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Biobased Polymers and Antioxidants in Cosmetic Formulations

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Biobased Polymers and Antioxidants in Cosmetic Formulations

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STATEMENT OF INTEGRITY

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Polímeros e Antioxidantes de Origem Biológica em Formulações Cosméticas

Cada vez mais, os consumidores optam por produtos que priorizam a saúde, mas também a sustentabilidade e a saúde ambiental. Em resposta a esta nova procura, a indústria cosmética tem vindo a adotar novas praticas, em particular no que diz respeito à economia circular. Como ingredientes-chave na cosmética sustentável, estão o ácido hialurónico, o poliglutamato e compostos fenólicos, reconhecidos pelas suas propriedades hidratantes e de antienvelhecimento. Estes ingredientes podem ser produzidos de forma sustentável a partir de resíduos agrícolas e industriais, apoiando assim a sustentabilidade ambiental e contribuindo para alcançar os Objetivos de Desenvolvimento Sustentável (ODS) da União Europeia. Tendo em consideração, as novas necessidades da indústria cosmética, este projeto focou-se na utilização de subprodutos da indústria do vinho, especificamente no extrato da mistura de caules de uva (GS) de diversas castas. O tema do trabalho seguiu o *moto* da economia circular em linha com os ODS. O extrato foi caracterizado em termos do seu conteúdo em compostos fenólicos e do seu potencial biológico.

Os principais compostos identificados foram a catequina, trímeros de proantocianidina, rutina, quercetina e tetrâmeros de resveratrol. Em termos de atividade antioxidante o extrato demonstrou ter capacidade antioxidante, e baixa toxicidade, sem alteração significativa da morfologia das células da pele (queratinócitos HaCaT e fibroblastos BJ-5ta). O extrato demonstrou potencial na gestão de cicatrizes ao inibir a proliferação celular excessiva, promovendo a apoptose, sugerindo a sua eficácia no tratamento de condições de cicatriz anormal, como queloides e cicatrizes hipertróficas. O extrato GS quando incorporado em formulações de creme, adicionou-lhe teor de compostos fenólicos, embora cerca de 52% dos compostos fenólicos do extrato GS tenham sido perdidos devido a problemas de solubilidade. Algumas alterações físicas nas formulações foram observadas como a cor e o aumento da viscosidade. O comportamento de diluição por cisalhamento para fácil aplicação, assim como atividade antioxidante, embora menor que o extrato puro (em entre 25–50%) e a capacidade de inibir a elastase foram preservados. Os cremes com a incorporação de concentrações mais elevadas de extrato GS (acima de 3,23%) mostraram-se menos estáveis. No entanto as concentrações mais baixas (0,83% a 1,64%) demonstraram maior estabilidade, com propriedades reológicas consistentes.

Este trabalhou demonstrou as diversas potencialidades do extrato GS como um ingrediente sustentável e eficaz, já que apresenta elevado potencial oxidativo e na gestão de cicatrizes.

Palavras-chave: Caule de Uva; Compostos Fenólicos; Economia Circular; Polímeros de Origem Biológica; Sustentabilidade

Biobased Polymers and Antioxidants in Cosmetic Formulations

Consumers are increasingly opting for products that prioritise not only health but also sustainability and environmental well-being. In response to this growing demand, the cosmetic industry has been adopting new practices, particularly in relation to the circular economy. Key ingredients in sustainable cosmetics include hyaluronic acid, polyglutamate, and phenolic compounds, recognised for their moisturising and anti-ageing properties. These ingredients can be sustainably produced from agricultural and industrial waste, thus supporting environmental sustainability and contributing to the achievement of the European Union's Sustainable Development Goals (SDGs). In light of the new needs of the cosmetic industry, this project focused on utilising by-products from the wine industry, specifically the extract from the blend of grape stems (GS) from various grape varieties. The subject of the work followed the principles of the circular economy in line with the SDGs. The extract was characterised in terms of its phenolic compound content and biological potential.

The main compounds identified were catechin, proanthocyanidin trimers, rutin, quercetin, and resveratrol tetramers. In terms of antioxidant activity, the extract demonstrated antioxidant capacity and low toxicity, with no significant alteration to the morphology of skin cells (HaCaT keratinocytes and BJ-5ta fibroblasts). The extract also showed potential in scar management by inhibiting excessive cell proliferation and promoting apoptosis, suggesting its efficacy in the treatment of abnormal scar conditions such as keloids and hypertrophic scars. When incorporated into cream formulations, the GS extract contributed to the phenolic content, although around 52% of the phenolic compounds from the GS extract were lost due to solubility issues. Some physical changes in the formulations were observed, such as colour alteration and increased viscosity. However, the shear-thinning behaviour for easy application was maintained, as well as antioxidant activity, although reduced compared to the pure extract (by 25–50%). The ability to inhibit elastase was preserved.

Creams incorporating higher concentrations of GS extract (above 3.23%) proved less stable. However, lower concentrations (between 0.83% and 1.64%) demonstrated greater stability, with consistent rheological properties.

This work demonstrated the diverse potential of the GS extract as a sustainable and effective ingredient, as it exhibits high antioxidant potential and efficacy in scar management.

Keywords: Bio-based Polymers; Circular Economy; Grape Stem; Phenolic Compounds; Sustainability

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List of Abbreviations

Abs	Absorbance
ABTS	(2,2′-Azino-Bis-(3-Ethylbenzothiazoline-6-Sulfonic) Acid) Diammonium Salt
Ala	Alanine
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
C/N	Carbon-To-Nitrogen
CAGR	Compound Annual Growth Rate
CAT	Catechin
CEGA	Crude Extract Of Glutamic Acid
CF-GS	Cosmetic Formulation Containing Grape Stem Extract
CFU	Colony-Forming Units
CIR	Cosmetic Ingredient Review
DAPI	4',6-Diamidino-2-Phenylindole
DIC	Instantaneous Controlled Pressure Drop
DMEM	Dulbecco's Modified Eagle Medium
DMR	Dry Mushroom Residues
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
DOPA	Dihydroxyphenylalanine
DPPH	2,2-Diphenyl-1-Picrylhidrazyl
dw	Dry Weight
EC	European Cosmetics
ECM	Extracellular Matrix
ELAS	Anti-Elastase Assay
ELISA	Enzyme-Linked Immunosorbent Assay
EPS	Extracellular Polymeric Substances
EU	European Union
FC	Flavonoid Content
FRAP	Ferric Reducing Antioxidant Power
FSC-A	Forward Scatter Area

FSC-H	Forward Scatter Height
GAE	Gallic Acid Equivalent
GMP	Good Manufacturing Practices
GS	Grape Stem
GTP	Green Tea Polyphenol
HA	Hyaluronic Acid
HA-MW	Hyaluronic Acid Molecular Weight
HEPES	2-(4-(2-Hydroxyethyl)-1-Piperazinyl)-Ethanesulfonic Acid
HPLC	High-Performance Liquid Chromatography
HVED	High Voltage Electric Discharge
IC50	Half-Maximal Inhibitory Concentration
IL	Interleukin
IPC	*Ipomoea Pes-Caprae*
IPTG	Isopropyl-B-D-Thiogalactoside
ISO	International Organization For Standardization
KLα	Gas–Liquid Volumetric Oxygen Transfer Coefficient
LPS	Lipopolysaccharides
MGPR	Monosodium Glutamate Production Residues
MS	Mass Spectrometry
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
MW	Molecular Weight
NHDF	Normal Human Dermal Fibroblasts
NMF	Natural Moisturizing Factors
ODC	Ortho-Diphenols Content
OIV	International Organisation Of Vine And Wine
PBS	Phosphate-Buffered Saline
PCA	Pyrrolidone Carboxylic Acid
PDGF	Platelet-Derived Growth Factor
PEF	Pulsed Electric Field
PGA	Poly-Γ-Glutamic Acid
Pj	Palmyra Palm Sugar
PLE	Pressurized Liquid Extraction

PP	Peptone
ROS	Reactive Oxygen Species
rpm	Revolutions Per Minute
RT	Room Temperature
Rt	Retention Time
SDA-C	Sabouraud Dextrose Agar – Chloramphenicol
SDG	Sustainable Development Goals
SHLE	Super-Heated Liquid Extraction
SOCS	Suppressor Of Cytokine Signaling
SPF	Sun Protection Factor
TBARS	Thiobarbituric Acid Reactive Substances
TEAC	Trolox Equivalent Antioxidant Capacity
TEWL	Transepidermal Water Loss
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
TP	Tryptone
TPC	Total Phenolic Content
TPTZ	2,4,6-Tripyridyl-S-Triazine
TROLOX	6-Hydroxy-2,5,7,8-Tetramethylchroman-2-Carboxylic Acid
TSA	Trypticase Soy Agar
TYR	Anti-Tyrosinase Assay
UAE	Ultrasound-Assisted Extraction
UDP	Uridine Diphosphate
UDP-GlcNAc	Uridine Diphosphate N-Acetylglucosamine
UDP-GlcUA	Uridine Diphosphate Glucuronic Acid
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor

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SCOPE AND OUTLINE

In an era of growing environmental awareness, industries worldwide are seeking innovative ways to reduce waste and maximize resource efficiency. The cosmetic industry is no exception, with companies working to create products that not only meet consumer demands for quality and effectiveness but also support a commitment to sustainability.

This doctoral thesis explored the intersection of environmental sustainability and cosmetic product development. This work was performed at Universidade of Minho and Universidade de Trás-os-Montes e Alto Douro in collaboration with **Mesosystem**, a leading medical cosmetics manufacturing and trading company. The thesis analysed the bioactive potential compounds derived from a sustainable sources, for incorporation into cosmetic formulations.

The structure of this thesis is presented in the Figure 1.

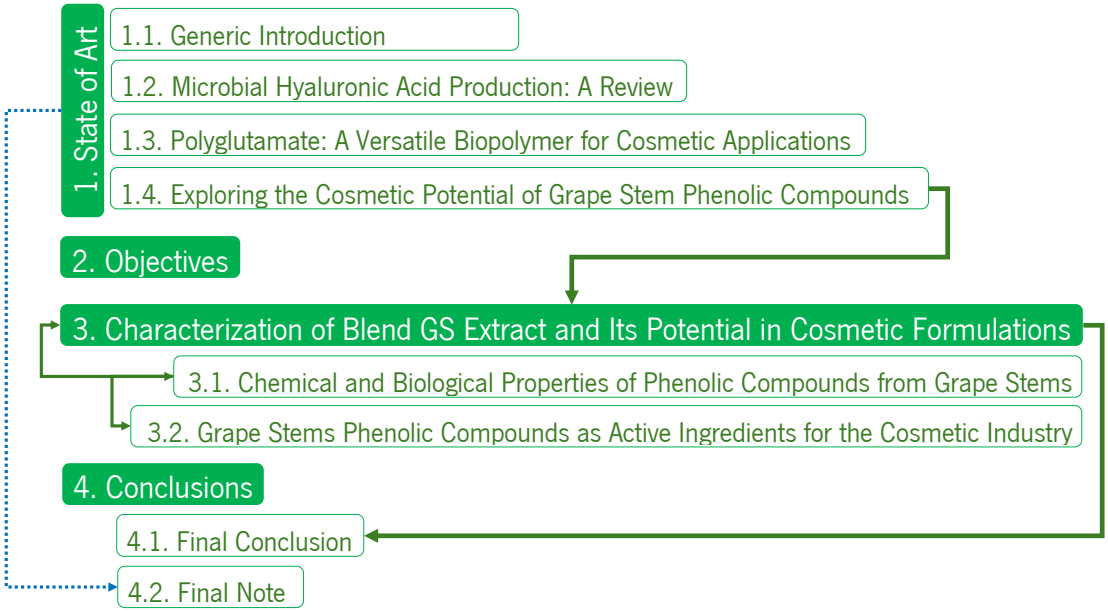

Figure 1. Schematic representation of the thesis.

The thesis begins with an overview of the state of art regarding biopolymers and antioxidants, highlighting their sustainable production and potential applications in cosmetics *(1. State of art)*. It presents a detailed analysis of the chemical composition of grape stem extracts, emphasizing phenolic compounds, which are known for their antioxidant and anti-ageing properties. By employing analytical techniques, this research characterizes these compounds and demonstrates their suitability for use in cosmetic products *(3.1. Chemical and Biological Properties of Phenolic Compounds from Grape Stems)*.

Following the chemical analysis, the thesis explores the development of a cosmetic cream with grape stem extract *(3.2. Grape Stems Phenolic Compounds as Active Ingredients for the Cosmetic Industry)*. This section covers a comprehensive characterization of the new formulation's physical, chemical, and biological properties. The thesis also describes the formulation stability tests. This research reflects **Mesosystem**'s commitment to innovation and quality.

Ultimately, this thesis highlights the transformative potential of integrating sustainable practices into the cosmetic industry. By embracing eco-friendly alternatives and utilizing natural resources, companies like **Mesosystem** can enhance product performance while contributing to a more sustainable future.

This research combines chemistry, biology, and business, highlighting the connection between eco-friendly practices and product innovation. As the cosmetic industry grows, the findings in this study can guide companies aiming to benefit both society and the environment.

1. State of art

1.1. Generic Introduction

In recent years, consumer preferences have shifted significantly towards products that emphasize health, safety, and environmental sustainability. This trend is particularly noticeable in the cosmetic industry, where increasing awareness of the risks associated with synthetic chemicals has heightened demand for natural alternatives [1,2]. Consumers are now more concerned about the long-term health effects and environmental impact of the ingredients in their beauty products [3–5].

Synthetic chemicals, such as preservatives, polymers and artificial fragrances, pose notable health risks. These substances can cause skin irritation, allergic reactions [6], and other serious health issues due to the lack of biocompatibility [7,8]. Endocrine-disrupting chemicals, which interfere with hormone functions, are particularly concerning, as they have been linked to hormone-related cancers, thyroid disorders, reproductive problems, and neurological issues [9].

Environmental concerns also arise from synthetic compounds. Many of these chemicals are not easily biodegradable and can persist in wastewater, leading to contamination of freshwater ecosystems. This pollution disrupts aquatic life, causing reproductive and behavioural changes, and contributes to habitat destruction and high carbon emissions during their production [10,11]. In response to these issues, there is increasing interest in the use of natural compounds in cosmetics [12]. This shift is not merely about replacing synthetic chemicals, but also about embracing the principles of the circular economy. Circular economy emphasizes resource efficiency by minimizing waste and reusing materials in a closed-loop system, contrasting with the traditional linear model of production and disposal [3]. In cosmetics, this approach involves repurposing natural waste materials (such as agricultural by-products and plant residues) into valuable bioactive compounds [12,13].

Bioactive compounds, including antioxidants, biopolymers, and essential oils, offer various skin benefits and can be used to create sustainable, high-performance cosmetic products [12,14]. Natural ingredients are generally more compatible with the skin, reducing the risk of irritation and allergic reactions. Additionally, sourcing ingredients from waste materials can enhance sustainability, lower production costs, and minimize environmental impact [13–16].

Focusing on sustainability and in alignment with Sustainable Development Goals (Figure 2), this work focused on two main areas: the production of biopolymers, hyaluronic acid and polyglutamate, through fermentation, and the extraction of bioactive compounds, particularly phenolic, from natural sources. Key sources of these compounds include by-products from the wine industry, especially grape stems.



Figure 2. United Nations Sustainable Development Goals. Adapted from https://www.un.org/pt/teach/SDGs (accessed on 21 September 2024)

Hyaluronic acid and polyglutamate are well-known in cosmetics for their moisturizing and anti-inflammatory properties, making them valuable in skincare formulations. Similarly, phenolic compounds are gaining attention for their antioxidant, anti-ageing, and anti-inflammatory benefits, positioning them as promising ingredients in the cosmetic sector.

These efforts are closely aligned with the principles of the circular economy, a concept promoted by the European Union's sustainability framework. Alternative methods for producing these biopolymers have been explored using agricultural residues and industrial waste, such as cheese whey, sheep wool peptone, molasses, corn steep liquor, and dry mushroom residues. This approach not only reduces waste but also offers a sustainable source of high-value materials for the cosmetics industry.

The extraction of phenolic compounds from agricultural by-products is another area of focus, adding value to materials that would otherwise be discarded. This approach aligns with the EU's circular economy goals, which aim to create a more sustainable, resource-efficient economy by reducing waste and maximizing the use of renewable resources. These initiatives contribute to Europe's Sustainable Development Goals (SDGs), particularly:

SDG 9 Industry, Innovation, and Infrastructure: Developing innovative methods for extracting and producing natural compounds supports the advancement of sustainable industrial practices.

SDG 12 Responsible Consumption and Production: By minimizing waste and maximizing the reuse of resources, these approaches promote more sustainable production methods in line with European targets.

SDG 13 Climate Action: Reducing the environmental impact of cosmetic production by utilizing waste and by-products helps lower carbon emissions and supports the EU's climate action objectives.

SDG 15 Life on Land: Sustainable sourcing of raw materials and minimizing habitat destruction contribute to the protection and restoration of terrestrial ecosystems.

The approach explored in this research centres on the application of bioactive compounds extracted from grape stems in the cosmetics industry.

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1.2. Microbial Hyaluronic Acid Production: A Review

Adapted from:

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1.2.1. Introduction

Hyaluronic acid (HA) is a naturally occurring biopolymer that is widely distributed in nature. It is a linear, non-sulphated glycosaminoglycan that is composed mainly of repeating units of N-acetylglucosamine and glucuronic acid linked by β-(1-4) and β-(1-3) glycosidic bonds making its structure energetically stable (Figure 3) [1]. Each disaccharide presents a molecular weight (MW) of around 400 Da, and a hyaluronic acid chain can be composed of 10,000 disaccharides, which means a molecular weight of around 4.0×10^3 kDa [2,3].

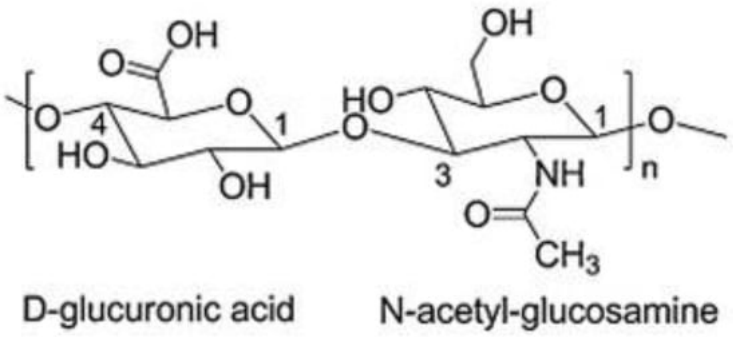

Figure 3. Chemical structure of HA adapted from [1].

Hyaluronic acid plays an important role in living organisms and is an attractive biomaterial for different applications due to its features, in particular moisturizing retention ability, viscoelasticity, resistance to mechanical damage, and lack of immunogenicity and toxicity. In many cases, it acts as a lubricant (joints), a structure stabilizer, an organ space filler (skin), and a shock absorber (cartilage) [4–6].

It is a naturally occurring polysaccharide found in the body that plays a vital role in skin health and beauty. It is a major component of the extracellular matrix (ECM) and is responsible for maintaining skin hydration, elasticity, and volume [5,7]. As we age, the levels of hyaluronic acid in our skin decrease, leading to wrinkles, dryness, and loss of firmness. Therefore, the use of products containing hyaluronic acid has become increasingly popular in the cosmetic industry to combat the signs of ageing and promote healthy, youthful-looking skin [8].

This biocompatible polymer immobilizes the water in the tissue, and it can change the dermal volume that influences cell proliferation, differentiation, and tissue repair. The biological functions depend on its molecular weight, for example, mucoadherence is a property of hyaluronic acid with a high molecular weight. This type of hyaluronic acid is used as space fillers, antiangiogenic and immunosuppressive, while medium-size hyaluronic acid chains are involved in ovulation, embryogenesis, and wound repair. Small chains of hyaluronic acid have inflammatory, immuno-stimulatory, angiogenic, and anti-apoptotic properties [3,7,9].

Hyaluronic acid is a humectant, which means it attracts and retains moisture. It can hold up to 1000 times its weight in water, making it an effective ingredient in moisturizers and serums. By keeping the skin hydrated, hyaluronic acid helps to plump up the skin and reduce the appearance of fine lines and wrinkles. It also improves skin elasticity, making it appear firmer and more youthful. In addition to its ability to hydrate and plump the skin, hyaluronic acid has anti-inflammatory properties that can help to reduce redness and irritation. This makes it a great ingredient for sensitive skin types, as well as for those with conditions such as rosacea or eczema [8,10].

The applications of hyaluronic acid are diverse such as the correction of facial folds and wrinkles, body contouring, and as a marker in the diagnosis of tumours. Hyaluronic acid can also be used in the supplementation of joint fluid, in eye surgery, regeneration of surgical wounds, and as a drug delivery agent for various administration routes [2,4,11,12].

Hyaluronic acid can also be used to enhance the effects of other skincare ingredients. For example, when combined with retinoids, it can help to reduce the dryness and irritation that can be caused by these powerful anti-ageing ingredients [13]. Similarly, when used in combination with antioxidants, it can help to protect the skin from environmental damage [10,14].

While hyaluronic acid is a naturally occurring substance in the body, topical products containing hyaluronic acid are typically derived from either rooster combs or fermentation. There are different types of hyaluronic acid, such as sodium hyaluronate and hyaluronic acid. Sodium hyaluronate is a salt form of hyaluronic acid that is smaller and can penetrate deeper into the skin [15].

The demand and the value of hyaluronic acid have increased over the years. According to R. G. Ferreira et al. [16], the estimated market of hyaluronic acid was EUR 7.6 billion in 2019. It is expected to have an annual growth of 8.1% from 2016 to 2027, which means that 20 MT of hyaluronic acid produced by that year will have an average price between EUR 1500/kg and EUR 4000/kg. The main market segments are derma fillers in cosmetics, osteoarthritis, and ophthalmology. Consequently, it is necessary

to understand the improvement points in the hyaluronic acid production process, including downstream processing [16].

1.2.2. Hyaluronic Acid Production Using the Fermentation Process

Microbial production of hyaluronic acid is an area of research that has been gaining attention in recent years. Hyaluronic acid is widely distributed in nature [1], and it has a wide range of properties such as viscoelasticity, lubrication, and hydration that make it an attractive material for various industrial applications [2,4,11,12].

Microorganisms such as bacteria and yeast have been used to produce HA through fermentation. *Streptococcus zooepidemicus*, a Gram-positive bacteria, is one of the most widely used organisms for the production of HA due to its high hyaluronic acid production rate and ease of cultivation [5,7,17].

Traditionally, hyaluronic acid is extracted from animal sources such as rooster combs and cocks' combs. However, the difficulty in controlling animal tissue, high costs, and ethical concerns associated with animal-derived HA have led to the development of microbial production methods [5,7,9].

During the production of hyaluronic acid, there are still challenges to be overcome, such as the limited production of hyaluronic acid due to the high viscosity of the broth, causing difficulties in the mixing and mass transfer rate of oxygen; competition for the same precursors for cell growth and hyaluronic acid production; and the accumulation of lactic acid, the main by-product of HA fermentation, causing the inhibition of cell growth and hyaluronic acid production [5,7,9].

