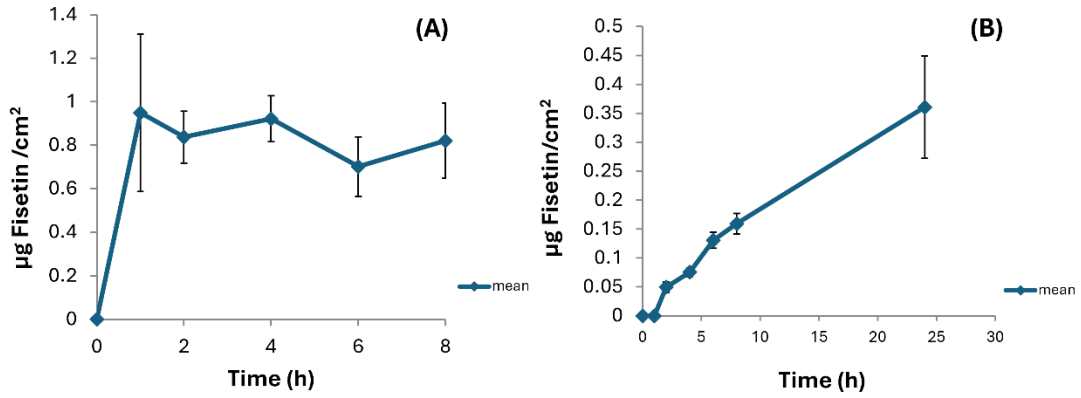

Figure 9: (A) Release profile of fisetin from formulation F8 over 8h (Mean ± S, n = 6); (B) Permeation profile of fisetin from formulation F8 over 24h (Mean ± S, n = 6).

The rapid initial release of fisetin followed by sustained release (burst-release profile), combined with time-dependent permeation across the membrane over 24h, is consistent with literature showing that fisetin-loaded phytosomal systems exhibit controlled release kinetics with initial burst effects due to surface-adsorbed fisetin followed by sustained diffusion, while quercetin's negligible release and permeation reflects its well-documented poor aqueous solubility and membrane permeability limitations that necessitate advanced formulation strategies to enhance bioavailability. These findings indicate that the formulation effectively delivers fisetin while suggesting that optimization strategies may be required to enhance quercetin's release and transmembrane diffusion in future optimisation of the formulation (78,79).

4.3.7 Cell growth

Fisetin (at a concentration of 10 µM) has been described to induce a change in the hair follicle by inducing proliferation of hair follicle bulge stem cells, ultimately promoting hair growth (40). In this study, fisetin was identified and quantified in the ROSE. To evaluate if treating HaCaT cells with ROSE induces cell proliferation, an assay based on cell viability measurement was performed (**Figure 11**).

Results show that the extract containing fisetin (diluted to 10 µM fisetin) significantly increased HaCaT cell growth on day 2 of the culture period. A decrease on viability was observed on day 3 as cell culture was conditioned. The results did not show the cell proliferation increase differing from previous studies using other proliferation assays (40). Discrepancy in results are most probably related to the evaluation method, however, having the same experimental conditions, ROSE exhibits a superior performance than the fisetin alone. The superior proliferative effect of ROSE on HaCaT cells is consistent with literature demonstrating that fisetin alone promotes HaCaT keratinocyte cell growth at 5-10 µM through

upregulation of TERT, IGF-1, and KGF expression, while the synergistic interaction between polyphenols present in ROSE exhibit significant synergistic effects in modulating cell proliferation and cell cycle regulation, providing enhanced bioactivity compared to individual compounds (80–82). Even considering this study a preliminary approach to assess cell proliferation stimulus, the results here presented are quite promising.

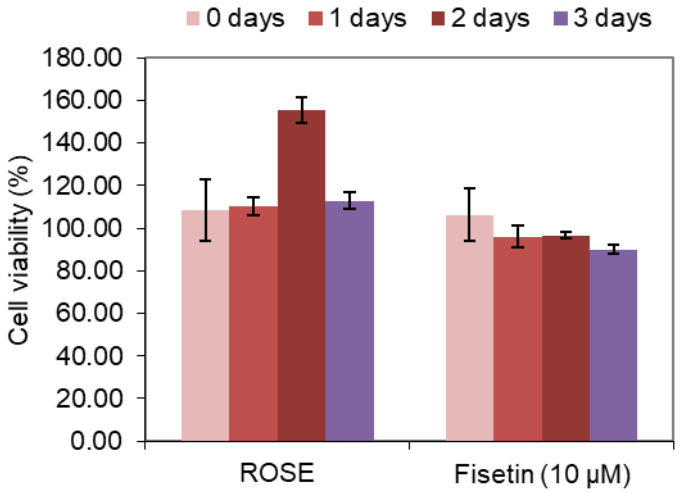

Figure 10: Effect of ROSE (diluted to 10 µM fisetin) and fisetin (10 µM) on the growth of HaCaT cells expressed as Mean ± Standard deviation of the viability (%) during 3 days of cell culture.