To overcome these challenges and also increase the microbial production of hyaluronic acid, several strategies have been studied: selection of producing strains and appropriate culture media, the establishment of culture methods, and determination of culture conditions, among others.

1.2.3. Selection of a Microorganism Producer and Its Cultivation Media

The selection of the appropriate microorganism to produce hyaluronic acid is an important factor in the microbial production process. Each microorganism has its own unique advantages and disadvantages in terms of production performance and profitability. When choosing a microorganism to produce hyaluronic acid, it is essential to consider the specific requirements of the final application of this molecule [5,7,9].

The microorganism must be able to produce high yields of hyaluronic acid, be easy to cultivate, and have a low cost of production. Several microorganisms have been used to produce hyaluronic acid through fermentation, including *Streptococcus zooepidemicus*, *Bacillus subtilis*, and *Escherichia coli* [18–21].

Streptococcus zooepidemicus produces a high molecular weight HA with excellent biocompatibility, making it suitable for medical applications such as wound healing and joint lubrication. However, *Streptococcus* is a slow-growing organism, and the production process is costly due to the need for expensive media and downstream processing. However, it does not require the use of toxic chemicals or solvents, resulting in a pure and safe end product [16,22].

Bacillus subtilis is another microorganism that has been used to produce hyaluronic acid. It is a Gram-positive bacterium that is known for its ability to produce high yields of HA and is also non-pathogenic. However, it is considered more difficult to cultivate than *Streptococcus zooepidemicus*. *Bacillus* produces a lower molecular weight hyaluronic acid that is suitable for cosmetic applications such as moisturizing creams and serums. *Bacillus* grows rapidly and is relatively cheap to produce, making it a more profitable option for cosmetic companies [18,23].

Escherichia coli is a Gram-negative bacterium that has been used for the production of hyaluronic acid. It is a well-studied organism and has a wide range of genetic tools available for use in genetic engineering. However, it produces a lower yield of hyaluronic acid compared to *Streptococcus zooepidemicus* and *Bacillus subtilis* [20,21,24].

Another microorganism that has been recently explored for hyaluronic acid production is the yeast *Pichia pastoris*. This organism produces a high yield of low molecular weight HA, making it suitable for use in the pharmaceutical industry. The production process is relatively cheap and can be scaled up easily, making it a promising candidate for large-scale production [25,26].

When considering the profitability and possible applications of the hyaluronic acid produced by each microorganism, it is essential to consider factors such as production costs, purity of the final product, and market demand. For example, the high-molecular-weight hyaluronic acid produced by *Streptococcus* has significant potential in the medical field due to its excellent biocompatibility, making it a high-value product. Meanwhile, the lower-molecular-weight hyaluronic acid produced by *Bacillus* has excellent potential in the cosmetic industry, with demand for hyaluronic acid-based cosmetics continuing to grow.

In conclusion, the selection of the appropriate microorganism for hyaluronic acid production is critical for determining the production performance and profitability of the process. By evaluating the advantages of the final application, producers can optimize their production process, enhance product quality, and increase profitability.

Group A and C *streptococci*, namely *Streptococcus zooepidemicus*, have been the most explored strain in the production process of microbial hyaluronic acid and have obtained the best results. Nevertheless, due to the pathogenicity of this natural HA-producer bacteria, other metabolically engineered microorganisms have been studied, such as *Bacillus subtilis* [18,19,23], *Corynebacterium glutamicum* [27–29], *Escherichia Coli* [20,21,24], *Lactococcus lactis* [30,31], *Pichia pastoris* [25], and *Kluyveromyces lactis* [32]. Among the cited strains, *Corynebacterium glutamicum* has presented the best results [28,29].

The cultivation medium to produce hyaluronic acid is also an important factor to consider. The medium must contain a source of carbon, nitrogen, vitamins, minerals, and other growth factors for the microorganism. Additionally, the medium should be optimized to promote the production of hyaluronic acid by the microorganism [5,26].

Concerning media composition for hyaluronic acid production, an important factor is the cultivation condition. *Streptococcus zooepidemicus* is a nutritionally demanding microorganism, and a nitrogen source is an essential nutrient for its growth. This bacterium does not synthesize some amino acids that are favourable for its growth and hyaluronic acid production. Therefore, media supplementation with nutrients, such as amino acids, has been studied [5,26].

1.2.3.1. Alternative Sources as Substrates for the Culture Media

One of the key factors in the production of hyaluronic acid is the culture medium that is used to grow the microorganism producers.

Traditionally, the culture medium for hyaluronic acid production has been based on complex sugars such as glucose and fructose, but these can be expensive and may not be readily available [33,34]. As a result, researchers have been investigating alternative sources as substrates for the culture medium. Some of the most promising alternative sources include:

- Agricultural waste: By-products of agriculture, such as sugar beet pulp, corn steep liquor, and wheat bran, have been found to be suitable substrates for hyaluronic acid production. These waste products are readily available and have the added benefit of being environmentally friendly [35].
- Industrial waste: Industrial waste products, such as distillery waste and molasses, have also been found to be suitable substrates for hyaluronic acid production. These waste products are often cheaper than traditional substrates, making them an attractive option for commercial production [35,36].

- Synthetic substrates: Some researchers have also investigated synthetic substrates, such as hydrolysates of starch and cellulose, as an alternative substrate for hyaluronic acid production. These substrates are relatively cheap and easy to produce, making them an attractive option for commercial production [37].

Overall, alternative sources as substrates for the culture media for hyaluronic acid production have been explored to reduce the costs of production and make the process more sustainable. While more research is needed to fully understand the potential of these alternative sources, they offer promising possibilities for the future of hyaluronic acid production.

Different agricultural resources and industrial wastes have been explored as alternative nutritive sources for microbial hyaluronic acid. The goal of the formulation of cost-effective culture media is to maintain low costs during microbial hyaluronic acid production and to reduce pollution problems, as well as to improve the efficiency of the fermentation processes.

Amado et al. [36] studied the optimization of a media containing cheese whey to produce hyaluronic acid by *Streptococcus zooepidemicus*. The major nutrients in cheese whey are lactose, soluble proteins (β-lactoglobulin, α-lactalbumin), lipids, and B-group vitamins. Using cheese whey protein and glucose as nitrogen and carbon sources, respectively, the maximum production rate was 0.87 g/L h (0.75 g/L h in control media with glucose and yeast extract), the HA production was 4.02 g/L (3.19 g/L in control media), and the HA average molecular weights (HA-MW) were 3.71×10^3 kDa.

In another study, Amado et al. [35] formulated a culture media containing corn steep liquor instead of tryptone as a nitrogen source. The highest hyaluronic acid production in this media was 3,48 g/L (comparable to the control media of 3.60 g/L) with a molecular weight of 3.8×10^3 kDa (higher than the control media of 3.0×10^3 kDa).

Arslan & Aydogan [38] tested the effectiveness of sheep wool peptone and molasses as nitrogen and carbon sources, respectively, in fermentation media to produce hyaluronic acid, using the same strain as Amado et al. They verified a higher HA production in media containing sheep wool peptone (3.54 g/L) than in media containing tryptone TP (2.58 g/L) and peptone PP (2.47 g/L). Sheep wool peptone has a lower protein content (70.6 g/100 g) than tryptone and peptone (83.1 and 83.3 g/100 g, respectively), and by contrast, sheep wool peptone has higher element contents (K, P, and Mg) than tryptone and peptone, and these elements promote hyaluronic acid production. Moreover, this nitrogen source contains high cystine and arginine contents, which are the main amino acids that affect hyaluronic acid

production. These are possible reasons for the higher production of hyaluronic acid in sheep wool peptone than in tryptone and peptone.

Similarly, Pan et al. [39] developed a media for hyaluronic acid production which contains sugarcane molasses. After molasses treatment with activated charcoal and using it as a substrate for *Streptococcus zooepidemicus*, the hyaluronic acid production was 19% higher than using molasses without treatment. This suggested pre-treatment decreased the excessive metal ions content that can inhibit hyaluronic acid production. After 24 h of fermentation with pH control (pH 8), the hyaluronic acid production was 2.83 g/L.

Pires et al. [40] investigated the use of cashew apple juice in the fermentation media, and the HA production was 0.89 g/L with a molecular weight of 18.4 kDa.

Vázquez et al. [41] explored a culture media using marine by-products as substrates for *Streptococcus zooepidemicus* when producing microbial hyaluronic acid. Firstly, glycogen from mussel processing wastewater and tuna peptone from viscera residue were used as a carbon source and a protein source, respectively. In this media, hyaluronic acid production and the cell growth rate were lower (2.46 g/L and 0.81 g/L/h, respectively) than in commercial media containing glucose and tryptone (3.07 g/L and 1.32 g/L/h, respectively); on the other hand, the hyaluronic acid produced in alternative media presented a higher molecular weight (2.50×10^3 kDa). Later, the same scientists explored a media formulated with peptones obtained from *Scyliorhinus canicula* viscera by-products. Using this alternative substrate as a nitrogen source, hyaluronic acid production was explored in fed-batch culture. Using this culture mode, the hyaluronic acid production (2.53 g/L) and molecular weight (2.11×10^3 kDa) of the polysaccharide were increased compared to production using the batch mode (2.26 g/L; 1.80×10^3 kDa). Compared with the commercial media (3.23 g/L; 0.930 g/L/h; 1.85×10^3 kDa), the hyaluronic acid production and cell growth rate were lower in the alternative media; however, the hyaluronic acid molecular weight was higher [42].

Benedini & Santana [43] studied the effect of replacing Brain Heart Infusion with soy peptone as a nitrogen source for *Streptococcus zooepidemicus*. In this case, there was an increment in hyaluronic acid production and hyaluronic acid molecular weight from 0.29 g/L and 3.09×10^3 kDa to 0.30 g/L and 3.60×10^3 kDa, respectively. The increase in hyaluronic acid molecular weight was related to the diminution of lactic acid production and hyaluronic acid exposition to a higher pH.

Another study was performed in which Ghodke et al. [44] evaluated palmyra palm sugar (Pj) and soya peptone to formulate a media for hyaluronic acid production by *Streptococcus zooepidemicus*. Palmyra palm sugar contains sucrose and vitamins (nicotinic acid, thiamine, riboflavin, and vitamin C) that are

essential to the growth of a microorganism, which led to the hyaluronic acid production and specific growth rate on palmyra palm sugar-based media (0.41 g/L; 0.54 h^{-1}) being higher than on pure sucrose-based media (0.31 g/L; 0.32 h^{-1}). After exploring the best initial substrate concentration (Pj, 30 g/L), the microorganism yielded a hyaluronic acid concentration of 1.22 g/L with 9.50×10^2 kDa.

Zhang et al. [37] proposed a serum-free starch media in which the cells could grow and produce hyaluronic acid. Replacing glucose with starch obtained hyaluronic acid with a yield of 6.7 g/L. The studied strain could grow in serum-free media but not in other carbohydrates such as glucose. This could be due to the reduction in lactic acid (metabolite from glucose catabolism) in the broth for decreasing glucose in the media.

In their work, Duffeck et al. [45] optimized the production of hyaluronic acid in sugarcane molasses pre-treated with active charcoal media (likewise explored by Pan et al. [39]) with nutrient supplementation (glutamine). They concluded that the hyaluronic acid production by *Streptococcus zooepidemicus* was higher in sugarcane molasses media (0.710 g/L) than in glucose media (0.469 g/L) due to the molasses media being rich in sugar and amino acids, and after treatment, this molasses contains fewer inhibitors to hyaluronic acid production. Regarding media supplementation, glutamine supplementation revealed positive effects on hyaluronic acid production, improving the yield of the product to 2.55 g/L, because this amino acid acts as the amino donor group to UDP-N-acetylglucosamine formation (one of hyaluronic acid precursors).

1.2.3.2. Supplementation of Culture Media

The process of media supplementation in the production of hyaluronic acid includes:

- Carbon source supplements: Carbon sources such as glucose, fructose, and maltose can be added to the culture medium to provide an additional energy source for the microorganisms. This can help increase the rate of hyaluronic acid production [46].
- Nitrogen source supplements: Nitrogen sources such as ammonium sulphate and yeast extract can be added to the culture medium to provide essential amino acids and other nutrients for the microorganisms. This can help increase the rate of hyaluronic acid production [33].
- Vitamin supplements: Vitamins such as thiamine, riboflavin, and pyridoxine can be added to the culture medium to promote the growth of microorganisms and increase the rate of hyaluronic acid production [47].

- Mineral supplements: Minerals such as sodium, potassium, and calcium can be added to the culture medium to provide essential minerals for the microorganisms and promote hyaluronic acid production [34].
- Growth factors: Growth factors such as yeast extract, soy peptone, and tryptone can be added to the culture medium to provide additional nutrients and growth factors for the microorganisms [33]. This can help increase the rate of hyaluronic acid production.

It is important to note that the appropriate supplements and the optimal concentration of the supplement in the culture medium can vary depending on the microorganism used for hyaluronic acid production. Researchers often use a combination of different supplements and tweak the concentration to find the best condition to produce hyaluronic acid. It is also important to note that the use of supplements in the culture medium can increase production costs; hence, it is important to find a balance between costs and the enhancement of production.

The substrates used more often in hyaluronic acid production are glucose and sucrose as the primary carbon and energy sources and yeast extract and peptones as nitrogen sources [33,34,48–52]. Other carbon sources have been explored as substrates for hyaluronic acid production, namely, lactose, maltose, galactose, mannose, and others; however, hyaluronic acid production is not as high [18,34,53–55].

In their investigation, Chen et al. [56] used glucose as a carbon source and yeast extract as a nitrogen source to explore the best carbon-to-nitrogen (C/N) ratio and concluded that the C/N ratio of 2:1 maximizes hyaluronic acid synthesis at 2.45 g/L. In other C/N ratios, such as 1,3:1 and 4:1, the hyaluronic acid production was 2.20 g/L and 1.52 g/L, respectively.

Concerning media supplementation, Aroskar et al. [46] also investigated the effect of different nutrients in the culture media for *Streptococcus zooepidemicus* production of hyaluronic acid. In this study, the carbon source used was dextrose for producing hyaluronic acid with a higher yield (0.70 g/L) than other carbon sources used, such as sucrose (0.51 g/L), maltose (0.50 g/L), and dextrin (0.55 g/L). The researchers explored the addition of L-arginine HCl and tannic acid to the fermentation media, which resulted in an increment in the yield of HA to 1.029 g/L. L-arginine HCl is a carbon and nitrogen donor for the synthesis of nucleotides that are needed for the growth and multiplication of microorganisms, thus saving energy consumption in the organism for its production. Tannic acid represses the synthesis of the hyaluronidase enzyme that acts by depolymerizing hyaluronic acid. In light of this, the use of tannic acid showed an increase in the molecular weight of hyaluronic acid.

In the same way, Shah et al. [33] explored the supplementation media for *Streptococcus zooepidemicus* with glutamine and sodium iodoacetate. Iodoacetate decreases lactic acid synthesis by inhibiting the glycolysis pathway; consequently, there was a redirection of the carbon flow from lactic acid formation to UDP glucuronic acid formation, one of the precursors of hyaluronic acid. Glutamine, as mentioned above, is an amino acid involved in the formation of one of the hyaluronic acid precursors. Accordingly, the HA concentration was increased from 2 g/L in media without supplementation to 5 g/L, the specific growth rate was decreased from 0.42 h^{-1} to 0.25 h^{-1}, and the hyaluronic acid molecular weight was increased from 2.4 × 10^3 kDa to 3.2 × 10^3 kDa.

Aiming to obtain HA with high yield and molecular weight, Im et al. [34] studied the optimization of media components using the strain *Streptococcus* sp. ID9102 (KCTC 11935BP), which is negative to haemolytic activity and hyaluronidase. Exploring the best carbon source, a higher hyaluronic acid production was reached when glucose was the carbon source (1.58 g/L) followed by lactose and sucrose, which reached similar results (≈1.3 g/L). According to the results, the investigators selected the best components media as glucose, yeast extract, casein peptone, K_2HPO_4, and $MgCl_2$ and obtained a hyaluronic acid production of 5.88 g/L with a molecular weight of 3.60 × 10^3 kDa. After supplementing the media with amino acids (glutamine and glutamate) and organic acid (oxalic acid), the HA titre and HA-MW were higher (6.94 g/L and 5.90 × 10^3 kDa, respectively).

Kim et al. [47] also studied the production of HA from *Streptococcus* equi mutant KFCC 10830. This mutant has nonhemolytic and hyaluronidase-negative characteristics. Furthermore, the scientists explored the effect of lysozyme addition during cultivation on the fermentation media. Lysozyme can damage the cell walls of hyaluronic acid producer microorganisms, which then produce more hyaluronic acid to protect themselves from the action of lysozyme. According to this, the addition of lysozyme increased the production and molecular weight of hyaluronic acid.

1.2.4. Determination of Culture Conditions

Another important factor to develop a successful process for producing microbial hyaluronic acid is identifying ideal fermentation parameters.

The culture conditions refer to the various factors that can affect the growth and production of the microorganisms used in hyaluronic acid production. Some of the key culture conditions that must be determined include:

- Temperature: The optimal temperature for the growth of the microorganisms and the production of hyaluronic acid will vary depending on the species used. The temperature range that is most suitable to produce HA is usually between 30 and 37 °C [50].
- pH: The pH of the culture medium is another important culture condition that must be determined. The pH must be maintained within a specific range that is suitable for the growth of the microorganisms and the production of hyaluronic acid. The optimal pH range for HA production is usually between 6.5 and 7.5 [57,58].
- Aeration: Adequate aeration is necessary for the growth of the microorganisms and the production of hyaluronic acid. The amount of air supplied to the culture medium can be adjusted to achieve the optimal aeration for the microorganisms used [59].
- Agitation: Agitation is the process of physically stirring the culture medium to ensure that the microorganisms are well-mixed and have access to nutrients and oxygen. The optimal agitation rate will depend on the microorganisms used and the culture medium [49].
- Substrate concentration: The concentration of the substrate in the culture medium will affect the rate of hyaluronic acid production. The optimal substrate concentration will depend on the microorganisms used and the culture medium [51].
- Supplementation: Supplementation of the culture medium with various nutrients and growth factors can also affect the rate of hyaluronic acid production. The optimal concentration of supplements will depend on the microorganisms used and the culture medium [48,49,60].

Determining the optimal culture conditions to produce hyaluronic acid will typically involve a process of experimentation and optimization. Researchers will often use a combination of different culture conditions and tweak the various factors to find the conditions that yield the highest hyaluronic acid production [57–61].

In their work, Johns et al. [57] identified the optimum pH as 6.7. A high agitation rate of 600 rpm was found to be advantageous to release the HA capsule from *Streptococcus zooepidemicus* bacteria cells into the medium; however, a higher shear rate can damage the polymer. In addition, the use of aerated cultures enhanced the hyaluronic acid concentration, maybe due to the highest energy yield.

David C. Armstrong & Johns [50] described the effect of different culture conditions on the molecular weight of HA produced by *Streptococcus zooepidemicus*. Concerning temperature, their results showed that lower temperatures (32 °C) resulted in higher molecular weight and higher yield of the produced hyaluronic acid than higher temperatures (at 40 °C: 1.85×10^3 kDa and 1.20 g/L, respectively), and by

decreasing the temperature, the specific growth rate also decreased, as the two processes (cell growth and HA production) are competitors for the same precursors. Their results also showed that higher aeration rates (1.0 vvm) enhanced HA production and hyaluronic acid molecular weight from 1.45 g/L and 2.10 × 10^3 kDa (0.2 vvm) to 4.20 g/L and 3.0 × 10^3 kDa, respectively. From the other culture conditions explored, the initial glucose concentration also presented an influence on hyaluronic acid production. A higher initial glucose concentration (60 g/L) in the fermentation broth enhanced the HA concentration and HA-MW, which could be due to HA precursors (UDP-GlcUA and UDP-GlcNAc) being derived from glucose. However, the initial glucose concentration is limited to 60 g/L because at higher concentrations, there are limitations in mass transfer due to the high broth viscosity.

Similarly, Don & Shoparwe [51] explored the effect of glucose in the fermentation media for S. zooepidemicus on HA production. The highest hyaluronic acid molecular weight (2.52 × 10^3 kDa) was reached when the initial glucose concentration was 40 g/L. Above these concentration levels, there was a reduction in hyaluronic acid molecular weight.

To achieve the highest concentration of hyaluronic acid (5.3 g/L), Zakeri & Rasaee [52] identified the best fermentation conditions. This way, after they explored various fermentation conditions, they proposed HA production with the following conditions: 37 °C, pH 7, 300 rpm, and DO (dissolved oxygen) 50%. Moreover, they also identified the best sources of carbon and nitrogen: glucose (70 g/L) and yeast extract (30 g/L).

Using the same substrates as Zakeri & Rasaee [52], Huang et al. [59] explored the role of dissolved oxygen and the effect of agitation on hyaluronic acid production using fermentation. They found an increment in hyaluronic acid yield (from 2.7 g/L to 3.1 g/L) when dissolved oxygen increased from 2.5% to 5%, and above these values of dissolved oxygen, the HA yield was not influenced. So, the investigators found a critical level of dissolved oxygen to produce HA, and they suggested dissolved oxygen to have a stimulant role in hyaluronic acid synthesis. Concerning agitation, its main function is to mix the broth; however, agitation can favour oxygen absorption by cells. At 400 rpm (0.74 g HA/g cell), the hyaluronic acid productivity was higher than at 200 rpm (0.65 g HA/g cell).

Under aerobic conditions at 37 °C and with 200 rpm agitation, L Liu et al. [58] explored an intermittent alkaline-stress strategy. Under stress conditions at pH 8.5 for 1 h to 6 h of fermentation, a hyaluronic acid concentration of 6.5 g/L was obtained (higher than control conditions: 5.0 g/L). This increase in productivity may be due to the redirection of the carbon flow (more availability of carbon source to produce hyaluronic acid rather than cell growth or lactic acid production) and/or the increase in dissolved oxygen that was found in stress conditions.

Oxygen mass transfer has an important role in hyaluronic acid production. Nonetheless, the high viscosity of the media caused by hyaluronic acid production can challenge ideal mixing and uniform oxygenation. To overcome this problem, Saharkhiz & Babaeipour [60] studied the effect of diluting the culture media controlling pH with a low concentration of NaOH. The dilution improved the mass transfer of oxygen, and the hyaluronic acid production was enhanced from 6.6 g/L to 8.4 g/L.