4.3.8 Voluntary sensory evaluation

A voluntary sensory evaluation of the serum formulation was conducted with 23 male participants, aged 14-50 years (mean ± SD: 28.4 ± 9.8).

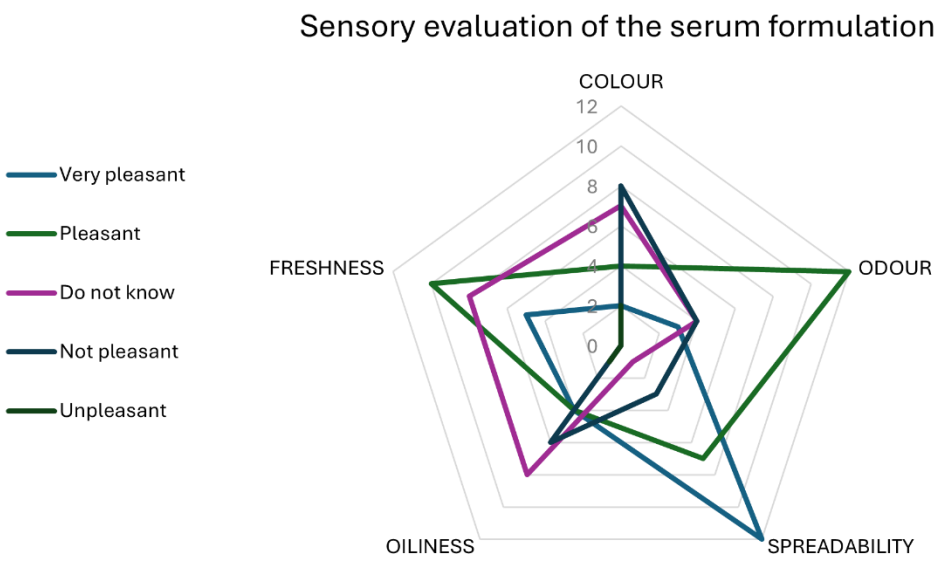

Figure 11: Sensory evaluation in terms of colour, odour, spreadability oiliness and freshness (n = 23).