Long Liu et al. [49] proposed three strategies to control dissolved oxygen in the production media. Firstly, they applied a three-stage agitation speed control culture model in which they varied the agitation speed between 200 rpm (0–8 h), 400 rpm (8–12 h), and 600 rpm (12–20 h). The HA productivity was enhanced from 5.0 g/L at constant agitation speed (200 rpm) to 5.5 g/L. This may be due to the increase in the dissolved oxygen level that is favourable to the growth of the cells, and the increase in agitation that induces shear stress to cells. Following the previous model, the investigators explored a two-stage dissolved oxygen control model. In this model, they controlled the dissolved oxygen level at the 10% level during the first 8 h of the fermentation process, and after, they decreased the dissolved oxygen level to 5% using agitation speed control. Once there was a difference in the critical dissolved oxygen levels for cell growth and hyaluronic acid production, with this strategy, it was possible to redirect the carbon flow in the first phase of the fermentation process for cell growth and, later, for the production of hyaluronic acid. As a result, the HA production and productivity increased to 6.3 g/L and 0.0984 g/L/h (from 5 g/L and 0.0694 g/L/h, respectively). Another strategy to improve dissolved oxygen levels in the broth is media supplementation with oxygen vectors. In this way, Long Liu et al. [36] explored the effect of PFC (perfluorodecalin) addition to the media at 8 h of the process. It was found that perfluorodecalin improved the dissolved oxygen levels from 0.5% to 5% at a low agitation speed (200 rpm), and the HA titre in the media was 6.6 g/L.

Likewise, Z. W. Lai et al. [48] investigated the effect of n-hexadecane as an oxygen vector. This oxygen vector enhanced the hyaluronic acid concentration from 2.45 g/L to 4.25 g/L and the hyaluronic acid molecular weight from 5.20×10^3 kDa to 1.54×10^4 kDa. So, this suggested n-hexadecane can be used as an oxygen vector and organic phase for increasing the molecular weight of hyaluronic acid. This investigation of Lai et al. [48] confirmed what was reported in the Duan et al. [62] studies. In their work, they explored the effect of dissolved oxygen levels on the molecular weight of hyaluronic acid and concluded that molecular weight is dependent on the balance of the effect of ATP and reactive oxygen species (ROS) in the culture media. Moreover, higher dissolved oxygen levels enhanced ATP levels and ROS in the media. As it is known, ATP and ROS levels have contrary effects, so the highest HA-MW (2.19

× 10^3 kDa) was reached on the balance of these two effects at the 50% dissolved oxygen level. Above this level (80% DO), the HA-MW was decreased (2.06 × 10^3 kDa).

To achieve a balance between a higher hyaluronic acid concentration and a higher hyaluronic acid molecular weight, J. Liu et al. [63] developed a two-stage fermentation strategy. The highest hyaluronic acid concentration and hyaluronic acid molecular weight were reached at the same conditions of aeration rate and agitation speed (1 vvm and 600 rpm, respectively). However, these values of concentration and molecular weight were achieved at different conditions of temperature and pH. The first stage of fermentation was performed at pH 8 and 31 °C allowing for the best conditions for molecular weight growth, and lastly, the final stage of the process was conducted at pH 7 and 37 °C to promote HA accumulation. The HA titre and hyaluronic acid molecular weight increased from 2.99 g/L and 2.26 × 10^3 kDa to 4.75 g/L and 2.36 × 10^3 kDa, respectively.

Li et al. [64] evaluated the production of hyaluronic acid with different MW from a single-producing bacterium, *Bacillus subtilis*. The scientists obtained high-, medium-, and low-MW HAs by adjusting the temperature (32 °C, 42 °C, and 47 °C, respectively); the highest molecular weight of hyaluronic acid (6.19 × 10^3 kDa) was obtained at 47 °C with a hyaluronic acid concentration of 1.88 g/L. However, the highest hyaluronic acid concentration (4.25 g/L) with 8.61 kDa was achieved at 32 °C.

1.2.5. Fermenter Configuration

Fermenter configuration refers to the design and setup of the equipment used in the fermentation process for the production of hyaluronic acid [65]. A typical fermenter setup includes the following components:

- The fermenter vessel: This is the main container where the fermentation process takes place. The vessel is typically made of stainless steel and can be designed in a variety of shapes and sizes depending on the scale of the production.
- Agitator: This is a mechanical device that is used to mix the culture medium and provide oxygen to the microorganisms. Agitators can be designed in a variety of forms, such as propellers, impellers, and turbines, and can be adjusted to provide the optimal agitation rate for the microorganisms used.
- Aeration system: This is a device that is used to supply oxygen to the culture medium. Aeration systems can be designed in a variety of forms such as spargers, air bubblers, and air diffusers.
- pH and temperature control: These devices are used to monitor and maintain the pH and temperature of the culture medium within the optimal range for the growth and production of hyaluronic acid.

- Control system: This is a device that is used to control and monitor the various parameters of the fermentation process such as temperature, pH, agitation rate, and aeration rate.

The design and configuration of the fermenter will depend on the scale of production and the microorganisms used. Additionally, a large-scale industrial fermentation system is equipped with additional features such as automatic sampling, automatic pH and temperature control, automatic foaming control, automatic sterilization, etc.

Several scientists have investigated agitation speed and impeller type to improve the oxygen transfer coefficient. Z. W. Lai et al. [48] explored the effect of the helical ribbon and the Rushton turbine impeller, and they verified that KLα (gas–liquid volumetric oxygen transfer coefficient) was enhanced by 25% using the helical ribbon as compared to the Rushton turbine impeller. A decrease in KLα is related to the increase in viscosity, which affects the oxygen transfer rate. Using the helical ribbon impeller, the gas–liquid volumetric oxygen transfer coefficient was improved to 1.047 m/s impeller tip speed; however, the fermentation performance improved at a 0.785 m/s impeller tip speed. This decrease in fermentation performance could be due to the high shear rate on bacterial cells.

In their work, Kim et al. [47] tested two different types of impellers: Rushton type and intermig type. The hyaluronic acid production was similar using the two impellers under the same fermentation conditions, but there was an increase in molecular weight of hyaluronic acid using the intermig type (4.8×10^3 kDa) instead of the Rushton type (3.8×10^3 kDa). Despite that, the Rushton-type impeller has been the most used system to agitate fermentation [49,58,66].

1.2.6. Establishment of Culture Modes

Establishing the modes of culture is an important step in the production of hyaluronic acid using microorganisms. The mode of culture refers to the method used to grow and maintain the microorganisms used in the fermentation process. Some common modes of culture include:

- Batch culture: In batch culture, the microorganisms are grown in a closed vessel for a specific period of time, after which the culture is harvested, and the hyaluronic acid is extracted. Batch culture is simple and easy to set up, but it has the disadvantage of being less efficient and more costly than other modes of culture [31,56].
- Fed-batch culture: In fed-batch culture, the microorganisms are grown in a closed vessel and are periodically fed with a specific substrate or supplement. This allows for a higher concentration of

microorganisms and a higher rate of hyaluronic acid production. Fed-batch culture is more efficient than batch culture, but it is more complex to set up and control [67].

- Continuous culture: In continuous culture, the microorganisms are grown in a closed vessel and are continuously supplied with a fresh medium. This allows for a high concentration of microorganisms and a high rate of hyaluronic acid production. Continuous culture is the most efficient mode of culture, but it is also the most complex to set up and control [56].

Each mode of culture has its own advantages and disadvantages, and the most appropriate mode of culture will depend on the microorganisms used and the scale of production. Researchers often use a combination of different modes of culture and tweak several factors to find the conditions that yield the highest hyaluronic acid production.

Fermentation process development includes the establishment of a culture mode. The most used culture mode to produce hyaluronic acid has been the batch mode [31,50,56,67]. In their work, Long Liu et al. [67] explored the effect of different culture modes on hyaluronic acid production, and they found higher hyaluronic acid concentration (5.0 g/L) using the batch mode than using the fed-batch mode (4.72 g/L); however, the cell concentration increased from 13.3 g/L in the batch mode to 14.7 g/L in the fed-batch mode.

These results suggest that the fed-batch mode is preferred for cell growth, and the batch mode is more suitable for producing hyaluronic acid. Consequently, investigators explored a two-stage cultivation strategy in which the cultivation was performed in the fed-batch mode in the early phase of the fermentation process and lastly in batch mode. As a result, the hyaluronic acid concentration was higher (6.6 g/L) than the process performed in batch (5 g/L) and fed-batch culture (4 g/L) [68].

However, the batch mode has some limitations such as the long turnaround time and decreasing hyaluronic acid production due to inhibition by a high carbon source concentration. Thus, Chen et al. [56] proposed a fill-and-draw operation to improve the batch mode culture, such as reducing the turnover time of the fermenter. The investigators found that the operation had better results when performed in the late exponential growth phase (8 h), and the best quantity of displaced media was one-third of the broth. The hyaluronic acid concentration was 1.60 g/L.

Huang et al. [69] suggested another mode to skip the turnaround time on the fermenter and the lag phase of the process: the repeated batch mode. Seeding with 5% of the broth, the cell and hyaluronic acid concentration (2.3 g/L) were maintained as in the batch culture for seven repeated cycles. When

the seed was increased, the hyaluronic acid production decreased, which suggested there were inhibitors for hyaluronic acid production in the broth.

To maintain the environmental and intracellular conditions in the fermentation media, Badle et al. [70] explored the chemostat mode. In this mode, they found an inverse relationship between the molecular weight of hyaluronic acid and the dilution rate of the media, and the best dilution rate found was 0.066 h^{-1} to produce HA with 2.6 × 10^3 kDa. Other dilution rates (0.1, 0.2, and 0.3 h^{-1}) lowered the molecular weight of the hyaluronic acid (2.2, 2.1, and 1.4 × 10^3 kDa, respectively). In this work, they also confirmed an inverse relationship between the molecular weight of hyaluronic acid and the specific cell growth rate because, since the doubling time of a cell is longer, the cells have more time to elongate the hyaluronic acid chain.

Jagadeeswara Reddy et al. [71] reported the optimization of fermentation conditions to produce hyaluronic acid by *Streptococcus* nonepidemic mutant. Using the batch mode with the following fermentation conditions: pH 7.2, 36 °C, 400 rpm, and 0.6 vvm, they achieved hyaluronic acid with a concentration of 1.84 g/L. In another study, using the same *Streptococcus* strain and the same fermentation conditions, scientists reached a higher hyaluronic acid concentration (2.34 g/L) with 2.5 × 10^3 kDa MW using the fed-batch mode [22].

The fed-batch mode also proved to be advantageous in hyaluronic acid production by *Corynebacterium glutamicum* mutant bacteria. Cheng et al., 2016b [28] explored the hyaluronic acid production in batch mode at 28 °C, pH 7.2, 600 rpm, and 1 vvm. The hyaluronic acid production reached 8.3 g/L at 48 h, and the molecular weight of the hyaluronic acid was 1.30 × 10^3 kDa. On the other hand, the authors performed the fermentation process using the fed-batch mode, and the hyaluronic acid concentration was enhanced to 21.6 g/L under the same fermentation conditions when produced by the same strain in the same production media concerning the process described above [29].

The culture conditions and culture media that have been explored for the microbial production of hyaluronic acid by various bacteria strains in different culture modes are shown in Table 1.

Table 1. Different fermentation conditions and culture modes for producing microbial HA by various microorganisms in different culture media.

Microorganism	Culture Media	Fermentation Conditions	Culture mode	[HA] / MW	Ref.
Streptococcus equi subsp. Zooepidemicus ATCC 35246	Cheese whey protein Glucose Yeast extract Tryptone	pH 6.7 T 37 °C 500 rpm 1 vvm	Batch mode 5 L-bioreactor	4.0 g/L -12 h 3.71 × 10^3 kDa 0.87 g/L h	[36]

Microorganism	Culture Media	Fermentation Conditions	Culture mode	[HA] / MW	Ref.
Streptococcus equi subsp. zooepidemicus ATCC 35246	Molasses Sheep Wool Peptone	pH 8 T 37 °C 200 rpm 1 vvm	250-mL flasks	3.54 g/L - 48 h	[38]
Streptococcus equi subsp. zooepidemicus ATCC 35246	Glucose Yeast extract Peptone from *Scyliorhinus Canicula* visceral treatment	pH 6.7 T 37 °C 500 rpm 0 vvm	Fed-batch mode 2-L bioreactor	2.53 g/L - 18 h 2.11×10^3 kDa	[42]
Streptococcus zooepidemicus NJUST01	Starch (5%) Glucose Peptone Yeast extract	T 37 °C 220 rpm	Batch mode 500-mL flasks	6.7 g/L - 36 h	[37]
Streptococcus sp. ID9102 mutant strain KCTC 1139BP	Glucose Yeast extract Casein Peptone K_2HPO_4 $MgCl_2$ Glutamine Glutamate Oxalic acid	pH 7 T 36 °C 400 rpm 0.5 vvm	Batch mode 75-L jar fermenter	6.94 g/L - 24 h 5.9×10^3 kDa	[34]
Streptococcus zooepidemicus WSH-24	Sucrose Yeast extract 3% PFC as an oxygen vector	pH 7 T 37 °C 200 rpm 0.5 vvm	Batch mode 7-L bioreactor	6.6 g/L - 20 h	[49]
Streptococcus zooepidemicus WSH-24	Sucrose Yeast extract	T 37 °C 200 rpm aeration 2L/min alkaline-stress fermentation	Batch mode 7-L bioreactor	6.5 g/L - 16 h	[58]
Streptococcus zooepidemicus WSH-24	Sucrose Yeast extract	pH 7 T 37 °C 200 rpm 0.5 vvm	fed-batch mode (0-8h) batch mode (8-20h) 7-L bioreactor	6.6 g/L - 20 h 0.023 g HA/g cell/h	[68]
Streptococcus equi, subsp. zooepidemicus ATCC 39920	Glucose Yeast extract BHI Ascorbic Acid	pH 7 T 37 °C 200 rpm 1 vvm dilution rate 0.066 h^{-1}	Chemostat mode 2,4 L Bioengineering reactor	2.6×10^3 kDa	[70]
Streptococcus thermophilus NCIM 2904	Whey Permeate Whey Protein hydrolysate	pH 8 T 37 °C 150 rpm	Batch mode 250-mL flasks	0.34 g/L - 20 h 9.22–9.46 kDa	[72]
Bacillus subtilis WmB	Sucrose	T 32 °C	5.0-L fermenter	3.65 g/L - 54 h 3.92×10^2 kDa	[61]

Microorganism	Culture Media	Fermentation Conditions	Culture mode	[HA] / MW	Ref.
*Corynebacterium glutamicum/Δldh-*AB	Corn Syrup Powder Glucose IPTG (isopropyl- β -D-thiogalactoside)	pH 7.2 T 28 °C 600 rpm 1 vvm	Fed-batch mode 5.0-L fermenter	21.6 g/L - 48 h 1.28×10^3 kDa	[29]

1.2.7. Conclusions

In conclusion, the production of hyaluronic acid using microorganisms involves a complex process that includes selecting the appropriate substrate, supplements, and culture conditions. Alternative sources, such as agricultural waste, industrial waste, and synthetic substrates can be used as substrates in the culture media, but the most appropriate substrate will depend on the microorganisms used and the scale of production. Supplementation of the culture medium with various nutrients and growth factors can also affect the rate of hyaluronic acid production. The optimal culture conditions will depend on the microorganisms used and include factors such as temperature, pH, aeration, agitation, and substrate concentration. Fermenter configuration is also important, and the design and configuration of the fermenter will depend on the scale of production and the microorganisms used. The establishment of the mode of culture is also crucial and because each mode has its own advantages and disadvantages, researchers often use a combination of different modes of culture to find the conditions that yield the highest hyaluronic acid production.

Fermentation is a widely used process to produce hyaluronic acid since it allows the optimization of yield and molecular weight due to the possibility of controlling all its main stages. Nonetheless, there are some challenges to be overcome in the microbial production of hyaluronic acid such as the limited production of hyaluronic acid due to the high viscosity of produced hyaluronic acid, competition for the same precursors between microorganism growth and HA production, accumulation of by-products in the production media, and consequently, the inhibition of hyaluronic acid production [5,7,9].

In a single process of hyaluronic acid microbial production, hyaluronic acid with different molecular weights can be obtained. Obtaining hyaluronic acid of low dispersity has been a motivation for scientists to continue to explore and optimize the process [5].

As stated above, the microorganism used most often in industrial hyaluronic acid production is *Streptococcus zooepidemicus*, although it is a pathogen. Thereby, the demand for safe hyaluronic acid producer microorganisms has also been a challenge. Investigators have used metabolic engineering to

obtain recombinant microorganisms capable of producing hyaluronic acid with desired concentration and MW, such as *Bacillus subtilis* and *Corynebacterium glutamicum* [19,23,27,28,29,61].

Finally, at the industrial scale, is important to maintain the low cost of hyaluronic acid production. To address this, researchers have studied alternative culture media, such as molasses, cheese whey, and tuna peptone, among others [35,36,39,45]. Furthermore, the most significant cost in hyaluronic acid production is the downstream process, so different downstream strategies have been explored to achieve hyaluronic acid with high purity to be applied in medical fields [16].

1.2.8. References

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1.3. Polyglutamate: A Versatile Biopolymer for Cosmetic Applications

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1.3.1. Introduction: *Polyglutamic Acid—An Overview*

Polyglutamate, also known as poly-γ-glutamic acid (PGA), was first discovered in "natto", a type of fermented soybean. This biopolymer is derived from glutamic acid and is characterized by its unique structure, featuring anionic natural polymer chains linking α-amino groups and γ-carboxyl groups through amide bonds (Figure 4). The molecular weight (MW) of this homo-polyamide γ-PGA ranges from 50,000 to 2 million Daltons. The polymer composition can include a homopolymer derived from either L- or D-glutamic acid, or a heteropolymer combining both L- and D- enantiomers [1,2].

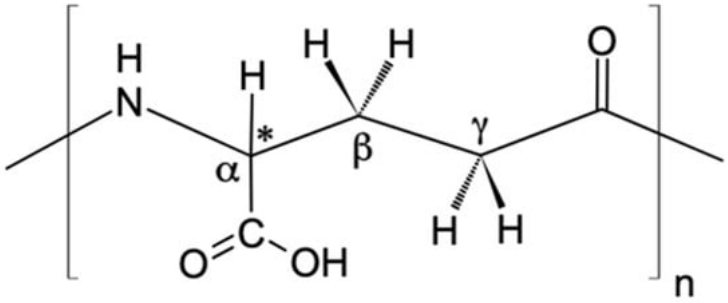

Figure 4. Chemical structure of polyglutamic acid.

Polyglutamate, or poly-γ-glutamic acid, is found in various natural sources, including specific micro-organisms and fermented foods [3]:

- Micro-organisms: Certain bacteria, particularly strains of Bacillus species, are known to produce PGA as a part of their metabolic processes. *Bacillus subtilis* is one of the most well-studied producers of PGA. These bacteria synthesize PGA intracellularly and excrete it into the surrounding environment. PGA production by micro-organisms is often stimulated under conditions of stress or nutrient limitation, suggesting a role for PGA in microbial adaptation and survival.
- Fermented Foods: PGA can also be found in certain fermented foods that undergo microbial fermentation processes. Fermented soybean products like natto, a traditional Japanese food, contain PGA produced by Bacillus subtilis during the fermentation of soybeans. Additionally, other fermented foods

such as fermented soy sauce and Korean kimchi may also contain PGA as a result of microbial activity during the fermentation process.

- Environmental Sources: Apart from microbial and food sources, PGA has also been detected in various environmental settings. For example, PGA-producing bacteria have been isolated from soil, water, and plant surfaces, indicating that PGA may play a role in microbial ecology and environmental interactions. The presence of PGA in environmental samples highlights its ubiquity and suggests its potential ecological significance beyond microbial metabolism.
- Biotechnological Production: In addition to natural sources, PGA can also be produced through biotechnological processes using recombinant micro-organisms or enzymatic synthesis. This approach allows for the controlled and scalable production of PGA for various industrial and biomedical applications, including cosmetics, pharmaceuticals [4–6], and biodegradable materials [7–9].

Overall, polyglutamate, or PGA, is derived from a variety of natural sources, including specific micro-organisms involved in fermentation processes and environmental samples. Understanding the diverse sources of PGA is essential for exploring its potential applications in various fields and harnessing its beneficial properties for human health and biotechnological advancements.

Bacteria, particularly strains of *Bacillus* species such as *Bacillus subtilis* and *Bacillus licheniformis*, are major producers of polyglutamate, often secreting it as a protective capsule [2].

Bacteria produce polyglutamic acid (PGA) as a strategy to adapt to their environment and enhance their survival. Some reasons why bacteria produce PGA include the following:

- Protection and Defence: PGA can act as a protective barrier around bacterial cells, shielding them from harsh environmental conditions such as extreme temperatures, pH fluctuations, and desiccation. This protective function helps bacteria withstand adverse conditions and enhances their chances of survival.
- Biofilm Formation: PGA is a key component of bacterial biofilms, which are complex communities of micro-organisms encased in a matrix of extracellular polymeric substances (EPS). Biofilms provide bacteria with protection against antimicrobial agents, immune responses, and other environmental stresses. PGA contributes to the structural integrity and stability of biofilms, facilitating bacterial colonization on surfaces and promoting community interactions.
- Nutrient Storage: Bacteria can utilize PGA as a reservoir for storing excess carbon and nitrogen. During periods of nutrient abundance, bacteria produce and accumulate PGA as a means of storing

surplus energy and resources. Later, when nutrient availability becomes limited, bacteria can degrade PGA and utilize it as a source of carbon and nitrogen for growth and metabolism.

- Virulence and Pathogenesis: In some pathogenic bacteria, PGA production is associated with virulence factors and contributes to the establishment and progression of infections. PGA can facilitate bacterial adherence to host tissues, evasion of host immune responses, and formation of biofilm-associated infections. By promoting bacterial colonization and persistence within the host, PGA enhances the pathogenicity of certain bacterial species [10,11].
- Environmental Adaptation: Bacteria inhabit diverse ecological niches with varying conditions, and the production of PGA allows them to adapt to different environments. PGA-producing bacteria are found in environments ranging from soil and water to the human body, highlighting the versatility of PGA as an adaptation mechanism.

Overall, the production of PGA by bacteria serves multiple functions that contribute to their survival, adaptation, and interaction with their surroundings. Understanding the roles of PGA in bacterial physiology and ecology is important for elucidating bacterial behaviour in natural environments as well as in the context of human health and disease [12–14].

Managing the complexities of PGA biosynthesis can be challenging, particularly in controlling the molecular weight of the chains and the variability of the D/L ratio. These factors significantly influence the polymer's physical structure and behaviour.

Polyglutamic acid (PGA) exhibits diverse applications due to its unique properties. For instance, PGA with lower molecular weight serves as a drug carrier and cryoprotectant, while the L-glutamic acid enantiomer, acting as a humectant, is preferred for cosmetic uses. The conformation of PGA can vary between an α-helix, a β-sheet, a helix-to-random coil transition, or an enveloped aggregate, depending on factors like pH and polymer concentration [15]. Given its versatility, PGA finds utility across multiple sectors, including food, cosmetics, pharmaceuticals, and agriculture. Cosmetics, defined as substances applied to external body parts, such as the skin, hair, nails, and mucous membranes within the oral cavity, are designed to enhance appearance, tactile sensation, and overall well-being. These products serve various functions, including cleansing, moisturizing, protecting, and enhancing the natural beauty of these tissues [1,2]. In cosmetics, its ability to retain moisture, form films, and stimulate collagen production is highly valued [16,17]. PGA is incorporated into skincare formulations to enhance hydration, reduce wrinkles, and strengthen the skin barrier against external stressors. In aesthetic procedures, it is used in dermal fillers to smooth wrinkles and other imperfections [18].

Furthermore, PGA has found utility in biomedicine for drug delivery and tissue engineering. It encapsulates and protects bioactive agents, ensuring controlled release and targeted delivery, owing to its biocompatibility and biodegradability. As a result, it can be easily assimilated by living organisms and naturally breaks down over time [19].

PGA's applications extend into agriculture and food science as well. It enhances nutrient uptake and retention in plants, promotes seed germination, and increases crop productivity. In the food industry, PGA serves as an edible and eco-friendly film-forming material for packaging [19].

In summary, polyglutamic acid (PGA) emerges as a versatile biopolymer with unique properties that make it adaptable to a wide range of industries.

1.3.2. Microbial Production of Polyglutamate

The fermentation process has emerged as a prominent technique for large-scale poly-γ-glutamic acid (PGA) production. Specific microorganisms, notably *Bacillus* species such as *Bacillus subtilis* [20–30] and *Bacillus licheniformis* [31–34], are involved in the biosynthesis of PGA. Other PGA-producing bacteria, including *Bacillus paralicheniformis* [35], *Bacillus velezensis* [36,37], and *Bacillus tequilensis* [38], have also been identified. These micro-organisms naturally secrete PGA as an extracellular polymer, often forming protective capsules. Genetic manipulation of these strains has facilitated increased PGA production by exploiting their metabolic pathways [32,38].

During the fermentation process, selected microbial strains are cultivated under controlled conditions in either solid- or liquid-state mediums. The choice of growth medium, temperature, pH, aeration, and other conditions significantly impacts PGA yield. Various researchers have investigated different variables in the fermentation process [39,40].

Solid-state fermentation for PGA production has been explored by Tang et al. [20], Zhang et al. [21], and Liu et al. [36]. Optimal carbon and nitrogen sources and their concentrations have been studied concerning culture media. For instance, Shi et al. [26] achieved a higher PGA concentration (54.4 g/L) using 60 g/L of sucrose and tryptone. Wang et al. [38] identified sucrose and ammonium sulphate as the best carbon and nitrogen sources, respectively, for improving PGA concentration.

Media supplementation has also been studied. Guo et al. [31] found that the addition of Fe^{2+} improved PGA concentration from 34.886 to 67.891 g/L. Glycerol supplementation was explored by Du et al. [32], who demonstrated that adding glycerol (70 g/L) enhanced PGA concentration from 9.7 g/L to 16.7 g/L, a finding supported by Richard et al. [27]. Moreover, PGA production has been studied with glutamic acid

dependency. Xu et al. [25] concluded that *Bacillus subtilis* NX-2 cells are dependent on glutamic acid for PGA production, obtaining a maximum concentration of 30.2 g/L with 30 g/L of glutamic acid in the culture media. Kongklom et al. [41] found similar results, where the addition and concentration of glutamic acid affected PGA production. However, Moraes et al.'s investigation showed PGA production without the need for glutamic acid in culture media [37]. Researchers have also explored the use of agro-industrial residues as carbon and nitrogen sources, including dry mushroom residues [20], chicken manure [21], soybean cake [21], molasses [29,31,37], corn steep liquor [29], and soy sauce [30].

The influence of fermentation conditions (pH, aeration, agitation, and temperature) on PGA production has been studied [27,33]. Cromwick et al. [33] identified pH 6.5 as optimal for PGA production and observed an increase in PGA concentration (from 6.3 to 23 g/L) when the aeration rate was increased from 0.5 to 2 L/min.

Several authors have explored alternative fermentation modes, such as fed-batch culture [28,29,34,38]. Huang et al. [28] reported a 36% increase in PGA concentration using this approach.

After PGA production, recovery and purification from fermentation broths are critical steps. Commonly employed methods include filtration, precipitation, and chromatography for separation and purification purposes.

The table presented below (Table 2) provides a summary of various fermentation conditions investigated for poly-γ-glutamic acid (PGA) production.

Table 2. Different fermentation conditions and culture modes for producing microbial PGA by various microorganisms in different culture media.

Microorganism	Culture Media	Fermentation Conditions	Results	Ref
Bacillus subtilis NX-2	95.6% *w/w* Glycerol Dry mushroom residues (DMR) Monosodium glutamate production residues (MGPR) [DSMR-to-MGPR ratio 12:8 (w/w)]	pH 7.0 35 °C 65% relative humidity 48 h	116.4 g/kg	[20]
Bacillus subtilis NX-2	30 g/L Glucose 30g/L L-glutamic acid	37 °C 200 rpm 24 h	30.2 g/L 1.26 g/L/h	[25]
Bacillus subtilis ZC-5	62.35 g Chicken manure 25.15 g Soybean cake 15.09 g Crude extract of glutamic acid after isoelectric crystallization (CEGA)	37 °C > 80% relative humidity 48 h	7 %	[21]
Bacillus subtilis (natto) strain MR-141	6% Maltose 7% Soy sauce 3% Sodium L-glutamate 3% NaCl	pH 8 40 °C 0.1 vvm: 0–18 h 1 vvm: 18–90 h 400 rpm 90 h	35 g/L	[30]
Bacillus subtilis subsp. *natto*	3% L-glutamic acid 2% Citric acid 1% $(NH_4)_2SO_4$	pH 7.0 37 °C 120 rpm 72 h	200 mg/L 10–50 kDa 17 kDa	[22]
Bacillus subtilis natto ATCC 15245	20 g/L L-glutamic acid 50 g/L Sucrose 50 g/L NaCl	pH 6.5 37 °C aeration rate 1 L/min 250 rpm 800 rpm, aeration rate 5 L/min to maintain DO > 40% 96 h	26–28 g/L 6.27×10^2 kDa	[24]

Microorganism	Culture Media	Fermentation Conditions	Results	Ref
Bacillus subtilis IFO 3335	30 g/L L-glutamic acid 20 g/L Citric acid 20 g/L Glycerol 10 g/L $(NH_4)_2SO_4$	pH 7 37 °C 2 vvm 700 rpm 32 h	23 g/L 3.89×10^3 kDa	[23]
Bacillus subtilis IFO 3335	30 g/L L-glutamic acid 20 g/L Citric acid 10 g/L $(NH_4)_2SO_4$ 20 g/L Glycerol	pH 7 37 °C 2 vvm 700 rpm 30 h	23 g/L	[27]
Bacillus subtilis ZJU-7	30 g/L Glutamate 20 g/L Glucose	pH 6.5 37 °C 1.5 vvm 300–800 rpm to maintain DO > 10% glucose < 3 g/L: fed with glucose solution 46 h	101.1 g/L 2.19 g/L/h	[28]
Bacillus subtilis ZJU-7	60 g/L Sucrose 60 g/L Tryptone 80 g/L L-glutamic acid	pH 7 37 °C 200 rpm 24 h	54.4 g/L 1.24×10^3 kDa	[26]
Bacillus subtilis 242	100 g/L Cane molasses 30 g/L L-glutamate 2 g/L Corn steep liquor	pH 7 37 °C 1.5 vvm 200–600 rpm to maintain DO = 10% fed at 24 h and 34 h with cane molasses and glutamate 48 h	32.14 g/L 27.99 kDa	[29]
Bacillus velezensis CAU263	100 g/kg Sucrose 150 g/kg L-Sodium glutamate	37 °C > 70% relative humidity 48 h	155.1 g/kg 3.23 g/kg/h 3.8×10^3 kDa	[36]

Microorganism	Culture Media	Fermentation Conditions	Results	Ref
Bacillus velezensis NRRL B – 23189	200 g/L Molasses 12.5 g/L Citric acid 8 g/L $(NH_4)_2SO_4$	pH 6.5 27 °C 200 rpm 72 h	4.82 g/L	[37]
Bacillus licheniformis CGMCC3967	Sugarcane molasses (9% soluble solids) 0.7 g/L $FeSO_4 \cdot 7H_2O$ 80 g/L Monosodium glutamate	pH 7.2–7.3 37 °C 1.2 vvm 450 rpm 72 h	76.848 g/L 1.07 g/L·h	[31]
Bacillus licheniformis WBL-3 mutant	10 g/L Citric acid 20 g/L L-glutamic acid 70 g/L Glycerol	pH 6.5 37 °C aeration rate 1.5 L/min 600–800 rpm to maintain DO > 20% 96 h	29.4 g/L	[32]
Bacillus licheniformis ATCC 994514	20.0 g/L L-glutamic acid 12.0 g/L Citric acid 80.0 g/L Glycerol	pH 6.5 37 °C 250 rpm and aeration rate 1.0 L/min: 0–20 h 800 rpm and aeration rate 2.0 L/min: 20–48 h	23 g/L	[33]
Bacillus licheniformis ATCC 9945	75 g/L L-glutamic acid 12 g/L Citric acid 80 g/L Glycerol 7 g/L NH_4Cl	pH 6.5 30 °C 250 rpm 72 h	12.64 g/L 98 kDa	[41]
Bacillus licheniformis ATCC 9945A	20 g/L L-glutamic acid 12 g/L Citric acid 80 g/L Glycerol 7 g/L NH_4Cl	pH 6.5 37 °C 2 vvm 1000 rpm 42 h	35 g/L	[34]
Bacillus tequilensis BL01 engineering strain	30 g/L Glucose 10 g/L Sodium citrate 5 g/L $(NH_4)_2SO_4$	pH 6.5 37 °C aeration rate 10 NL/min 400–700 rpm to maintain DO > 5% sugar < 10 g/L: fed with sucrose solution	25.3 g/L 2.06×10^3 kDa	[38]

Microorganism	Culture Media	Fermentation Conditions	Results	Ref
		citric acid < 5 g/L: fed with citric acid solution 30 h		
Bacillus paralicheniformis NCIM 5769	50% Sucrose 7% L-glutamic acid monosodium salt monohydrate 1% Citric acid monohydrate 1.5% Ammonium nitrate	pH 7.5 28 °C 1 vvm 250 rpm 72 h	284 g/L 3.94 g/L/h 785 kDa	[35]

1.3.3. The Potential of Polyglutamate in Cosmetic Applications

Polyglutamic acid (PGA) is emerging as a potent ingredient in cosmetic formulations, with Regulation (EC) No. 1223/2009 defining cosmetics as products utilized for personal grooming, hygiene, and aesthetic enhancement. This classification encompasses substances applied to various external body parts, including the skin, hair, nails, lips, external genital organs, teeth, and mucous membranes within the oral cavity [14,42].

Cosmetics are formulated to enhance the visual appearance, tactile sensation, and overall well-being of the skin, hair, and nails. These products serve multiple functions such as cleansing, moisturizing, protecting, and enhancing the natural beauty of these tissues. PGA's versatile capabilities have garnered significant attention in the cosmetic industry. Its ability to moisturize the skin, improve skin elasticity, and act as a whitening agent make it an intriguing candidate for incorporation into various cosmetic products. Thus, PGA has been proposed for use in moisturizing creams, anti-ageing solutions, and depigmentation formulations [14,42].

Researchers have conducted numerous studies to explore the efficacy of PGA in cosmetic applications, with highly promising results. These studies contribute to the growing body of evidence supporting PGA's potential as a valuable ingredient in cosmetic formulations aimed at enhancing skin health and appearance [14,43].

Polyglutamic acid (PGA) exhibits remarkable versatility in forming films, making it a valuable component in various applications. When dissolved in water, PGA molecules have the ability to self-assemble and form cohesive films on different surfaces. These films possess unique properties that make them suitable for a wide range of uses. PGA films are known for their excellent water solubility and biodegradability, making them environmentally friendly alternatives to traditional plastics. They can be used as coatings to protect surfaces from moisture or as barriers to prevent the penetration of gases and other substances. In the cosmetic industry, PGA films are often utilized in skincare products as occlusive layers to enhance the efficacy of active ingredients and improve skin hydration [11,14,44,45].

Moreover, PGA films have been explored for their potential in drug delivery systems. By encapsulating active compounds within PGA films, controlled release formulations can be developed, allowing for targeted and sustained delivery of therapeutic agents. This application is particularly promising in the pharmaceutical industry for the development of transdermal patches and wound dressings [46,47].

Additionally, PGA films can be engineered to have specific mechanical properties, such as flexibility and strength, depending on the processing conditions and additives used during film formation. This versatility makes PGA films suitable for various industrial applications, including packaging materials, agricultural

coatings, and biomedical devices [48–51]. Overall, the films formed from PGA offer a wide range of possibilities in terms of functionality and application, making them a promising material for innovation in multiple industries [52,53].

In fact, the biocompatibility, water solubility, and film-forming characteristics make it an ideal ingredient for cosmetic formulations. Its ability to retain moisture and form protective barriers on the skin enhances skincare and haircare products, offering hydration, protection, and improved texture. Additionally, PGA's compatibility with other ingredients allows for synergistic effects, further amplifying its efficacy in cosmetic applications. Overall, PGA's efficacy stems from its versatile properties, making it a valuable component in various products aimed at enhancing beauty and well-being [14,42,54].

1.3.3.1. The Potential of Poly-γ-Glutamic Acid as a Moisturizing and Anti-ageing Agent

Extensive research has delved into exploring the moisturizing potential of poly-γ-glutamic acid (PGA), comparing it with other polymers like hyaluronic acid and collagen. Studies consistently position PGA as an exceptional moisturizing agent, potentially owing to its capacity to stimulate the production of natural moisturizing factors (NMFs) such as pyrrolidone carboxylic acid (PCA), lactic acid, and urocanic acid [28]. These NMFs, synthesized within the deeper layers of the stratum corneum, play a pivotal role in maintaining skin health by ensuring hydration, preventing excessive dryness, and facilitating the natural exfoliation process crucial for smooth and healthy skin [55–59].

Research by Guan-Huel Ho et al. [60], has focused on incorporating gamma-polyglutamate into cosmetics and personal care items to develop cost-effective solutions with superior moisturizing attributes. Their studies evaluated the effectiveness of water retention after applying various cosmetic formulations to volunteers' skin, with propylene glycol and hyaluronic acid used as control and reference substances, respectively. The results showcased that gamma-polyglutamate formulations exhibited enhanced water retention (45%) for up to 120 min, surpassing both the control (40%) and the reference (36%). Moreover, PGA formulations demonstrated superior effectiveness in enhancing skin hydration compared to glycerol formulations (13.5 vs. 7.5). Importantly, the application of PGA formulations did not cause irritation or rashes, indicating the safety of poly-γ-glutamic acid for human skin [60].

Further studies, such as those conducted by Shin-An Yang et al. [61,62], explored the formulations of cosmetic products containing poly-γ-glutamic acid within specific concentrations and molecular weights. Their results demonstrated that PGA formulations promoted cell growth in fibroblast and keratinocyte cells, indicating biocompatibility and potential wound-healing properties. Additionally, PGA exhibited a

significant photoprotective effect against light-induced damage and improved skin texture, elasticity, and collagen content over time [62–65].

Additional investigations by Na-Ri Lee et al. [36], unveiled PGA's potential as a moisturizing agent in facial and hand creams, showing promising results in terms of hydration and antibacterial activity against Gram-positive bacteria. Furthermore, studies by Wen Liangliang [66] shed light on the collaborative impact of PGAs with different molecular weights on skin hydration and barrier enhancement. Results suggested that polyglutamic acid of varying molecular weights could effectively stimulate the production of NMFs and enhance skin barrier function synergistically [67–69].

The impact of different molecular weights of polyglutamic acid (PGA) on the condition of the skin is a subject of interest in cosmetic research. PGA molecules can vary significantly in their molecular weight, and this variation plays a crucial role in determining their effects on the skin [70,71].

- Skin Hydration: Studies have shown that PGA of varying molecular weights can influence skin hydration differently. Higher-molecular-weight PGAs tend to form a more occlusive barrier on the skin surface, reducing transepidermal water loss (TEWL) and enhancing skin hydration by preventing moisture evaporation. On the other hand, lower-molecular-weight PGAs may penetrate deeper into the skin layers, where they can bind to water molecules and increase skin hydration from within [10,71].
- Barrier Function: The skin barrier is essential for maintaining skin health and protecting against external aggressors. Different molecular weights of PGA can impact the skin barrier function in distinct ways. Higher-molecular-weight PGAs may form a protective film on the skin surface, reinforcing the skin barrier and preventing the entry of harmful substances. Lower-molecular-weight PGAs, meanwhile, may have the ability to penetrate the skin more effectively, where they can interact with skin cells to promote barrier repair and regeneration [72,73].
- Stimulation of NMFs: Natural moisturizing factors (NMFs) are substances present in the skin that help maintain hydration levels and support skin barrier function. Studies suggest that PGA of varying molecular weights can stimulate the production of NMFs in the skin. Higher-molecular-weight PGAs may trigger the synthesis of NMFs on the skin surface, while lower-molecular-weight PGAs may penetrate deeper into the skin to promote the production of NMFs within the epidermal layers [10,74].

Overall, the use of PGA with different molecular weights in skincare formulations allows for tailored approaches to address specific skin concerns. By selecting PGA molecules of the appropriate molecular weight, cosmetic formulators can optimize skin hydration, improve barrier function, and promote overall skin health effectively.

Collectively, these findings endorse the use of PGA as an active ingredient in moisturizing and anti-ageing creams, underscoring its ability to retain skin moisture, improve elasticity, and promote overall skin health without causing adverse reactions [11,75].

Poly-γ-glutamic acid (PGA) demonstrates significant potential as a moisturizing and anti-ageing agent in skincare formulations. Here are some key details about its potential in these areas:

- **Moisturizing Properties:**

PGA possesses an exceptional water-binding capacity, enabling it to attract and retain moisture in the skin. This hydration helps to keep the skin supple, soft, and plump, reducing the appearance of fine lines and wrinkles.

The ability of PGA to stimulate the production of natural moisturizing factors (NMFs) such as pyrrolidone carboxylic acid (PCA), lactic acid, and urocanic acid further enhances its moisturizing properties. These NMFs play a crucial role in maintaining skin hydration and preventing dryness.

PGA's water-soluble nature allows it to form a protective barrier on the skin's surface, minimizing water loss and improving overall skin hydration levels [76].

- **Anti-ageing Benefits:**

Studies have shown that PGA can stimulate collagen production in the skin, leading to improved skin firmness, elasticity, and smoothness. Collagen is a structural protein that helps maintain the skin's youthful appearance by providing support and structure.

PGA's antioxidant properties help to neutralize free radicals, which are molecules that can damage skin cells and contribute to premature aging. By protecting the skin from oxidative stress, PGA helps to prevent the formation of wrinkles, fine lines, and other signs of aging [77,78].

PGA has been found to promote cell growth and regeneration in the skin, accelerating the turnover of old, damaged skin cells and promoting the growth of new, healthy cells. This renewal process helps to improve skin texture, tone, and overall radiance [79,80].

Overall, poly-γ-glutamic acid shows promise as a multifunctional ingredient in skincare formulations, offering both moisturizing and anti-ageing benefits. Its ability to hydrate the skin, stimulate collagen production, and protect against oxidative damage makes it a valuable addition to skincare products aimed at promoting youthful, healthy-looking skin [7,9,81–83].

1.3.3.2. The Potential of Poly-γ-Glutamic Acid as a Depigmenting Agent

The potential of poly-γ-glutamic acid (PGA) as a skin lightening agent has been a subject of focused investigation. Researchers have found that PGA exhibits the ability to inhibit the activity of the enzyme tyrosinase, which plays a key role in regulating melanin production [55]. The ability of poly-γ-glutamic acid (PGA) to inhibit the activity of the enzyme tyrosinase can vary in percentage depending on factors such as concentration, experimental conditions, and the specific study being referenced [84,85].

In a study by Liu et al. [38], the inhibitory effects of PGA of various molecular weights on tyrosinase enzyme activity and melanogenesis were explored. Their findings revealed that PGA could effectively inhibit tyrosinase activity and melanogenesis in B16 melanoma cells. Notably, the polymer was observed to decrease levels of reactive oxygen and nitric oxide species while increasing the activity of catalase in these cells. The authors suggested that low-molecular-weight PGA could be particularly promising as a depigmenting agent, as it demonstrated the ability to suppress melanogenesis without stimulating B16 cell proliferation. This suggests a potential role for PGA in addressing hyperpigmentation concerns and promoting even skin tone.

1.3.3.3. Poly-γ-Glutamic Acid's Potential in Sunscreens

In the realm of cosmetics, sunscreens play a crucial role in safeguarding skin health and preserving its youthful appearance. They serve as a vital defence against sun-induced damage, premature aging, and other undesirable effects, significantly contributing to the overall vitality and aesthetics of the skin [86–88].

Recognizing the importance of sunscreen safety, Guo Tian [39] embarked on the development of a novel formulation incorporating polyglutamic acid sunscreen peptides. This innovation stemmed from concerns regarding the potential toxicity associated with traditional small-molecule sunscreens. These molecules, characterized by their low molecular weight, possess the ability to penetrate the skin, raising the possibility of adverse effects. Guo Tian's invention addresses this concern by designing sunscreen peptides with a higher molecular weight, thereby minimizing the risk of skin absorption and toxicity [89,90].

Despite the higher molecular weight, the sunscreen peptides retain their efficacy in absorbing UV radiation, aligning with the UV absorption range of conventional small-molecule sunscreens. This ensures that their protective capabilities against harmful UV rays remain uncompromised, offering a viable and safer alternative for shielding the skin from the sun's detrimental effects. By prioritizing safety without

compromising effectiveness, this innovative formulation represents a significant advancement in sunscreen technology, promising enhanced skin protection and peace of mind for consumers [91].

1.3.3.4. Exploring Poly-γ-Glutamic Acid's Potential in Treating Skin Damage

The concept of skin damage encompasses a wide array of conditions, spanning from the adverse effects of allergies and inflammation to the regenerative processes involved in wound healing. In this context, the potential use of poly-γ-glutamic acid (PGA) as an active agent in treating skin damage has been extensively explored.

Moon Hee Sung et al. [60] conducted research highlighting PGA's ability to ameliorate allergic responses. Their findings indicated that PGA effectively prevented the permeability of inflammatory cells, a critical step in attenuating allergic manifestations. Sensory tests involving 15 children with atopic dermatitis further validated these findings, with the use of PGA-infused shampoo providing noticeable relief for 8 of the children.

In parallel, research by Zeng Rong provided insights into PGA's anti-inflammatory potential. Gel film formulations incorporating crosslinked PGA demonstrated significant efficacy in suppressing the expression of TNF-α, a key regulator of inflammatory reactions [32]. This underscores the multifaceted utility of PGA, suggesting its potential as a modulator of crucial molecular mechanisms associated with skin inflammation.