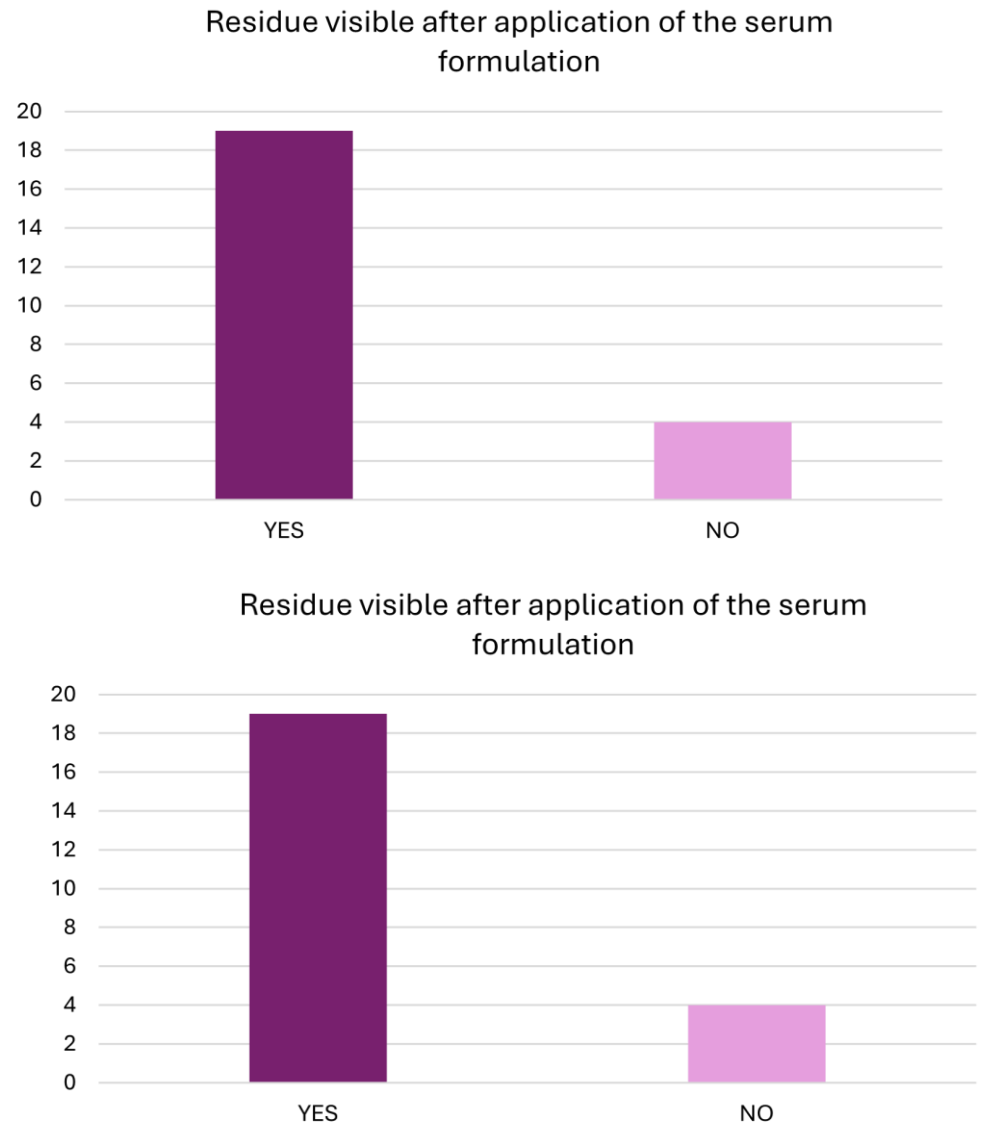

Figure 12: Participant responses regarding visible residue after application (n= 23).

Figure 13: Participant responses for likelihood of using the serum as a facial product (n = 23).

The serum was generally well-received by the 23 male participants. As can be seen in **Figure 12** odour, spreadability, and freshness were the most positively rated attributes, with most participants reporting “very pleasant” or “pleasant” scores. Colour and oiliness received more mixed responses, indicating that some participants found these attributes less appealing. No participants rated the serum as “unpleasant” for odour, spreadability, or freshness.

Most participants (83%) reported visible residue after application, which could influence acceptability in future use (**Figure 13**). Despite this, the majority expressed interest in using the serum as a facial product (**Figure 14**).

Overall, the sensory evaluation suggests that the formulation has a favourable sensory profile in terms of application and overall feel, however certain attributes like oiliness and residue may require optimization.

5. Conclusions

This thesis explored the development of a phytosomal serum incorporating red onion skin extract, obtained from an abundant agri-food by-product, for potential use in beard stimulation. The formulation demonstrated a favourable combination of physiochemical stability, delivery properties and biological compatibility. The phytosomal system effectively encapsulated quercetin and fisetin, supported fisetin release and permeation while maintaining keratinocyte viability and enhancing cellular proliferation, suggesting synergistic effects of ROSE flavonoids. Together with the positive sensory feedback obtained from volunteers, these findings indicate that the formulation possesses promising characteristics for topical use and highlights its potential as a sustainable and innovative cosmetic approach.

Further work is needed to strengthen the evidence and support product development. Microbiological and preservative efficacy testing were not performed, analytical methods require optimisation and replication, and sensory attributes such as colour, oiliness and residue need refinement. Additionally, tolerance and efficacy studies in human volunteers are essential to confirm safety and preliminary performance.

Overall, this study proposes a sustainable cosmetic approach that transforms an agricultural by-product into a value-added active ingredient delivered through a phytosomal system. The formulation aligns with green-chemistry principle, in design and excipient selection, highlighting the potential of eco-conscious design strategies in advancing next-generation cosmetic formulations.

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Annex I – Onion selection

The skins of four onion types: shallots, red onion, sweet onion, and white onion, were used to select the one that produces an extract with the highest TPC (**Figure 1**). The GAE/g contents were 1.97, 1.98, 2.82, 4.03 for extracts of shallots, red onion, sweet onion, and white onion, respectively. During rotary evaporation, more than half of the red onion extract was lost. Due to this loss, the measured TPC of red onion was lower than its actual value. Considering this loss, and in agreement with previous studies reporting the highest phenolic content in red onion (41), it would be expected that red onion would have the highest TPC among the onion types tested. Based on this rationale, red onion was selected for subsequent experiments. Future work could repeat this measurement to determine its exact TPC.