Moreover, PGA has shown promise in wound healing applications [61], which entail sequential stages including haemostasis, inflammation, proliferation, and tissue reconstruction. Studies have revealed PGA's effectiveness in promoting the growth of fibroblasts and collagen production, thus positioning it as a viable option for wound healing interventions. Wai-Ching Liu et al. [92] explored the use of PGA hydrogels for wound healing treatment, observing accelerated wound contraction, formation of new blood vessels, collagen fibres, granulation tissues, hair follicles, and capillaries.

Chi Bo et al. [93] investigated the potential applications of multi-polyglutamic acid cross-linked polymers in skin repair. Their comprehensive experiments evaluated the antioxidant attributes, biocompatibility, and blood compatibility of these polymers, revealing significant moisturizing, antioxidant, and tissue compatibility properties. These findings suggest the broad applicability of cross-linked polyglutamic acid polymers in various skin tissue-related applications.

Furthermore, Mei-Hua Huang et al. [94] explored a novel approach to wound care by developing sodium alginate/polyglutamic acid hydrogels for use as wound dressings. The hydrogel exhibited non-toxicity

against fibroblast and pulmonary cells, promoted blood coagulation and platelet adhesion, and demonstrated notable fluid retention, making it suitable for moist wound healing.

In conclusion, the multifaceted potential of poly-γ-glutamic acid (PGA) in addressing various aspects of skin damage is increasingly evident through rigorous scientific investigations. This underscores its potential as a versatile therapeutic agent in the field of dermatology and wound care.

1.3.3.5. The Potential of Poly-γ-Glutamic Acid in Haircare

The versatility of poly-γ-glutamic acid (PGA) extends beyond skincare into the realm of hair care, offering promising benefits for enhancing hair health and appearance. This biopolymer's unique characteristics, including its humectant properties, moisture retention abilities, and biocompatibility, position it as a compelling candidate for addressing various hair-related concerns. The multifaceted advantages of PGA in hair care have garnered significant attention, prompting thorough investigations and cementing its role in this domain [81,95].

An innovative study by Hasebe Kohei et al. [46] introduced a novel cosmetic product that combined polyhydric alcohol with poly-γ-glutamic acid (PGA), designed for application on both hair and skin. While polyhydric alcohol is renowned for its humectant and hydrating effects, its application to hair has often been associated with discomfort due to stickiness and tingling sensations. To overcome these drawbacks, researchers formulated blends of polyhydric alcohols and PGA. Subsequent sensory evaluations and safety assessments revealed no instances of skin irritation or inflammation post-application. Moreover, these formulated blends effectively alleviated the stickiness and tingling sensations commonly experienced with polyhydric alcohols when applied to hair, while demonstrating notable moisturizing benefits for both hair and skin. Thus, this research underscored the potential of incorporating polyhydric alcohol and PGA into cosmetic formulations to address hair and skin care needs simultaneously [96–99].

Furthermore, studies have explored PGA's potential in stimulating cell growth and promoting healing processes, particularly in the management of alopecia. Alopecia, characterized by hair loss, poses significant challenges with physical and psychological ramifications. Choi et al. [100] found that high-molecular-weight PGA exhibited inhibitory effects on the 5-alpha reductase enzyme, suggesting its potential utility in addressing androgenetic alopecia in men. Notably, PGA demonstrated efficacy in promoting hair growth, with more than 50% of shaved skin exhibiting hair regrowth within four weeks, outperforming the control group treated with minoxidil. These findings highlight the promising role of PGA in addressing hair loss concerns, offering potential solutions for individuals grappling with alopecia.

1.3.3.6. The Potential of Poly-γ-Glutamic Acid in Dental Care

The exploration of Gamma-PGA-based toothpastes represents a significant area of research, offering promising avenues for innovative, natural, and effective oral care solutions. The inclusion of PGA in toothpaste formulations holds immense potential due to its multifaceted benefits, ranging from its anti-allergenic properties to its whitening and oral health-enhancing effects, thereby making it a compelling ingredient for modern oral care products. A seminal study by Qiao Changsheng et al. [101] delved into the development of an oral hygiene toothpaste aimed at nurturing the oral cavity, enhancing teeth whitening, restoring oral microbial balance, and preventing oral health issues. Beyond these primary objectives, the innovation also boasted additional advantages, including prolonged action time and the non-toxic nature of glutamic acid, a byproduct of PGA degradation.

The formulated toothpaste exhibited notable efficacy in promoting oral health and hygiene, contributing to the maintenance of overall oral well-being. By addressing various aspects of oral care, such as microbial balance, teeth whitening, and oral cavity nourishment, the PGA-infused toothpaste emerged as a comprehensive solution for individuals seeking effective and natural oral care alternatives.

Moreover, the extended action time of the toothpaste ensured sustained benefits, providing prolonged protection and oral care support throughout the day. Importantly, the non-toxic nature of glutamic acid, derived from PGA degradation, underscored the safety profile of the toothpaste formulation, further enhancing its appeal as a reliable and health-conscious oral care option.

Overall, the study shed light on the potential of Gamma-PGA-based toothpastes to revolutionize oral care practices, offering a holistic approach to oral hygiene that combines effectiveness, safety, and natural ingredients [102,103].

1.3.3.7. Polyglutamic Acid Skincare Products in the Market

According to a report by the QYResearch Group [104], the global polyglutamic acid (PGA) market reached a value of approximately USD 441.19 million in 2023. Projections suggest a Compound Annual Growth Rate (CAGR) of 7.41% from 2023 to 2029, leading to an estimated market valuation of around USD 677.54 million by 2029. This growth trend is primarily fuelled by the increasing demand for PGA across various industries, particularly in skincare.

Polyglutamic acid (PGA) has garnered rising popularity in skincare due to its exceptional hydrating and moisturizing properties. It serves as an active ingredient in skincare products aimed at improving skin hydration, refining texture, and enhancing overall appearance. PGA is frequently incorporated into serums and moisturizers for its ability to retain water, ensuring sustained skin hydration. Its inclusion in makeup primers and setting sprays helps create a smooth makeup base and prolongs makeup wear while preserving skin moisture. Moreover, PGA's efficacy in enhancing skin texture and moisture makes it a valuable component in anti-ageing products targeting fine lines and wrinkles.

One notable skincare product featuring PGA is Charlotte's Magic Serum Crystal Elixir, which underwent testing on 209 individuals aged 18–80 over a 4-week period. User trial results revealed positive outcomes, with 93% reporting younger-looking skin, 97% experiencing intense hydration, 91% noticing refined pores, 91% observing reduced lines and wrinkles, 94% experiencing firmer skin, and 91% noting lifted contours. Clinical studies, conducted over 8 weeks with 31 participants aged 18–80, further demonstrated significant benefits, including a 172% increase in skin hydration after 1 h, a 43% reduction in water loss after 1 h, 24 h moisturization, a 122% improvement in skin elasticity, a 49% increase in firmness, a 34% reduction in wrinkles, and a 39% decrease in pore appearance [105].

Another noteworthy raw material is Twainmoist by Sollice Biotech (Toulouse, France), known for its significant effects on the skin when incorporated into cosmetic formulations. This ingredient has been shown to elevate hyaluronic acid levels and enhance natural moisturizing factors. Clinical efficacy tests have demonstrated its effectiveness in increasing skin hydration and firmness, reducing water loss, and even decreasing wrinkle depth by up to 35% [106].

The table provided below (Table 3) outlines several skincare products containing PGA currently available in the cosmetic market.

Table 3. Skincare products containing PGA available in the cosmetic market and their functions.

Trademark	Skin care Product	Benefits
REN Clean Skincare	Perfect Canvas Smooth, Prep And Plump Essence	Offers anti-pollution defence for fortified skin barrier; acts as skin primer.
Dr.Jart	Cicapair Tiger Grass Re.Pair Serum	Soothes redness, moisturizes, protects from environmental stressors; minimizes pore visibility.
TULA SKINCARE	24-7 Ultra Hydration Triple-Hydra Complex Day & Night Serum	Aids in smoothing wrinkles and fine lines.
dermalogica	Circular Hydration Serum	Promoting long-lasting hydration.
111Skin	The Hydration Concentrate	Moisturizes and prevents excess water loss through skin.

Trademark	Skin care Product	Benefits
The INKEY List	Polyglutamic Acid Serum	Promotes hydration and reduces the visibility of fine lines.
The INKEY List	Polyglutamic Acid Dewy Sunscreen Spf30	Offers full UVA and UVB protection against sun's rays.
Collistar	Hyaluronic And Polyglutamic Acid	Offers multi-level hydration.
Charlotte Tilbury	Charlotte's Magic Serum Crystal Elixir	Enhances hydration, elasticity, and firmness; diminishes wrinkles and pores.
Viviology	Ceramide Moisturiser	Moisturizing, soothing and calming product.
KATE SOMERVILLE	Dermalquench Wrinkle Warrior Advanced Hydrating And Plumping Treatment	Combats wrinkles, sagging, uneven tone; ensures unmatched hydration.
Sollice Biotech	Twainmoist®	Delivers hydration, firmness, and vitality.
Good Molecules	B5 Hydrating Body Serum	Preserves moisture, fortifies skin barrier.

1.3.4. Conclusions

In conclusion, the integration of polyglutamic acid (PGA) in the cosmetic industry represents a promising frontier, owing to its remarkable biocompatibility, water solubility, and film-forming properties. These characteristics render PGA an attractive ingredient for a wide array of cosmetic formulations, offering significant potential for innovation and product development.

Looking ahead, the trajectory of PGA research encompasses a multifaceted approach aimed at maximizing its utility and efficacy in cosmetic applications. Further optimization of fermentation processes, exploration of novel microbial strains, and advancements in genetic engineering techniques present opportunities for enhancing PGA production yields. By refining these methodologies, manufacturers can meet the escalating demand for PGA in skincare, haircare, and other cosmetic products.

In the realm of cosmetic applications, comprehensive studies are essential to elucidate PGA's performance across various formulations, ensuring its stability and compatibility with other cosmetic ingredients. Understanding the synergistic interactions between PGA and other components can unlock its full potential, enabling the creation of innovative and effective skincare and haircare solutions.

Moreover, as sustainability becomes increasingly pivotal in the cosmetics industry, future research endeavours should prioritize environmental considerations and sustainable practices. Aligning PGA

production processes with eco-friendly principles not only mitigates environmental impact but also resonates with consumer preferences for sustainable and responsibly sourced ingredients.

Navigating the intersection of biotechnology and cosmetics, the ongoing exploration of PGA's capabilities holds significant promise for meeting industry demands for sustainable, effective, and eco-conscious cosmetic ingredients. By leveraging PGA's unique properties and advancing scientific understanding, the cosmetic industry can continue to innovate and evolve, offering consumers products that are both efficacious and environmentally responsible.

1.3.5. References

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1.4. Exploring the Cosmetic Potential of Grape Stem Phenolic Compounds

Adapted from:

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1.4.1. Introduction

The winery industry in Portugal and Europe embraces a crucial position in the economy, culture, and heritage of these regions, positioning itself as one of the world's foremost wine-producing areas [1]. However, wine production yields a variety of by-products, including pruning wood, grape pomace, seeds, skins, and stems, among others [2–8]. Improper management of these by-products can lead to adverse environmental effects, such as water pollution, soil degradation, and greenhouse gas emissions [9,10]. Recognizing these concerns, the wine industry has increasingly prioritized the adoption of sustainable practices to mitigate its environmental impact and effectively handle the by-products generated during winemaking processes [10–17]. One approach gaining significant impetus is the implementation of circular economy principles, which aim to minimize waste and maximize resource utilization [18].

Scientists have been actively studying these wine residues to discover their composition and explore potential applications [2]. By discovering novel uses for these by-products, the wine industry can significantly reduce its environmental footprint, contribute to the principles of a circular economy, and potentially unlock new sources of revenue. This represents a pivotal step towards achieving greater sustainability within the wine industry while minimizing its overall environmental impact.

In recent years, there has been a growing interest in exploring the beauty potential of grape stems in the cosmetics industry [19]. Grape stems, typically discarded as waste during winemaking, contain a wealth of valuable phenolic compounds that offer numerous benefits for skincare and cosmetic products.

Phenolic compounds are highly desirable ingredients in the cosmetic industry due to their antioxidant, anti-inflammatory, and anti-ageing properties. Grape stems are particularly rich in these compounds, including stilbenes, flavonoids, anthocyanins, and tannins, extensively studied for their ability to protect the skin against oxidative stress, promote collagen synthesis, and improve overall skin health [14]. The use of grape stem extracts in cosmetics holds great promise. These extracts have shown potential in combating skin aging by reducing the appearance of wrinkles, fine lines, and age spots. Their antioxidant properties neutralize free radicals, known contributors to premature aging and skin damage. Grape stem

extracts can also enhance skin elasticity and firmness, giving the skin a more youthful and rejuvenated appearance [20].

Furthermore, the anti-inflammatory properties of grape stem phenolic compounds make them beneficial for soothing and calming irritated skin, alleviating redness, inflammation, and sensitivity, suitable for products targeting sensitive or reactive skin types. Additionally, these compounds reduce hyperpigmentation and improve skin tone, providing a more even complexion [16].

Cosmetic formulations incorporating grape stem extracts can vary from serums and creams to masks and toners. These extracts serve as active ingredients or supportive components, not only offering skin benefits but also meeting the rising demand for natural and sustainable beauty solutions [21].

By exploiting the phenolic compounds present in grape stems, the cosmetics industry can contribute to reducing waste generated by the winemaking process while unlocking a new source of valuable ingredients. This utilization of grape stems aligns with the principles of a circular economy, emphasizing resource optimization and sustainability [22].

In conclusion, the beauty potential of grape stems in cosmetics is a fascinating area of exploration. The phenolic compounds found in these by-products possess beneficial properties for skincare, including antioxidant, anti-inflammatory, and anti-ageing effects. Incorporating grape stem extracts into cosmetic formulations offers consumers natural and sustainable beauty solutions that promote healthy, youthful-looking skin. By recognizing and utilizing the hidden potential of grape stems, the cosmetics industry can contribute to a more eco-friendly and innovative approach to skincare.

1.4.2. Wine Industry in Portugal and Europe

The wine industry holds significant economic importance in Portugal and Europe, boasting a rich heritage and a diverse array of wine styles and regions. It plays a vital role in the global economy, supporting numerous jobs across various sectors, from grape growers and winemakers to processors, distributors, and retailers. Additionally, the industry serves as a significant cultural and tourist attraction, with vineyards and wineries offering immersive tours and tastings to visitors [1].

In Europe, the wine industry stands as a major economic sector, with several countries renowned for producing exceptional wines. According to the OIV [23], European countries dominate global wine production, with France, Italy, Spain, Germany, and Portugal emerging as the top wine-producing nations in Europe, as depicted in Figure 5. Each of these countries possesses its own distinctive wine styles and

regions, encompassing a wide spectrum from light and refreshing whites to full-bodied reds and succulent dessert wines [24].

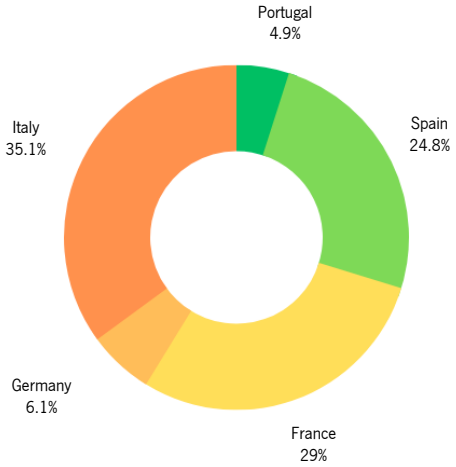


Figure 5. Percentual distribution of the main European wine producer countries.

The wine industry holds great historical and economic importance in Portugal, making a significant contribution to the country's economy. Portugal is renowned for its production of Port wine, a fortified wine exclusively produced in the picturesque Douro Valley region [24,25]. This region's unique terroir and winemaking traditions have positioned Portugal as a notable player in the global wine market.

Moreover, the wine industry in Portugal and Europe at large is deeply intertwined with the regions' economy, culture, and heritage. The production of wine has shaped the cultural fabric of these regions, with vineyards and wineries often serving as popular tourist destinations. The industry plays a pivotal role in preserving and showcasing the rich heritage associated with winemaking, offering visitors the opportunity to experience the traditions, craftsmanship, and diverse wine styles that define these regions.

Additionally, the wine industry contributes significantly to the economies of Portugal and Europe, providing employment across various sectors, from viticulture to production, distribution, and hospitality. The export of wines from these regions further strengthens their economic impact and promotes their unique wine offerings to international markets.

In summary, the wine industry in Portugal and Europe serves as a cornerstone of their economies, cultures, and legacies. The production of world-renowned wines, such as Port wine in Portugal, adds to the allure of these regions and continues to attract wine enthusiasts and connoisseurs from around the globe.

1.4.2.1. By-Products from the Winery Industry and Their Valorisation

The wine production process yields a diverse array of by-products, with Europe alone estimated to produce 14.5 million tons [2,3,9]. To obtain a thorough understanding of these by-products, it is essential to examine the different stages of winemaking, as outlined below.

Vines are pruned annually to enhance grape production and stimulate vine growth, yielding substantial amounts of pruning wood as a primary by-product. Regarding the winemaking process, it begins with the harvesting of grapes, which are then transported to the winery for crushing and pressing. At this stage, the grape berries are broken to combine the juice, pulp, seeds, skins, and stems, after which the juice is separated from the solid components. Following steps like fermentation and clarification facilitate the conversion of grape sugar into alcohol and the elimination of any suspended insoluble matter from the wine. Consequently, by-products, such as grape pomace and stems, are generated at each step of the process, as illustrated in Figure 6 (A).

Recognizing the environmental impact associated with the substantial production of winemaking by-products, the wine industry has increasingly focused on adopting sustainable practices to mitigate its environmental footprint and effectively manage these by-products [10,17]. The generation of by-products during winemaking is an inherent outcome that cannot be avoided. While these by-products themselves are not toxic, their high organic matter content can pose environmental challenges if not managed properly, contributing to issues such as water pollution, soil degradation, and greenhouse gas emissions [9,10].

Efforts to develop sustainable practices and adopt circular economy principles in winemaking are crucial for reducing the industry's environmental footprint and fostering a more sustainable future.

The circular economy model encourages minimizing waste and maximizing resource utilization, as illustrated in Figure 6 (B). Within the context of winemaking, this involves implementing strategies to reduce waste generation, optimize by-product management, and explore new avenues for their utilization. The wine industry aims to reduce waste generation and explore innovative ways to utilize by-products such as pruning wood, seeds, skins, stems, and lees by embracing the principles of the circular economy [18]. This entails implementing strategies to minimize waste, optimize the management of by-products, and discover new avenues for their utilization. By finding novel uses for these by-products, the wine industry can contribute to a more sustainable and circular approach, wherein materials are kept in use for as long as possible, thus reducing the strain on the environment. By valuing and effectively utilizing

by-products, the wine industry can move towards a more environmentally conscious and resource-efficient approach to winemaking.

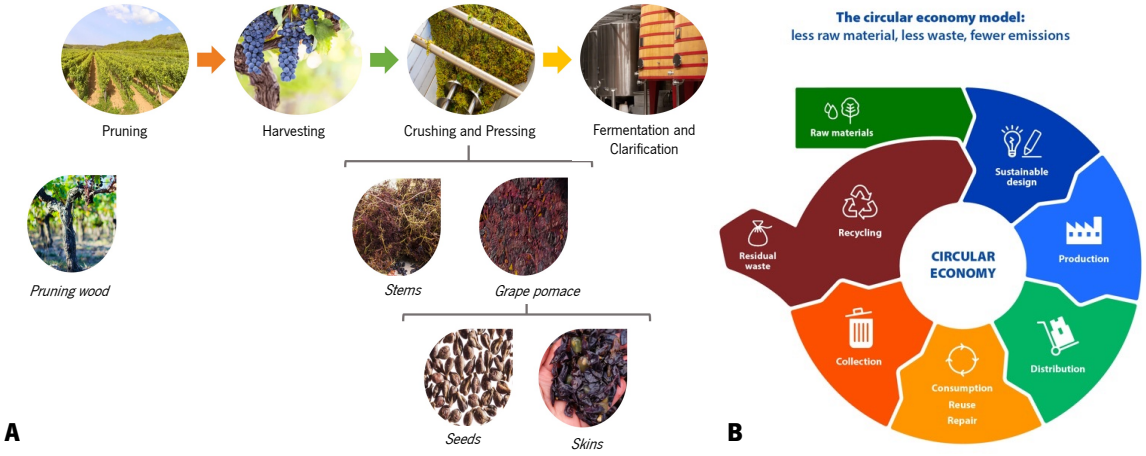


Figure 6. (**A**). Representation of the winemaking process and generated by-products. (**B**). The circular economy model according to European Parliament [18].

In line with the principles of the circular economy, scientists have extensively studied the composition and potential applications of winemaking residues [14,26].

The main by-products of the wine industry include pruning wood and grape marc (or grape pomace), which comprises stalks (or stems), skins, and seeds [5,7,17,27,28]. The composition of these residues can be influenced by several factors such as climate, soil type, fertilization, grape variety, maturation, and harvest time [5,29,30].

These residues exhibit complex compositions, encompassing polysaccharides, proteins, fibres, minerals, lignin, cellulose, hemicelluloses, vitamins, amino acids, and phenolic compounds. Each residue from the winemaking process possesses a distinct and characteristic composition [8,17,28,30–32].

Pruning wood, for instance, is primarily composed of lignin, cellulose, hemicellulose, and minerals like potassium and calcium. This wood can be directly utilized as organic material in vineyards or subjected to composting processes [4,9]. Moreover, studies have explored its potential in diverse applications. Research suggests that vine pruning possesses a high heating value and can serve as solid fuel in domestic stoves, exhibiting efficiency similar to wood pellets [6]. Additionally, attempts have been undertaken to transform vineyard residues into binderless particleboards, resulting in an environmentally friendly product for general use and furniture manufacturing [33]. Due to its composition, it can also be

bioconverted into raw materials for cultivating microorganisms that yield value-added products such as biodiesel, alcohol, and lactic acid [6,13,30].

Grape pomace, on the other hand, is recognized for its high content of phenolic compounds, including phenolic acids, flavonoids, tannins, and stilbenes [8,17,34–37]. These compounds are widely distributed in the plant kingdom and possess various significant biological and pharmacological properties, such as anti-inflammatory and antioxidant activities, cardioprotective and neuroprotective potentials, and anticancer and antibacterial properties [5,9,27,30,31,36–40]

The in-depth understanding of the composition of these winemaking residues opens avenues for their potential utilization in various applications. By exploiting the valuable components present in these residues, it becomes possible to extract and develop novel products with beneficial properties, contributing to the sustainable and circular utilization of winemaking by-products.

1.4.2.2. Grape Stems: Chemical and Phenolic Composition and Their Biological Potential

Grape stems, also known as grape stalks, are robust branches extending from the vine trunk, providing essential support to grape bunches or clusters [5,41]. These stems play a crucial role in the vine's structure by aiding in the distribution of water, nutrients, and hormones to all grape berries [41]. Their structural features are depicted in Figure 7.