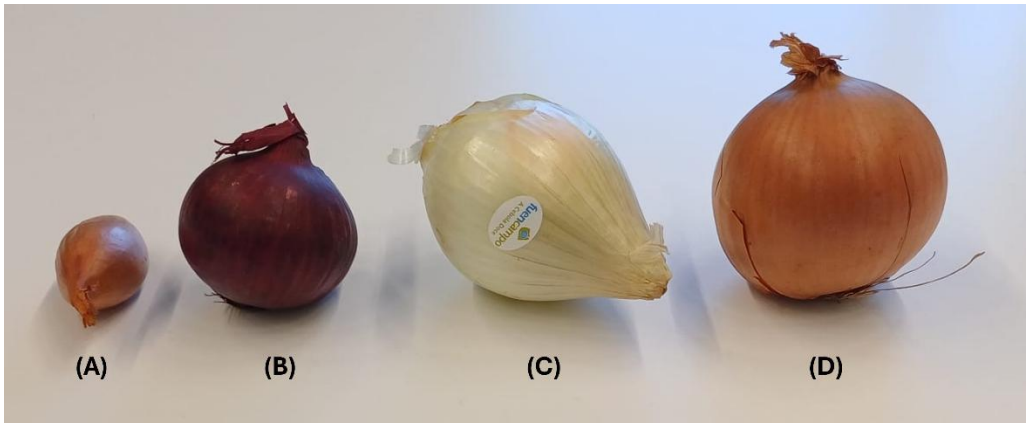


Figure 1: The four types of onions selected for testing: shallots (A), red onion (B), sweet onion (C), and white onion (D).

Annex II – Sensory analysis of a serum for application on male facial skin

Study design and participants

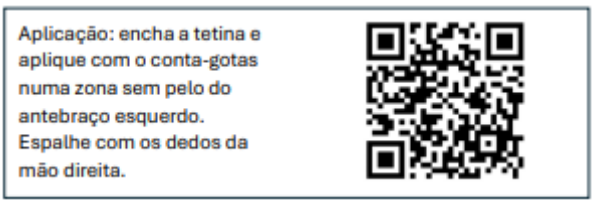
The study enrolled thirty (30) healthy male volunteers for a single evaluation of the test formulation, which was applied to a defined area on the anterior region of the left forearm. The specific inclusion criteria, as established in the study protocol, comprised male individuals aged over 18 years, with no history of dermatological disorders. Participants presenting any of the following characteristics during the selection phase were excluded: burns, allergy to any medication or food, skin infection, or use of any topical medication on the test site within 7 days prior to the start of the study.

Area and conditions for the application of the tested product

The study consisted of a single application of a formulation developed at the laboratories of the Faculty of Pharmacy, University of Lisbon. The application site corresponded to the anterior region of the left forearm, an area with reduced hair density. The applied volume was approximately 0.2 mL, corresponding to the dropper volume of a 5 mL dropper bottle. The product is to be evenly spread using the fingers of the right hand.

Cosmetic evaluation – Data collection

Each volunteer should evaluate several characteristics of the product and its application, recording their observations in a questionnaire provided with the product. The product label contained the application instructions and a QR code linking to an online questionnaire.



By scanning the QR code, the volunteer accessed the following questionnaire on an internet-connected electronic device:

Age: _____ years

Application: Fill the dropper and apply the product using the dropper onto a hairless area of the left forearm. Spread evenly using the fingers of the right hand.

How do you rate the following characteristics of the product?

Product colour:

1	1	3	4	5
Very pleasant	Pleasant	Indifferent	Slightly unpleasant	Unpleasant

Product odour:

1	1	3	4	5
Very pleasant	Pleasant	Indifferent	Slightly unpleasant	Unpleasant

Product spreadability:

1	1	3	4	5
Very pleasant	Pleasant	Indifferent	Slightly unpleasant	Unpleasant

Product greasiness:

1	1	3	4	5
Very pleasant	Pleasant	Indifferent	Slightly unpleasant	Unpleasant

Product freshness:

1	1	3	4	5
Very pleasant	Pleasant	Indifferent	Slightly unpleasant	Unpleasant

After application, was any visible residue left on the skin?