During winemaking, grape stems are commonly removed from the grapes before fermentation begins. This practice is necessary due to their composition, which can impart an undesirable bitterness to the wine if they remain in contact with the juice during fermentation [9,41–44]. Notably, grape stems contribute to up to 14% of the total weight of solid waste generated during wine production [28].

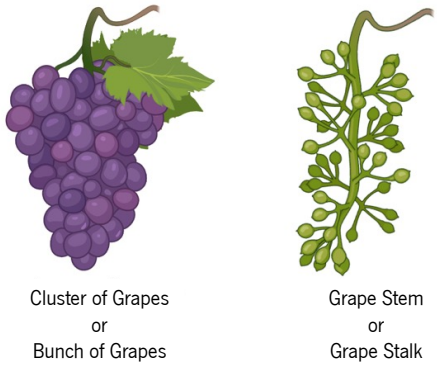


Figure 7. Schematic representation of the bunch and stem of the grape.

Grape stems, despite being undesired in wine production, possess a distinct chemical and phenolic composition that has attracted scientific attention for alternative applications.

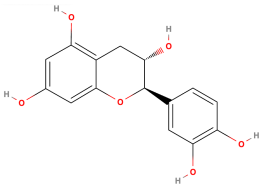
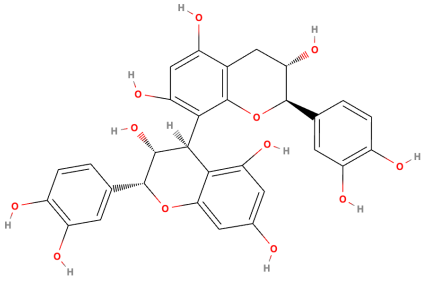
The composition of grape stems varies due to factors such as grape variety, maturation state, size, and extraction methods, leading to differences in their biological characteristics [30,32,41,45].

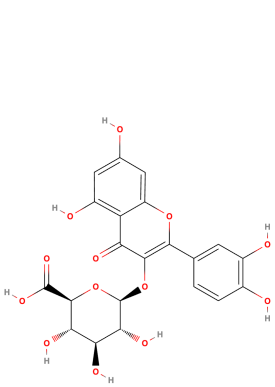
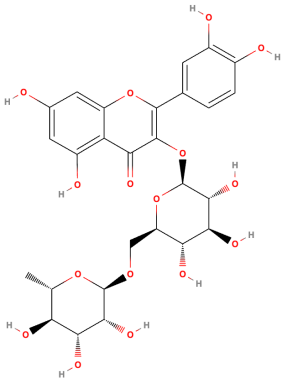
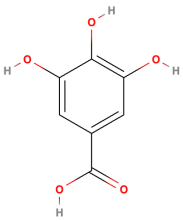
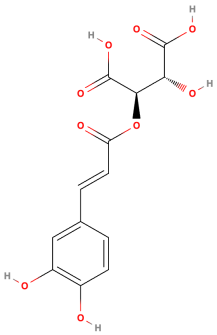
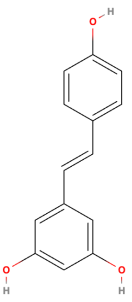
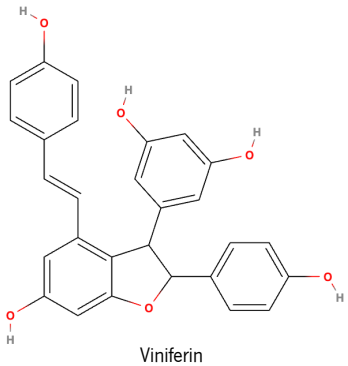
Chemically, grape stems are primarily composed of lignocellulosic materials like lignin, cellulose, hemicelluloses, and ash [32,41,43,46]. They also contain significant levels of monosaccharides, particularly glucose and xylose, along with smaller quantities of mannose, arabinose, and galactose [45,47,48]. Additionally, grape stem extracts are rich in essential minerals such as sodium (Na), magnesium (Mg), calcium (Ca), and potassium (K) [49].

In terms of their phenolic composition, grape stems contain bioactive phenolic compounds produced by plants in response to environmental stressors such as UV radiation, biotic and abiotic stress, and pathogens [2,43]. These compounds encompass various classes, including flavonoids (flavanols, flavonols, flavones, anthocyanins), phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids), stilbenes, and tannins, each with distinct chemical structures and biological activities [2,27,50].

Table 4 provides some examples of these phenolic compounds.

Table 4. Chemical structure of some examples of phenolic compounds.

Class of Phenolic Compounds	Examples
Flavan-3-ols	 Catechin
	 Procyanidin B1

Class of Phenolic Compounds	Examples
Flavonols	 Quercetin-3-O-glucuronide  Rutin
Hydroxybenzoic acids	 Gallic acid
Hydroxycinnamic acids	 *Trans*-caftaric acid
Stilbenes	 *Trans*-resveratrol  Viniferin

The biological potential of phenolic compounds extracted from grape stems has been extensively studied, revealing various beneficial activities, including antioxidant, antimicrobial, anti-inflammatory, anti-ageing,

and anticancer effects [27,31,41,43,51]. Researchers have conducted in-depth analyses of grape stem extracts to identify their phenolic compound composition and explore their biological potential.

For instance, Makris et al. [52] identified several phenolic compounds in grape stem extract, including gallic acid (a hydroxybenzoic acid), *trans*-resveratrol and ε-(or α-)viniferin (stilbenes), flavonols such as quercetin-3-O-glucuronide and quercetin-3-O-rutinoside (rutin), and the flavanonol astilbin.

Similarly, Nieto et al. [53] found that optimal grape stem extracts are rich in flavonoids, particularly catechin, epicatechin, and procyanidin B1 from the flavan-3-ols subclass. They also observed the presence of quercetin-3-O-glucuronide from the flavonols subclass, as well as significant amounts of phenolic acids, including gallic acid, vanillic acid, syringic acid (hydroxybenzoic acids), and *trans*-caftaric acid (a hydroxycinnamic acid). The extract also contained substantial levels of stilbenes, such as ε-viniferin, *trans*-resveratrol, and *trans*-resveratrol tetramer, along with lower concentrations of anthocyanins, namely malvidin-3-O-glucoside.

Jara-Palacios et al. [54] conducted a similar study on grape stem composition and reported comparable findings. They identified flavan-3-ols, including catechin, epicatechin, procyanidin B1, procyanidin B4, and procyanidin B2 3-O-gallate, as the main phenolic compounds in the extract. Flavonols, like quercetin 3-O-glucuronide and quercetin 3-O-glucoside, as well as phenolic acids, such as gallic acid and caftaric acid, were also present.

In addition, the exploration of stilbenoids in grape stem extracts using ultrasound-assisted extraction revealed the presence of other stilbenes beyond resveratrol and viniferin. These included piceatannol, isorhapontigenin, and vitisin-B [55]. These studies collectively demonstrate the diverse range of phenolic compounds present in grape stem extracts and their potential biological activities, further highlighting the value of grape stems as a source of bioactive compounds.

Phenolic compounds extracted from grape stems are renowned for their robust antioxidant properties, extensively examined by researchers. R. Domínguez-Perles et al. [56] specifically researched the phenolic content of grape stem extracts from the red variety Touriga Nacional. They optimized extraction conditions and determined the total phenolic content to be 64.25 mg GAE/g dw, with total ortho-diphenols at 42.61 mg GAE/g dw, total flavonoids at 71.96 mg CE/g dw, and total anthocyanin at 1.99 mg/g dw. Furthermore, their investigation revealed a higher scavenging capacity by the extracted phenolic compounds.

Other researchers exploring extraction conditions discovered extracts abundant in phenolic compounds, with catechin (flavan-3-ol), gallic acid (hydroxybenzoic acid), a derivative of quercetin (flavonol), and stilbenes (resveratrol and viniferin) among the most prominent. In a related study, Jiménez-Moreno et al.

[57] correlated the phenolic composition of the extract with its antioxidant potential, concluding that stilbenes do not significantly contribute to the antioxidant properties of grape stem extracts. Instead, malvidin-3-glucoside and quercetin emerged as the primary compounds responsible for the extracts' antioxidant capacity.

Anastasiadi et al. [58] and Spatafora et al. [59] also investigated the extraction of phenolic compounds from grape stems, revealing rich sources of natural antioxidant compounds. Consequently, numerous investigations have highlighted the antioxidant effects of phenolic compounds derived from grape stems. Scientists have delved into the antioxidant properties of grape extracts, particularly in endothelial and muscle cells, analysing compositions from various grape varieties. While the phenolic composition remained consistent across all extracts, the levels of individual compounds differed, affecting antioxidant activity. Notably, the Mandilaria variety stood out for its superior antioxidant effect, attributed to higher concentrations of specific phenolic compounds like *trans*-resveratrol, gallic acid, quercetin, catechin, epicatechin, and rutin [60].

In another study, Domínguez-Perles et al. [61] investigated the impact of phenolic compounds from grape stems on redox imbalance in human keratinocytes (HaCaT cells). Their findings highlighted the potential of these compounds in regulating oxidative stress and identified malvidin-3-O-glucoside and isorhamnetin-3-O-(6-O-feruloyl)-glucoside as key players in modulating redox equilibrium. Moreover, the study revealed the anti-apoptotic properties of grape stem phenolics, reducing apoptotic cell occurrences in human keratinocytes.

Furthermore, Manca et al. [62] explored the topical application potential of grape stalk extracts, assessing their compatibility with human keratinocytes (HaCaT) and mouse embryonic fibroblasts (3T3). Their investigation demonstrated antioxidant activity in the extract, maintaining cell viability between 88% and 92% under oxidative stress. Additionally, the extract exhibited excellent biocompatibility with skin cells, with cell viability ranging between 100% and 120%.

Phenolic compounds are recognized for their potent antimicrobial activity, making them useful in various applications for controlling microbial growth, including bacteria [51].

To evaluate the phenolic profile and assess the antioxidant and antimicrobial properties, Silva et al. [63] characterized extracts from grape stems of two varieties, Touriga Nacional and Preto Martinho. They determined a total phenolic content of 45.9 mg/g and 226.8 mg/g, and a total tannin content of 7.76 mg/g and 22.15 mg/g, respectively. Flavan-3-ols were identified as the main phenolic compounds in the extracts, with catechin, epicatechin, and gallocatechin gallate being expressed. Phenolic acids, including p-coumaric acid and ferulic acid (hydroxycinnamic acids), as well as vanillic acid, gallic acid, and

protocatechic acid (hydroxybenzoic acids), were also highly present. *Trans*-resveratrol was identified as a stilbene compound. In terms of biological activity, the results demonstrated a good antioxidant capacity, and microbiologically, the extracts were effective in inhibiting gram-positive bacteria.

Dias et al. [64] conducted a study to identify the phenolic compounds responsible for this activity and evaluated the antimicrobial activity of extracts against digestive pathogens. They discovered that extracts from the Fernão Pires variety were more effective against *Listeria monocytogenes* and *Pseudomonas aeruginosa*, while extracts from the Tinta Amarela variety showed stronger activity against *Pseudomonas aeruginosa*. The phenolic compounds responsible for the antimicrobial activity were identified as kaempferol-3-O-rutinoside (flavonol), kaempferol-3-O-glucoside (flavonol), and caftaric acid (hydroxycinnamic acid).

Grape stem extracts have demonstrated various biological activities, suggesting potential applications in both the cosmetic and pharmaceutical industries. Leal et al. [20] conducted an analysis of extracts from different grape varieties, identifying catechin (flavan-3-ol), caftaric acid (hydroxycinnamic acid), and quercetin-3-O-rutinoside (flavonol) as the major phenolic compounds. Particularly substantial was the significant content of anthocyanidins, notably malvidin-3-O-galactoside and malvidin-3-O-glucoside, found in the grape stem extracts of the Tinta Roriz variety. The authors further investigated the antioxidant, antimicrobial, anti-inflammatory, and anti-ageing activities of these extracts. Grape stem extracts exhibited robust antioxidant potential, demonstrating effective scavenging capacity, consistent with previous findings [65]. In terms of antimicrobial activity, the extracts displayed efficacy in inhibiting Gram-positive bacteria in the gastrointestinal segment and diabetic foot ulcers, particularly *Enterococcus faecalis* and *Staphylococcus aureus* [65].

The anti-inflammatory properties of grape stem extracts were assessed by measuring the inhibition of nitric oxide (NO) production. All the examined extracts showed a strong ability to inhibit NO production by macrophages, with the Arinto variety extracts proving to be the most effective [20]. Additionally, Khadher et al. [66] investigated the anti-inflammatory properties of extracts derived from grape stems, with a specific focus on the white Sauvignon variety. Their study revealed a significant inhibition of inflammatory activity, with a reduction of 64.5%. Baicalein (a flavone) and resveratrol (a stilbene) were identified as potential phenolic compounds responsible for this anti-inflammatory action.

Grape stem extracts have shown promise as potential agents in combating skin aging due to their anti-elastase and anti-tyrosinase activities, which are associated with the overexpression of enzymes contributing to skin aging. Leal et al. [20] conducted studies on various grape stem extracts, revealing

that those from the Syrah variety exhibited the highest effectiveness in inhibiting tyrosinase and elastase activity, suggesting their viability for cosmetic applications.

Moreover, phenolic compounds found in grape stem extracts have displayed potential in preventing or inhibiting cancer cell growth, indicating anticancer activity. Quero et al. [67] investigated the phenolic composition of grape stems and assessed their effects on breast cancer cell lines, identifying catechin, quercetin, ε-viniferin, and *trans*-resveratrol as abundant compounds. These extracts showed promising results in reducing breast cancer cell growth and providing antioxidant effects on intestinal barrier cells. Similarly, Sahpazidou et al. [68] explored stem extracts from various grape varieties and their inhibitory effects on human cancer cell growth, particularly in renal and thyroid cancer cells.

Additionally, individual phenolic compounds within grape stem extracts have been studied for their specific biological activities. For instance, resveratrol has been suggested to have neuroprotective effects, potentially benefiting individuals with neurological disorders like Alzheimer's disease [69]. Furthermore, catechins and epicatechins found in grape stem extracts have been investigated for their potential neuroprotective effects [70].

In summary, grape stem extracts are abundant in phenolic compounds, particularly flavan-3-ols like catechin, although anthocyanins are present in relatively low amounts. The composition of these extracts can vary depending on factors such as grape variety, cultivation region, and environmental conditions [29,71–75]. Grape stem extracts exhibit diverse biological activities, including antioxidant, antimicrobial, anti-inflammatory, and anti-ageing properties, as summarized in **Figure 5**.

These findings highlight the potential applications of grape stem extracts in both the cosmetic and pharmaceutical industries. However, further research is necessary to elucidate the mechanisms of action and explore potential therapeutic applications of grape stem extracts and their phenolic compounds.

Table 5. Summary of biological activity of grape stems extracts according to grape variety, extract composition, and sample processing.

Grape Variety	Identified compounds	Biological Activity	Ref.
Moscatel White variety	Quercetin-3-O-glucoside Caftaric acid Unidentified hydroxycinnamic acid ε-viniferin Isohamnetin-3-O-(6-O-feruloyl)-glucoside Quercetin-3-O-rutinoside Kaempferol-3-O-rutinoside Kaempferol-3-O-glucoside	Antioxidant activity	[65]

Grape Variety	Identified compounds	Biological Activity	Ref.
Moscatel White variety	Caftaric acid Quercetin-3-O-glucoside Unindentified hydroxycinnamic acid ε-viniferin Isorhammetin-3-O-(6-O-feruloyl)-glucoside Quercetin-3-O-rutinoside Kaempferol-3-O-rutinoside Kaempferol-3-O-glucoside	Antioxidant activity Antibacterial activity	[73]
Malvasia Fina White variety	Quercetin-3-O-glucoside Caftaric acid Unidentified hydroxycinnamic acid Isorhammetin-3-O-(6-O-feruloyl)-glucoside Kaempferol-3-O-rutinoside Quercetin-3-O-rutinoside ε-viniferin Kaempferol-3-O-glucoside	Antioxidant activity Antimicrobial activity	[65]
Malvasia Fina White variety	Quercetin-3-O-glucoside Σ-viniferin Caftaric acid Isorhammetin-3-O-(6-O-feruloyl)-glucoside Quercetin-3-O-rutinoside Kaempferol-3-O-rutinoside Kaempferol-3-O-glucoside	Anti-apoptosis activity	[61]
Sauvignon White variety	Gallic acid Methyl 3,4-dihydroxybenzoate 7,3′-dihydroxyflavone 7-Hydroxy-4-methylcoumarin-3-acetic acid	Antioxidant activity Anti-inflammatory activity Antidiabetic activity Anti-Alzheimer activity	[66]
Preto Martinho Red variety	Catechin p-coumaric acid Quercetin-3-O-rutinoside Vanillic acid Gallocatechin gallate Epicatechin Ferulic acid Gallic acid Protocatechic acid *trans*-Resveratrol Catechin gallate	Antioxidant activity	[63]
Mazuelo Red variety	(+)-Catechin Quercetin-derivative *trans*-ε-viniferin *trans*-Resveratrol Gallic acid Unknown anthocyanin Caftaric acid Malvidin-3-glucoside Quercetin	Antioxidant activity Antioxidant capacity Antiproliferative effect	[67]

Grape Variety	Identified compounds	Biological Activity	Ref.
Touriga Nacional Red variety	Catechin Caftaric acid Quercetin-3-O-rutinoside Protocatechuic acid Epicatechin *trans*-Cinnamic acid Resveratrol ε-viniferin Malvidin-3-O-glucoside	Antioxidant activity Antimicrobial activity	[20]
Arinto White variety	Catechin Protocatechuic acid Caftaric acid Quercetin-3-O-rutinoside *trans*-Cinnamic acid Resveratrol ε-viniferin Gallic acid Epicatechin	Antimicrobial activity Anti-inflammatory activity	[20]
Syrah Red variety	Catechin Quercetin-3-O-rutinoside Caftaric acid Malvidin-3-O-glucoside Epicatechin *trans*-Cinnamic acid ε-viniferin Resveratrol Gallic acid	Antimicrobial activity Anti-tyrosinase activity Anti-elastase activity	[20]
Mavrodafne Red variety	Procyanidin B1 Gallic acid Caffeic acid Catechin Procyanidin B2 Epicatechin Quercetin *trans*-Resveratrol Ellagic acid Quercetin-3-O-glucoside	Antioxidant activity Antimutagenic activity	[76]

1.4.2.3. Extraction Procedures for Phenolic Compounds

The selection of an extraction method plays a pivotal role in recovering bioactive compounds from grape stems and other botanical sources. Various extraction techniques are available, each presenting its own set of advantages and drawbacks [7,30,38,43,77].

Among these methods, solvent extraction stands out as a widely employed approach, wherein a suitable solvent is utilized to extract compounds from the plant material. However, despite its common usage, solvent extraction comes with inherent limitations, including prolonged extraction times, substantial labour

requirements, and the consumption of large quantities of solvents. These factors not only contribute to increased costs but also pose environmental concerns [38,53]. Typically, the extraction process involves multiple sequential steps, as depicted in Figure 8.

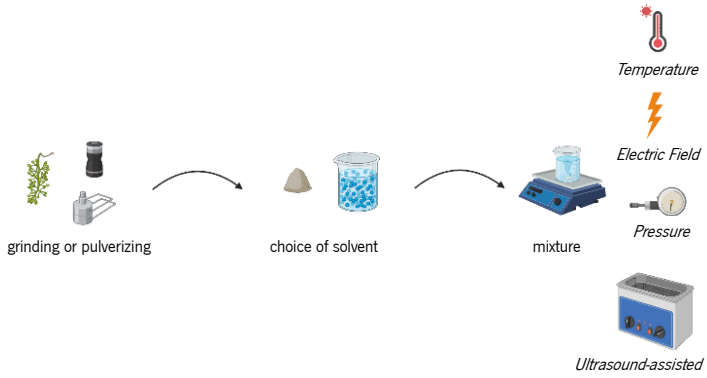


Figure 8. Schematic representation of the extraction procedure.

Researchers have explored various conditions and solvents to enhance the extraction efficiency of phenolic compounds from grape stems. For example, optimal conditions using a solid–liquid extraction with an ethanol–water mixture, along with specific temperature and time settings, were established to extract antioxidant compounds effectively [56]. Temperature and solvent selection were also identified as crucial parameters in solvent extraction, with ethanol at 60°C proving to yield superior results [78]. Moreover, the concentration of ethanol in the solvent was found to significantly impact the extraction of specific compounds like gallic acid, catechin, and *trans*-resveratrol [57]. Additionally, acetone, ethanol, and ethyl acetate were investigated as solvents, with each demonstrating better efficiency for extracting particular phenolic compounds [79].

To further optimize the extraction process, researchers have explored pretreatment techniques such as instantaneous controlled pressure drop (DIC) and ultrasound-assisted extraction (UAE). DIC pretreatment, when combined with conventional solvent extraction, enhances the extraction of phenolic compounds by disrupting plant cell walls, thereby increasing extraction efficiency [80]. UAE, which utilizes ultrasound waves to break down cell walls, offers advantages such as minimal instrumental requirements, cost-effectiveness, and shorter extraction times [63].

Other extraction techniques, including pressurized liquid extraction (PLE) and super-heated liquid extraction (SHLE), have also been investigated. PLE employs high pressure and temperature to extract bioactive compounds, offering benefits such as high extraction efficiency and low solvent consumption. However, determining optimal extraction temperatures is crucial [38,81]. SHLE, similar to PLE, has

shown promise, particularly when using superheated ethanol as the solvent, resulting in higher antioxidant content [82]. Novel extraction methods like high voltage electric discharge (HVED) have been explored as well. HVED disrupts cell walls through electrical discharges, thereby improving the extraction of target compounds, especially flavan-3-ols and flavonols [83].

Researchers have also explored combinations of different techniques. For instance, ultrasound-assisted extraction combined with pulsed electric field (PEF) pretreatment has demonstrated increased yields of phenolic compounds [84].

In conclusion, selecting an appropriate extraction method is critical for recovering bioactive compounds from grape stems. Researchers continue to investigate various conditions, solvents, and pretreatment techniques to enhance the efficiency and yield of phenolic compound extraction. Continued research and development in extraction methods are essential for improving the extraction process and obtaining higher yields of bioactive compounds from grape stems.

1.4.3. Cosmetic Industry

The cosmetic industry is a dynamic sector with a profound influence on the global economy, offering a diverse range of products designed to enhance the skin, hair, and overall appearance of individuals. It encompasses various product categories, including makeup, skincare, hair care, fragrances, and personal care items [85–87].

In the global market, the European cosmetic industry stands out for its size and innovative contributions. As reported by the European Cosmetics Association (Cosmetics Europe), the European cosmetics and personal care market reached an impressive value of EUR 78.6 billion in 2019. This industry significantly contributes to the European Union's economy by generating employment for over 1.7 million people and exporting products worldwide.

Competition within the cosmetic industry is intense, compelling companies to continuously innovate and differentiate themselves. This endeavour involves offering unique products, maintaining high-quality standards, and ensuring affordability. In the European Union, cosmetic products are subject to strict regulations that establish rigorous safety standards for both products and ingredients. This regulatory framework prioritizes consumer safety and underscores the industry's commitment to compliance [86,88].

An emerging trend in the cosmetic industry is the escalating demand for natural and organic products. Consumers are increasingly conscious of the ingredients in their cosmetic formulations and seek options

that are free from harmful chemicals while also being environmentally sustainable. In response, many companies have begun offering natural and organic cosmetic products that avoid synthetic fragrances, preservatives, and other potentially harmful substances. This trend reflects consumers' heightened awareness of the impact of personal care products on their health and the environment [85,89–94].

In summary, the cosmetic industry is a rapidly expanding and fiercely competitive domain catering to individuals' grooming and hygiene needs. It thrives on innovation, effective marketing strategies, and adherence to regulatory standards. As consumer preferences shift towards safety, sustainability, and environmental consciousness, the industry evolves to meet the demand for natural and organic products, highlighting its adaptability and responsiveness to changing market dynamics.

1.4.3.1. The Skin: Aging Process and Wound Healing

The skin, the body's largest organ, covers about 2 square meters in adults and serves as a protective barrier against external threats while regulating temperature and hydration [95]. Structurally, it comprises three main layers: the epidermis, dermis, and subcutaneous tissue (Figure 9) [96–98].

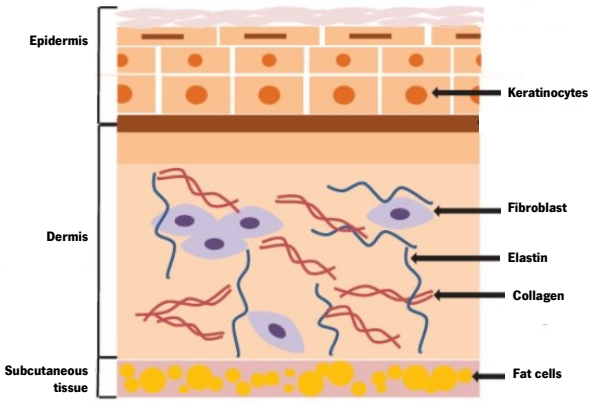


Figure 9. Skin structure. Adapted from [99].

The epidermis, the outermost layer of the skin, is primarily composed of keratinocytes, cells that produce keratin, a structural protein that gives the skin its strength and resilience [100–103]. These keratinocytes originate in the basal layer, the deepest part of the epidermis, where continuous cell division generates new skin cells. As these cells gradually move upward through the epidermal layers, they flatten, lose moisture, and become less viable. By the time they reach the skin's surface, known as the stratum corneum, they have died and transformed into tightly packed, flattened structures. This process, known as keratinization (Figure 10), results in the formation of the keratinized layer [98,104,105].

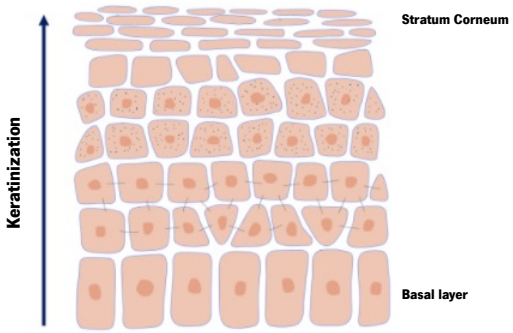


Figure 10. Keratinization process. Adapted from https://skintypesolutions.com/blogs/skincare/exfoliation-and-desquamation-of-skin (accessed on 01 September 2024).

This keratinized layer, composed of dead cells rich in keratin, acts as a vital protective barrier for the body. It shields the skin from mechanical forces such as pressure, friction, and impacts, as well as from harmful environmental factors like UV radiation, chemicals, and pathogens, including bacteria and fungi. In addition to protection, this layer also plays a crucial role in maintaining the body's internal balance by preventing excessive water loss [98,100].

The dermis, located beneath the epidermis, is a dense layer that contains a variety of structures, including cells, collagen and elastin fibres, blood vessels, nerves, sebaceous glands, hair follicles [98,106]. The primary cell in the dermis is the fibroblast, which produces the essential components of the dermal layer, such as collagen and elastin fibres [98,106,107]. Collagen fibres provide the skin with strength, while elastin fibres, which are thinner, give the skin its elasticity, allowing it to stretch and return to its original shape [98,107].

The subcutaneous tissue (or hypodermis) is the deepest layer of the skin, primarily composed of adipocytes (fat cells). This layer plays a crucial role in storing energy, regulating body temperature, and providing cushioning. Additionally, it serves as a connective layer, linking the skin to underlying structures such as muscles and bones [98,108].

Skin Aging Process

Skin aging is a complex biological process influenced by both intrinsic (genetic) and extrinsic (environmental) factors. Intrinsic aging is driven by genetic factors and hormonal changes. With the age, the production of sex hormones such as oestrogens and progesterone gradually decline, continuing through menopause [98,109]. This hormonal decline affects the skin in several ways. For example, it slows down cell turnover within the epidermis, leading to a thinner stratum corneum [110,111]. This

reduction in the outermost skin layer increases water loss, resulting in dryness and flakiness [85,112]. Additionally, the decrease in hormone levels leads to reduced collagen synthesis and increased collagen degradation. Consequently, the skin loses its firmness and elasticity, resulting in wrinkles and sagging (Figure 11) [85,112–114].

Extrinsic aging, on the other hand, is caused by external environmental factors, with UV radiation being a major contributor [98,114,115]. UV radiation accelerates skin aging by stimulating the overproduction of enzymes such as matrix metalloproteinases, which break down collagen in the dermis. Furthermore, UV exposure causes the denaturation and aggregation of elastic fibres, which diminishes dermal elasticity and leads to wrinkle formation. These changes result in skin that appears older and less resilient [116,117].



Figure 11. Skin aging. Adapted from https://dreamplasticsurgery.com/early-signs-of-aging-how-to-treat-them/ (accessed on 01 September 2024)

Wound Healing Process

When tissue is damaged, the body initiates the complex process of wound healing. This process depends on the coordinated actions of various cells, cytokines, and signalling molecules, as well as the vascular system, all of which work together to restore the injured tissue [118,119].

Wound healing begins with the release of lymphatic fluid and blood, leading to clot formation and haemostasis. Both the extrinsic and intrinsic coagulation pathways are activated to stop bleeding. Initially, vasoconstriction reduces blood flow, followed by platelet aggregation at the injury site. This process is brief and soon transitions to vasodilation, allowing white blood cells and more platelets to enter the area for further repair [118,120].

During the inflammatory phase of wound healing, TGF-β1 and platelet-derived growth factor (PDGF) play crucial roles in attracting and activating fibroblasts [121]. TGF-β1 promotes fibroblast proliferation and collagen production, which is essential for tissue repair [118]. At the same time, inflammatory cells like neutrophils and macrophages clear debris and bacteria [120]. These cells release inflammatory

cytokines, such as TNF-α, IL-1, and IL-6, which recruit additional immune cells and stimulate the next phase of healing, that is proliferation [118,120,121]. In this stage, fibroblasts and keratinocytes proliferate to rebuild the damaged tissue. Fibroblasts produce collagen, forming the extracellular matrix, while keratinocytes migrate across the wound to restore the epidermis (re-epithelialization) [122]. Angiogenesis (formation of new blood vessels) also occurs to provide nutrients to the regenerating tissue [121]. The wound also begins to contract and is facilitated by continued deposition of fibroblasts and myofibroblasts [118,120].

For last, the remodelling, the final phase, involves the reorganization of collagen fibres and the maturation of the newly formed tissue. Over time, the wound contracts, the excess collagen degrades, and the scar becomes less noticeable [118,120,121].

The process is illustrated in the Figure 12.

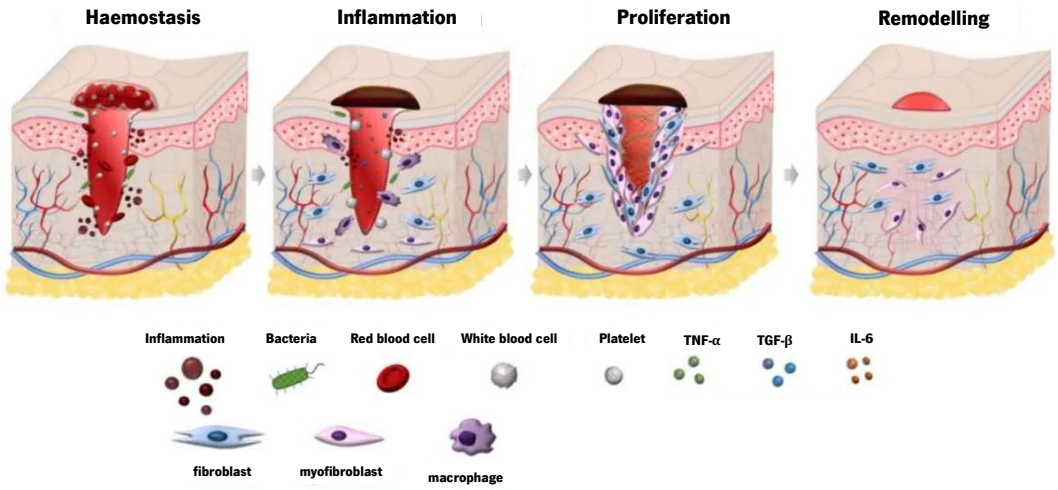


Figure 12. Wound healing process. Adapted from https://www.credihealth.com/blog/stages-of-healing-a-wound (accessed on 01 September 2024)

The dysregulation of the wound healing process, often caused by prolonged inflammation and excessive cytokine production, can lead to abnormal scarring, such as keloids and hypertrophic scars. In these types of scars, fibroblasts exhibit increased proliferation and resistance to apoptotic signals, which results in excessive and disorganized collagen production, along with an imbalance in extracellular matrix proteins. Both hypertrophic scars and keloids show heightened signalling of TGF-β1, a key driver of collagen and ECM overproduction in these conditions [123–125]. Additionally, excessive angiogenesis is a notable factor in the pathophysiology of these scar types, further perpetuating the inflammatory response [126].

Current treatment strategies mainly focus on inhibiting fibroblast growth by promoting apoptosis, suppressing TGF-β1 expression, and reducing angiogenesis [127]. Moreover, treatments aim to stimulate collagenase activity and inhibit signalling pathways involved in keloid formation, such as those associated with vascular endothelial growth factor (VEGF), TGF-β1, and IL-6 [128].

1.4.3.1.1. Effect of Phenolic Compounds in the Skin

Phenolic compounds exhibit a broad spectrum of effects on the skin, their actions varying depending on the compound and the specific skin area targeted [22,89,93,129–131]. The Cosmetic Ingredient Review Expert Panel (CIR), an independent scientific body, has thoroughly evaluated the safety of 24 grape-derived ingredients commonly used in cosmetics. According to their assessment, these ingredients are deemed safe for cosmetic use, serving various functions such as skin conditioning, antioxidant protection, flavouring, and colour enhancement [132].

Within the skin, phenolic compounds can interact with different structures, including keratinocytes and fibroblasts. Some polyphenols possess the ability to hinder melanin production in the epidermis, thereby aiding in the reduction of hyperpigmentation and enhancement of skin tone. A key mechanism of action for phenolic compounds is their capacity to neutralize free radicals and mitigate oxidative stress. Given that oxidative stress significantly contributes to skin aging, the antioxidative properties of phenolic compounds play a vital role in shielding the skin from damage induced by reactive oxygen species and other free radicals.

Researchers have increasingly focused on the potential of phenolic compounds in cosmetic formulations due to their anti-inflammatory and protective effects on the skin against environmental stressors such as UV radiation and pollution.

Czemplik et al. [133] examined the effects of flax shives extract on normal human dermal fibroblasts (NHDF), finding increased cell viability and modulation of genes related to extracellular matrix remodelling and inflammation, such as IL-6, IL-10, and SOCS1. Similarly, Illescas-Montes et al. [134] observed that phenolic compounds promote wound healing without disrupting the cell cycle.

Melguizo-Rodríguez et al. [135] and Panichakul et al. [136] investigated olive oil and *Ipomoea pes-caprae* (IPC) extracts, respectively, and reported enhanced fibroblast activity and improved gene expression related to wound healing. Notably, Panichakul et al. [136] also found that IPC extracts increased collagen synthesis, suggesting their potential use in anti-ageing formulations.

Mittraphab et al. [137] explored phenolic compounds derived from *Entada phaseoloides* leaves and highlighted their potential anti-photoaging and anti-inflammatory properties. Additionally, Silva et al. [138] studied *Melaleuca leucadendron* (L.) L. flower extract and revealed its antioxidant and photoprotective effects against UV radiation-induced damage.

Phan et al. [139] investigated the impact of various phenolic compounds on keloid and hypertrophic scars. They found that quercetin demonstrated significant potential in inhibiting both the proliferation and contraction of fibroblasts derived from excessive scar tissue. Similarly, Park et al. [140] also found that epigallocatechin-3-gallate reduces the pathological attributes of keloid fibroblasts.

Together, these studies underscore the promising role of phenolic compounds in skincare formulations, offering potential benefits for enhancing skin health.

1.4.3.2. Cosmetics

Cosmetics, as defined by Regulation (EC) No. 1223/2009 of the European Parliament and of the Council dated 30 November 2009 [141], encompass a broad spectrum of products designed for personal care, hygiene, and beautification purposes. This definition includes substances or mixtures applied externally to various parts of the body, including the skin, hair, nails, lips, external genital organs, teeth, and mucous membranes of the oral cavity [88].

The primary objective of cosmetics is to enhance the appearance, texture, and overall health of the skin, hair, and nails. These products serve purposes such as cleansing, moisturizing, protecting, and accentuating the natural beauty of these tissues [85]. Additionally, cosmetics effectively address pigmentation concerns, such as hyperpigmentation (darkening of the skin) and hypopigmentation (lightening of the skin), which can result from factors like sun exposure, hormonal changes, and aging [85,142].

Moreover, numerous cosmetic products are specially formulated to diminish the appearance of fine lines and wrinkles, refine skin texture, and promote a more youthful appearance. They also play a role in preventing acne while providing protection against harmful UV rays from the sun. This preventive measure aids in prevention sunburn, minimizing skin damage, and reducing the risk of skin cancer [85,115].

1.4.3.2.1. EU Cosmetic Regulation

Cosmetics in Europe fall under the regulatory purview of the European Union (EU) through the Cosmetics Regulation (EC) No 1223/2009 [141]. This comprehensive framework, aimed at standardization and

consumer safety, oversees the production and marketing of cosmetic products in the EU. Good Manufacturing Practices (GMP) guidelines, including ISO 22716, play a crucial role in maintaining consistent quality during production [143].

EU regulations stipulate that cosmetic products must meet specific requirements, prioritizing safety, quality, and efficacy [143,144]. Safety is paramount, requiring products to be safe for human health under normal usage conditions and not pose risks to health or the environment. Evaluation for toxicity, irritation, and sensitization is mandatory. Additionally, products must effectively fulfil their intended functions, such as moisturizing or providing anti-ageing effects, while meeting stability and microbiological quality standards [145]. This entails assessing stability over time, including resistance to environmental factors, and ensuring microbiological safety.

1.4.3.2.1.1. Evaluation of Safety, Quality, and Efficacy of Cosmetic Formulations Containing Phenolic Compounds

Numerous scientific studies have delved into the efficacy and safety of cosmetic formulations incorporating phenolic compounds across a spectrum of products [129].

Wagemaker et al. [146] examined a topical antioxidant formulation with green tea polyphenol (GTP), demonstrating its ability to penetrate the skin, restore the skin barrier, and suppress inflammatory cytokines. Maluf et al. [147] investigated grape pomace extract, finding significant radical scavenging activity and no cytotoxicity, suggesting its viability for antioxidant cosmetics.

Taofiq et al. [148] explored mushroom ethanolic extracts, determining their safety for keratinocytes and fibroblasts in cosmetic formulations. Rafique et al. [149] focused on a cream with grape seed extract, observing enhanced skin hydration, elasticity, and reduced pore size and roughness, indicating its potential as an anti-ageing agent. Similarly, Sharif et al. [150] tested an emulsion with grape seed extract on male cheek skin, witnessing decreased melanin content, sebum, and erythema, along with improved elasticity.

Waqas et al. [151] developed a grape seed extract-based cosmetic, showcasing reduced melanin levels, increased moisture content, and improved skin elasticity over 12 weeks. These formulations highlight phenolic compounds' potential as anti-ageing and moisturizing agents.

Moreover, phenolic compounds have been found to inhibit collagenase and elastase, crucial enzymes in collagen and elastin breakdown. This inhibition aids in wrinkle prevention [152]. Some compounds also

absorb UV and blue light, acting as physical filters. However, grape seed extracts alone may not suffice for sun protection, necessitating combination with other formulations for enhanced efficacy [153,154]. However, Hubner et al. [154] evaluated a sunscreen formulation incorporating grape pomace extract, achieving high SPF values and superior UVA protection without adverse effects.

In vitro and *in vivo* studies have elucidated the biological activity and effectiveness of phenolic compounds, underscoring their potential in cosmetics. Green tea polyphenol, grape pomace extract, and grape seed extract stand out for their antioxidant, anti-ageing, anti-inflammatory and photoprotective properties, contributing to the growing understanding of phenolic compounds' role in cosmetic formulations.

1.4.3.2.1.2. Evaluation of Penetration of Phenolic Compounds in the Human Skin

Penetration of active substances into the skin involves a series of intricate steps. Initially, the active substance diffuses within the topical formulation, influenced by its physicochemical properties and the formulation itself. Subsequently, the release of the active substance from the formulation occurs, a process influenced by factors like viscosity, pH, and temperature. However, the most challenging step is the actual penetration of the active substance through the skin due to its heterogeneous nature [96]. Studies have delved into the skin permeation of phenolic compounds.

Marti-Mestres et al. [155] investigated the percutaneous penetration of natural antioxidant compounds into excised pig skin in vitro. Their findings revealed differential penetration, with some compounds reaching all skin layers (caffeic acid and chlorogenic acid) while others were primarily detected in the epidermis (oraposide).

The penetration of phenolic compounds into deeper skin layers may vary depending on the delivery system employed. Butkeviciute et al. [156] examined the release and penetration of phenolic compounds from various formulations into human skin layers. They discovered that specific compounds exhibited penetration into both dermal and epidermal layers, with variations based on the formulation used. Additionally, they evaluated the antioxidant potency of these compounds in the skin, highlighting the importance of formulation in achieving effective skin penetration and antioxidant activity. Similarly, Rafique et al. [157] developed cosmetic formulations (emulgel and emulsion) using grape seed extract (GSE) and assessed their efficacy. The formulations effectively improved skin elasticity and hydration while reducing roughness, scaliness, and wrinkles, with emulgel formulations yielding superior results.

In summary, evaluating penetration is crucial for assessing the efficacy of cosmetics, as the ability of active ingredients to penetrate the skin directly influences their effectiveness. Understanding the

penetration mechanisms and optimizing formulations can enhance the delivery of active compounds, ensuring desired cosmetic effects.

1.4.3.2.1.3. Evaluation of the Stability of Cosmetic Formulation with Phenolic Compounds

Studying the stability of cosmetic formulations is imperative according to EU regulation [141,145], ensuring product safety, efficacy, and aesthetic appeal throughout its shelf life. Cosmetic products comprise diverse ingredients prone to interaction, chemical or physical changes, or degradation over time, potentially compromising effectiveness, sensory properties, and consumer safety.

Typically, cosmetic product stability is assessed through various physical, chemical, and microbiological tests, encompassing pH measurement, viscosity determination, colour and odour assessment, microbial contamination testing, and accelerated aging studies. These evaluations determine changes in appearance, texture, and chemical composition over time, estimating the product's susceptibility to degradation or microbial growth [158].

Rafique et al. [149] examined their formulation's stability by monitoring physical attributes such as liquefaction, coalescence, phase separation, pH, colour, and conductivity over 12 weeks. Results indicated stability at room temperature with minimal pH variation within the acceptable range for human skin.

Wagemaker et al. [146] evaluated their formulations' preliminary stability through centrifugation, pH measurements, microscopy, visual observation, and viscosity assessments. Their formulations exhibited stability without phase separation or crystalline structure formation, with pH values and viscosity remaining within acceptable limits.

Following the development of cosmetic formulations containing grape seed extracts, Rafique et al. [157] assessed stability, observing consistent viscosity and pH levels over 12 weeks without phase separation.

In the study by Yarovaya et al. [153], stability of sunscreens with grape seed extracts was examined post heat/cold cycles. The formulations demonstrated robust stability, maintaining white colour, light texture, and smoothness post-test.

Ensuring stability is paramount to preserve product quality and efficacy throughout its shelf life, guaranteeing consumers receive the intended benefits of phenolic compounds for skin health and beauty.

1.4.4. Conclusions

In conclusion, the use of grape stem extracts in cosmetics shows immense potential for unlocking a multitude of beauty benefits. Abundant in phenolic compounds, these extracts offer antioxidant, anti-inflammatory, antimicrobial, and anti-ageing properties, promising to shield the skin from oxidative stress, reduce inflammation, and enhance skin elasticity and firmness for a more youthful appearance.

Although research on grape stem extract application in cosmetics is in its promising stages, the results thus far are highly promising. Integration of these extracts presents a natural and sustainable alternative to conventional synthetic cosmetic ingredients, supporting the circular economy by repurposing a by-product of the wine industry that would otherwise be discarded. With ongoing research, the incorporation of grape stem extracts into cosmetics is expected to become more widespread.

Furthermore, recent advancements have addressed challenges related to delivering phenolic compounds to the skin. Innovative approaches, such as encapsulating these compounds in nanoparticles, liposomes, and specialized carriers, show potential for enhancing their efficacy and delivery to target areas.

Cosmetic companies can capitalize on the benefits of grape stem extracts in their formulations, offering consumers safe, effective, and sustainable products that promote healthy and beautiful skin. As research progresses, the future holds promising prospects for grape stem extracts to revolutionize the cosmetics industry and contribute to skin health and well-being.

1.4.5. References

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2. Objectives

2.1. General Objectives

The primary objective of this doctoral thesis is to explore the potential of wine industry by-products, specifically extract from blend grape varieties (blend GS extract), for use in the cosmetics industry. This work aims to apply the concept of the circular economy in line with the United Nations Sustainable Development Goals, contributing to more sustainable and resource-efficient practices in cosmetics production.

2.2. Specific Objectives

- **Characterization of the blend GS extract:** Quantification and identification of phenolic compounds, along with the determination of biological activities, including antioxidant, anti-ageing, and depigmenting properties.
- **Study of the Influence of blend GS Extract on skin cells:** Evaluation of the effects on HaCaT keratinocytes, BJ-5ta fibroblasts, and J774A.1 macrophages, focusing on morphology, metabolic activity, anti-inflammatory effects, cell cycle, and cell migration.
- **Incorporation of GS Extract Blend into Cosmetic Formulations:** Assessment of formulations with varying concentrations of the extract, including characterization and stability studies (colour, odour, appearance, pH, biological monitoring, rheology, and textural properties), along with the quantification and identification of phenolic compounds and evaluation of antioxidant and anti-ageing activities.