☐ Yes ☐ No

If the product were a facial serum, would you use a product with these characteristics?

1	1	3	4	5
Very likely	Likely	Uncertain	Unlikely	Never

After completing the application and evaluation, the volunteer should wash the application area with water and a mild soap. The product is not expected to leave any visible marks. If any change in skin colour or appearance, or sensations of itching or burning occurred, the volunteer should contact the Principal Investigator of the study at the following number: 965095260.

Instrumental evaluation

The volunteers are not subjected to any instrumental evaluation. The evaluation is purely sensory and observational and is carried out by the participants themselves.

Instrumental evaluation

The safety assessment of the product and its individual components was conducted through an in vitro assay using HaCaT cells to determine potential cytotoxic effects. Neither the formulation nor its isolated constituents reduce cell viability.
The potential for adverse reactions resulting from improper or accidental use of the product is considered low, given the limited quantity of product provided and the nature of its ingredients. Prior to the beginning of the study, all volunteers sign an Informed Consent Form.

Informed Consent Form

CONSENTIMENTO INFORMADO – Avaliação da cosmeticidade de um sérum para aplicação na pele do rosto masculina.

Por favor, leia com atenção todo o conteúdo deste documento. Não hesite em solicitar mais informações se não estiver completamente esclarecido.
Se entender que tudo está em conformidade, então assine este documento.
Leia e rubrique cada um dos pontos de forma a demonstrar a sua compreensão e aceitação em cada um a das afirmações.
No âmbito da elaboração de uma tese do Mestrado em Cosmetologia Avançada em curso na Faculdade de Farmácia da Universidade de Lisboa, orientada pela Doutora Sandra Simões, e co-orientada pela Doutora Manuela Carvalheiro, venho por este meio solicitar a sua participação no estudo abaixo referido.

	Afirmação
1	As propriedades cosmetológicas (nomeadamente o odor e a cor, a espalhabilidade e a sensação de untuosidade e de frescura) de formulações tópicas são fatores essenciais para a aceitabilidade de um produto cosmético.
2	A presença de um extrato de casca de cebola com propriedades antioxidantes nas formulações confere uma cor característica ao produto final.
3	O estudo pretende avaliar se a formulação tópica apresenta características cosmetológicas adequadas para comercialização de um produto cosmético.
4	Autorizo a Doutora Sandra Simões e, ou a quem ela designe como seu representante, a realizar o questionário que permite avaliar as propriedades cosméticas de uma formulação tópicas (contendo extrato de casca de cebola roxa)
5	Este estudo é não invasivo e não acarreta risco para a minha saúde.
6	Este estudo requer a avaliação observacional e sensorial, não sendo necessária instrumentação de análise.
7	Compreendo e foram-me explicadas as razões da necessidade deste estudo.
8	A investigadora respondeu às minhas questões e entendi que vou participar voluntariamente num estudo de investigação.
9	Foi-me garantido que não haverá prejuízo para os meus direitos assistenciais se eu recusar a participação no estudo.
10	Foi ainda salvaguardado, que todos os dados a serem recolhidos serão para uso exclusivo ao nível da investigação e que será mantido o anonimato.

Qualquer esclarecimento adicional ou contacto posterior, deverá ser realizado presencialmente antes da assinatura do consentimento ou para Doutora Sandra Simões, investigadora da Faculdade de Farmácia da Universidade de Lisboa, 965095260, ssimoes@ff.ulisboa.pt.

Declaro ter lido e compreendido este documento, bem como as informações verbais que me foram fornecidas pela/s pessoa/s que abaixo assina/m. Foi-me garantida a possibilidade de, em qualquer altura, recusar participar neste estudo sem qualquer tipo de consequências. Desta forma, aceito participar neste estudo e permito a utilização dos dados que de forma voluntária forneço, confiando em que apenas serão utilizados para esta investigação e nas garantias de confidencialidade e anonimato que me são dadas pelo/a investigador/a.

Nome

_____

Assinatura _____

Data ____ /____ /_____

Foram explicados ao voluntário os procedimentos decorrentes deste estudo.

ASSINATURA DO INVESTIGADOR:_____

ASSINATURA DO INVESTIGADOR:_____

Data: ____ /____ /_____

ESTE DOCUMENTO É COMPOSTO DE 2 PÁGINAS E FEITO EM DUPLICADO: UMA VIA PARA O /A INVESTIGADOR/A, OUTRA PARA A PESSOA QUE CONSENTE.