3. Characterization of Blend GS Extract and Its Potential in Cosmetic Formulations

3.1. Chemical and Biological Properties of Phenolic Compounds from Grape Stems

3.1.1. Introduction

The European wine sector embraces a prominent position in the global economy, with countries such as France, Italy, Spain, Germany, and Portugal identified by the International Organization of Vine and Wine (OIV) as key contributors to worldwide wine production [1].

The global wine industry, immersed in tradition and economic importance, has attracted increasing attention for its potential contributions to sustainable practices, particularly within the scope of circular economy principles [2,3]. Grapes, essential for wine production, offer not only a base for the winemaking process but also a source of valuable by-products such as grape stems (or stalks), seeds, and skins, which have the potential for circularity and economic sustainability [4–7].

In the context of the circular economy [8], where the emphasis is on minimizing waste and maximizing resource efficiency, the valorisation of by-products becomes fundamental. The grape stem, one of the most generated by-products (up to 14%) in winemaking, is often overlooked and represents a significant by-product with natural bioactive compounds, particularly phenolic compounds [9].

Phenolic compounds, as secondary metabolites synthesized by plants in response to various environmental challenges, serve as pivotal defence mechanisms against pathogens, parasites, UV radiation, and both biotic and abiotic stressors [9,10]. Grape stems represent a rich source of these compounds, including flavanols (epicatechin, catechin), flavonols (quercetin-3-o-rutinoside), hydroxycinnamic acids (caftaric acid, coumaric acid), hydroxybenzoic acids (gallic acid), and stilbenes (viniferin), among others [11–16]. While the phenolic compounds present in various extracts from grape stems remain consistent, the distinguishing factor resides in the varying quantities of each compound across different grape varieties. In addition to grape variety, several other factors, including soil type, maturation state, size, and harvest time, exert influence on the composition of the extract [17–20].

These differences significantly impact the extract's biological activities, which are increasingly recognized for their potential health benefits, encompassing antioxidant, anti-inflammatory, anti-ageing, anti-cancer, and antimicrobial properties [10,19,21–23].

In the field of antioxidant efficacy, phenolic compounds derived from grape stems, particularly from Touriga Nacional [15], Sousa [12], Cabernet Moravia [14], and Mazuelo [16] varieties, have exhibited notable scavenging capacities.

Additionally, these metabolites, extracted from Mandilaria variety grape stems, have demonstrated antioxidant properties in both endothelial and muscle cells [24].

Exploring anti-inflammatory potential, grape stem extracts from the Arinto variety have exhibited the ability to inhibit NO production by macrophages [25]. Furthermore, the antiaging attributes of grape extracts, particularly from the Syrah variety, have been assessed through elastase and tyrosinase enzyme inhibition, revealing promising outcomes [25].

In the context of antimicrobial activity, extracts from grape stems of Fernão Pires and Tinta Amarela varieties have been investigated against diverse microorganisms, including *Listeria monocytogenes* and *Pseudomonas aeruginosa*, demonstrating notable effectiveness [13].

These findings underscore the diverse and promising bioactive properties of phenolic compounds extracted from grape stems, emphasizing their potential applications in health and wellness.

Previous investigations have predominantly focused on studying isolated grape varieties. However, the current study takes a different approach by exploring the extraction of phenolic compounds from combined grape stems of various varieties. This exploration offers the prospect of enhancing various properties due to the potential for synergies among diverse varieties. These synergies not only enrich the overall bioactive profile but also expand the spectrum of health-related benefits associated with the extracted compounds. The collaborative effects of these compounds may significantly contribute to improved antioxidant activity, providing robust protection against oxidative stress. Furthermore, recognizing that each grape variety may contribute distinct major compounds, this approach adopts a holistic strategy to address various aspects of aging.

Considering the principles of the circular economy and the valorisation of by-products, this study primarily focuses on the chemical and biological analysis of grape stem extract from different varieties and explores their biological properties, including antioxidant, anti-elastase, anti-tyrosinase and anti-inflammatory activities. Additionally, the study is designed to provide practical insights applicable to the cosmetic and pharmaceutical industries.

3.1.2. Materials and Methods

3.1.2.1. Chemicals and Reagents

Gallic acid (3,4,5-trihydroxybenzoic acid), Folin-Ciocalteau reagent and acetic acid (all extra pure >99%) were obtained from Panreac (Panreac Química S.L.U., Barcelona, Spain). Sodium hydroxide (98%),

sodium nitrite, sodium carbonate (purity levels >99%), aluminum chloride (purity levels >99%), and ethanol were acquired from Merck (Merck, Darmstadt, Germany).

Sodium molybdate (99.5%) was purchased from Chem-Lab (Chem-Lab N.V., Zedelgem, Belgium). Additionally, catechin (98%), TROLOX (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, purity ≥98.0%), $ABTS^{\bullet+}$ ((2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid) diammonium salt, purity ≥98.0%), $DPPH^{\bullet}$ (2,2-diphenyl-1-picrylhidrazyl radical, 100.0%), potassium persulfate (purity ≥ 99.0%), TPTZ (2,4,6-Tripyridyl-s-Triazine, purity ≥ 98.0%), iron (III) chloride (purity ≥ 99.9%), tyrosinase and elastase enzymes, and the reagents employed in all enzymatic activities were sourced from Sigma Aldrich (Steinheim, Germany), while hydrochloric acid (≈37%) was obtained from Honeywell, Fluka, (Germany).

All reagents used in the cell assays were obtained from PAN-Biotech.

Distilled water was generated using a Millipore water purification system (Millipore, Bedford, MA, USA).

3.1.2.2. Preparation of blend GS Extract

Grape stems from various varieties were dried in an oven (Memmert, Schwabach, Germany) at 40°C for 72 hours. After drying, the stems were ground, and the resulting samples from the different varieties were combined in equal proportions to create a blend extract.

The extraction of phenolic compounds followed the methodology defined by Dias Costa et al. [26] with certain modifications.

The sample weighed at 1 g, were homogenized and stirred in 50 mL of extraction solvent (Ethanol:Water, 70:30) for 30 minutes in an orbital shaker (GFL 3005, GEMINI, Apeldoorn, The Netherlands). Post-homogenization, the mixture was subjected to centrifugation (Sigma 2-16KL Refrigerated Centrifuges, Sigma Laborzentrifugen, Berlin, Germany) at 10000 × g for 15 minutes at 4 °C, a process repeated three times. The resulting supernatant volumes from the repeated centrifugations were adjusted to 50 mL in a volumetric flask with extraction solvent.

3.1.2.3. Determination of Phenolic Content

The determination of the phenolic content in blend GS extract involved adapted spectrophotometric methodologies designed for 96-well microplates (PrimeSurface MS-9096MZ, Frilabo, Maia, Portugal), according to Gouvinhas et al [27]. The absorbances were subsequently measured using microplate readers (Multiskan GO Microplate Photometer, Thermo Fisher Scientific, Vantaa, Finland).

3.1.2.3.1. Determination of Total Phenolic Content

To determine the total phenolic content (TPC) of the blend GS extract, 1 mL of the sample was mixed with 0.5 mL of Folin–Ciocalteau reagent. Next, 2.0 mL of Na_2CO_3 (7.5% w/v) and 6.5 mL of water were added to the mixture. The reaction was incubated at 70 °C for 30 minutes, protected from light, and then cooled under running water. The absorbance was measured at 750 nm. An analytical curve was constructed using various concentrations of gallic acid, and the total phenolic content was quantified and expressed as milligrams of gallic acid equivalent per litre of the sample (mg GAE/g sample).

3.1.2.3.2. Determination of ortho-diphenols content

The ortho-diphenols content (ODC) of the blend GS extract was assessed by adding 1 mL of Na_2MoO_4 (50 g/L) to 4 mL of the appropriately diluted samples. After vortexing, the mixtures were left to stand at room temperature for 15 minutes, shielded from light. The absorbance was then measured at 370 nm. Quantification was performed using gallic acid as the standard, and the results were expressed in milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g dw).

3.1.2.3.3. Determination of Flavonoids Content

The method employed for determining flavonoid content (FC) involved the formation of a flavonoid-aluminum complex. In this process, 0.5 mL of the diluted sample was added to a test tube, followed by the addition of 150 µL of a 5% sodium nitrite solution. After allowing a 5-minute reaction, 150 µL of a 10% aluminum chloride solution was introduced. Following a 6-minute reaction period, 1 mL of a 1M sodium hydroxide solution was added to the well. The microplate was then stirred for 30 seconds, and the absorbance was measured at 510 nm. To construct the calibration curve, various concentrations of catechin were used. The results were expressed as milligrams of catechin per gram of dry weight of the sample (mg CAT/g dw).

3.1.2.4. Identification of Phenolic Compounds by HPLC-MS

The quantitative (poly)phenolic profile of the grape stem extract was achieved by applying the methodology described by Costa-Pérez et al. [25]. The chromatographic separation of (poly)phenols was developed on a Luna C18 column (150.0 × 4.6 mm, 5.0 µm particle size, Phenomenex, Macclesfield, UK) using an Agilent HPLC 1100 series (Agilent Technologies, Waldbronn, Germany) that consisted of a binary pump (model G1312A), an autosampler (model G1313A), a degasser (model G1322A), a photodiode array (PDA) detector (model G1315B), and an ion trap spectrometer (model G2445A) with an electrospray

ionization interface, all controlled by LCMSD software, v. 4.1 (Agilent Technologies), operated according the specifications detailed by Barros et al. [12]. The mobile phases used were H_2O/formic acid (99.0:1.0, v/v) (solvent A) and acetonitrile/formic acid (9.0:1.0, v/v) (solvent B). Spectral data from all peaks were detected in the 200–600 nm range, with chromatograms recorded at 280 nm for proanthocyanidins, 330 nm for phenolic acids and stilbenes, and 360 nm for flavonols. Mass spectrometry data were acquired in the negative mode. Phenolic compound identification was carried out by examining retention time (min), parent ions, and MS2 fragmentation patterns. Phenolic compounds were quantified using PDA chromatograms recorded at 280 nm for proanthocyanidins, 330 nm for phenolic acids, and 360 nm for flavonols, applying daily prepared calibration curves with catechin (proanthocyanidins and catechin derivatives), chlorogenic acid (phenolic acids), and quercetin glucoside (flavonols), and the concentration expressed as µg/g dw.

3.1.2.5. Determination of Antioxidant Capacity

The antioxidant capacity of the blend GS extract was assessed using spectrophotometric methods, specifically the ABTS ((2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid) diammonium salt), DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (Ferric Reducing Antioxidant Power) assays, according to Santos et al [28]. Both methods are based on the fundamental principle of antioxidants within the sample donating electrons to reduce the oxidized forms of the reagents.

In the ABTS and DPPH assay, the reaction centres around a radical formed by the oxidation of ABTS and DPPH, respectively. On the other hand, the FRAP assay is centred on the reduction of a ferric complex.

3.1.2.5.1. ABTS assay

The initial phase of this assay involved oxidizing the ABTS salt, achieved by introducing 148 mM potassium persulfate solution (88 µL) to the 7 mM ABTS solution (5 mL). The mixture was left to rest for 12 to 16 hours, protected from light at room temperature, to achieve its most stable oxidative state.

Subsequently, the $ABTS^{\cdot+}$ working solution was prepared by diluting the cationic $ABTS^{\cdot+}$ solution in a 20 mM sodium acetate buffer (pH 4.5) until an absorbance of 0.700 ± 0.020 at 734 nm was achieved.

Then, the evaluation of sample antioxidant capacity involved adding 188 µL of $ABTS^{\cdot+}$ working solution to each microplate well, followed by the introduction of 12 µL of the diluted sample and blank. After a 30-minute incubation period at room temperature, protected from light, the absorbance was measured at 734 nm [Abs (734 nm)]. For this assay, TROLOX at various concentrations served as the standard for constructing the analytical curve, with water working as the blank.

The ABTS assay measured the scavenging capacity of $ABTS^{\cdot+}$ by the samples, and the inhibition percentage was calculated using the formula:

$$\%\ \text{inhibition}_{\text{Sample}} = \frac{\text{Abs (734 nm)}_{\text{Blank}} - \text{Abs (734 nm)}_{\text{Sample}}}{\text{Abs (734 nm)}_{\text{Blank}}} \times 100$$

The results were presented in TROLOX Equivalent Antioxidant Capacity (TEAC) and were expressed in millimoles of TROLOX per gram of dry weight of the sample (mmol TROLOX/g dw).

3.1.2.5.2. FRAP assay

The evaluation of antioxidant activity using the FRAP method began with the preparation of the FRAP working solution. This solution was prepared by combining 10 volumes of 220 mM acetate buffer (pH 3.6), 1 volume of 40 mM TPTZ (2,4,6-Tripyridyl-s-Triazine) solution dissolved in 40 mM HCl, and 1 volume of 20 mM ferric chloride. The FRAP working solution, prepared daily, was preheated to 37 °C 10 minutes before utilization.

Subsequently, 20 µL of the sample was introduced into the microplate well, followed by the addition of 280 µL of the FRAP working solution. The microplate was then shaken and incubated at 37 °C in darkness for 30 minutes, after which the absorbance was measured at 593 nm. To determine the calibration curve, varying concentrations of TROLOX were used. The results were presented in millimoles of TROLOX per gram of the sample dry weight (mmol TROLOX/g dw).

3.1.2.5.3. DPPH assay

In the initial phase of this assay, the DPPH stock solution (8.87 mM) was diluted in ethanol: water (70:30, v/v) until an absorbance close to 1.000 at 520 nm was achieved, creating the DPPH working solution. To evaluate the antioxidant capacity of the samples, 190 µL of the DPPH working solution was added to each microplate well, followed by 10 µL of the diluted sample and blank (ethanol: water, 70:30 v/v). The mixture was incubated at room temperature for 30 minutes, protected from light, and the absorbance was measured at 520 nm [Abs (520 nm)]. TROLOX, at various concentrations, was used as the standard to construct the analytical curve. The inhibition percentage was calculated using the following formula:

$$\%\ \text{inhibition}_{\text{Sample}} = \frac{\text{Abs (520 nm)}_{\text{Blank}} - \text{Abs (520 nm)}_{\text{Sample}}}{\text{Abs (520 nm)}_{\text{Blank}}} \times 100$$

The results were presented in TROLOX Equivalent Antioxidant Capacity (TEAC) and were expressed in millimoles of TROLOX per gram of dry weight of the sample (mmol TROLOX/g dw).

3.1.2.6. Evaluation of anti-ageing and depigmenting activities

The evaluation of anti-ageing activity was carried out through assays measuring the inhibition percentage of elastase and tyrosinase enzymes, according to Taghouti et al [29].

3.1.2.6.1. Tyrosinase inhibition assay

The tyrosinase inhibition assay was conducted to measure the percentage of tyrosinase inhibition. In this procedure, 20 µL of 1000 U/mL tyrosinase and 170 µL of a mixture containing 1 mM L-tyrosinase solution, 50 mM phosphate buffer (pH 6.5), and water (in a 10:10:9 proportion) were added to the sample. Subsequently, the microplate was incubated at 37 °C for 10 minutes, and the absorbance was read at 490 nm. Positive and negative controls, 1 mg/mL kojic acid, and 10% DMSO, respectively, were used.

The percentage of inhibition was calculated using the following equation:

$$\%\ \text{inhibition}_{Sample} = \frac{\text{Abs (490 nm)}_{Negative\ Control} - \text{Abs (490 nm)}_{Sample}}{\text{Abs (490 nm)}_{Negative\ Control}} \times 100$$

3.1.2.6.2. Elastase inhibition assay

The elastase inhibition assay was performed to determine the percentage of elastase inhibition.

50 µL of the sample was combined with 160 µL of 0.20 mM Tris-HCl buffer (pH 8) and 20 µL of 0.80 mM N-Succinyl-Ala-Ala-Ala-p-nitroanilide substrate (in Tris-HCl buffer). This mixture was incubated for 10 minutes at room temperature. Subsequently, 20 µL of 1 U/L elastase enzyme (in Tris-HCl buffer) was added, and the microplate was incubated for an additional 20 minutes at room temperature. The absorbance was then measured at 410 nm. Tris HCl-buffer served as the negative control.

The percentage of inhibition was calculated using the following equation:

$$\%\ \text{inhibition}_{\text{Sample}} = \frac{\text{Abs (410 nm)}_{\text{Negative Control}} - \text{Abs (410 nm)}_{\text{Sample}}}{\text{Abs (410 nm)}_{\text{Negative Control}}} \times 100$$

3.1.2.7. Cell maintenance and assays

3.1.2.7.1. Cell morphology assay

HaCaT and BJ-5ta cells were first plated in a 48-well plate at the following optimized densities (3.5×10^4 cells/mL) and incubated for 24 hours.

After 24 hours, the extracts were added to the wells, and the incubation continued for an additional 24 hours. The medium was then removed, and the cells were washed once with PBS. To fix the cells, 100 µL of 4% paraformaldehyde was added, and the cells were incubated for 40 minutes at room temperature (RT). Following fixation, the cells were washed once more with PBS.

For permeabilization, 100 µL of 0.1% Triton X-100 in PBS was added, and the cells were incubated for 30 minutes at RT. The permeabilization solution was prepared by mixing 5 µL of Triton X-100 with 4995 µL of PBS. After permeabilization, the cells were washed again with PBS.

Phalloidin staining was performed by diluting 0.25 µL of Alexa Fluor 568 Phalloidin (Invitrogen, A12380) from a 400x stock solution into 100 µL of PBS. The staining solution was prepared by combining 3.8 µL of the Phalloidin stock solution, 1496.2 µL of PBS, and 15 mg of BSA. The cells were then incubated with this Phalloidin solution for 60 minutes at RT, protected from light.

After staining, the cells were washed twice with PBS. Subsequently, 50 µL of DAPI solution was added, and the cells were incubated for 15 minutes. Finally, the cells were washed twice with PBS and examined using a microscope (Leica Inverted Microscope DMI 3000B).

3.1.2.7.2. Cell metabolism assay: Resazurin Assay

Cells were monitored until they reached 80% confluency. Once ready, the DMEM medium was removed, and cells were washed with filtered PBS. To detach the cells, 500 µL of trypsin was added to T25 flasks. Cells were incubated for 5–10 minutes, and detachment was confirmed by microscopy or gentle shaking. After detachment, double the volume of medium was added to neutralize the trypsin.

Cells were sub cultured by adding 1000 µL of the cell suspension to 5 mL of medium in a T25 flask. The flasks were then incubated under standard conditions.

3.1.2.7.2.1. Cell Counting for Resazurin Assay

After subculturing, the cell suspension was transferred to 15 mL Falcon tubes. A 10 µL sample was used for cell counting with a Neubauer Chamber. The average cell count from four quadrants was used to adjust the cell concentration to 0.32×10^4 cells/mL and 4.0×10^4 cells/mL.

For the assay, 200 µL of the cell suspension was plated in triplicate in a 96-well microplate and incubated for 24 hours. After incubation, the medium was removed, and 200 µL of extract at various concentrations was added, along with controls (medium alone and medium with 0.5% DMSO). Cells were incubated for another 24 hours.

The following day, the cells were washed with PBS and incubated with resazurin solution (10 mL resazurin in PBS + 40 mL medium) for 2 hours in the dark. Cell viability was then measured by reading absorbance at 560 nm (excitation) and 590 nm (emission) using a spectrometric microplate reader (BioTek Cytation 3).

3.1.2.7.3. Anti-inflammatory evaluation

The macrophage cell line J774A.1 was utilized to assess cell viability and cytokine secretion following incubation with the extracts. This cell line was routinely cultured in DMEM high glucose medium, supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 1 mM sodium pyruvate, and 25 mM HEPES buffer. Cultures were maintained in tissue culture flasks (Nagle Nunc, Int., Hereford, UK) within a humidified atmosphere containing 5% CO_2 at 37 °C (Binder CB150; Tuttlingen, Germany).

Upon reaching confluence, the macrophage cells were washed with fresh medium and harvested by scraping. Viable cells were counted using Trypan blue exclusion in a hemacytometer and resuspended in DMEM to a final concentration of 1×10^6 cells/mL. A volume of 300 µL of the macrophage suspension was then cultured in 48-well tissue culture plates. Cells were incubated overnight with or without lipopolysaccharides (LPS, Sigma) at a final concentration of 1 µg/mL. Following incubation, supernatants were collected, and the cells were treated with the blend GS extract at a specified final concentration. After 24 hours of treatment, the supernatants were stored at -20 °C for subsequent cytokine quantification. The metabolic viability of the cells was determined using the MTT assay, as described by Madesh et al. [30]. Formazan crystals formed were dissolved in a DMSO:ethanol (1:1) solution, and absorbance was measured at 570 nm. Control conditions included cells incubated with DMEM alone, the extract solvent (DMSO), and LPS at a final concentration of 1 µg/mL.

The concentrations of TNF-α, IL-1β, IL-6, IL-10, and TGF- β1 in cell culture supernatants were quantified using the respective Mouse Uncoated ELISA kits (Invitrogen), following the manufacturer's instructions.

3.1.2.7.4. Evaluation of cell cycle by flow cytometry: Abcam AB287852

For cell cycle analysis, cells were cultured to a confluency of over 90%. The medium was removed, and cells were washed with PBS. Cells were then detached with 1 mL trypsin, incubated for 10 minutes at 37°C with 5% CO_2, and neutralized with 2 mL of DMEM medium. After cell counting, cells were plated in a 6-well plate in triplicate at the following densities: 2.64×10^5 cells/well for HaCaT, and 1.40×10^5 cells/well for BJ-5ta cells. Cells were incubated for 24 hours to allow adherence, followed by treatment with blend GS extract for an additional 24 hours.

Post-treatment, the medium was removed, and cells were washed with PBS. Cells were detached with 500 µL trypsin, incubated for 10 minutes, and neutralized with 1 mL of medium. Cells from three wells per condition were transferred into 15 mL Falcon tubes. After cell counting, cells were centrifuged at 400 × g for 7 minutes. The cell pellet was resuspended in 2 mL of ice-cold binding buffer and centrifuged again at 400 × g for 7 minutes. Cells were then fixed by adding 2 mL of ice-cold 70% ethanol dropwise while vortexing, followed by incubation on ice for 40 minutes.

After fixation, cells were centrifuged at 400 × g for 7 minutes and resuspended in binding buffer, followed by another centrifugation step. The final cell pellet was resuspended in 500 µL of Staining Solution (100 µL Enzyme A, 400 µL Nuclear Dye, and 10 mL binding buffer) and incubated in the dark for 30 minutes. Stained cells were analysed for cell cycle distribution using flow cytometry (CytoFLEX, Beckman Coulter).

3.1.2.7.5. In vitro cell migration assay

The cell lines were seeded into a 24-well plate at a density of 8.0×10^4 cells/mL and incubated at 37 °C in 5% CO_2 atmosphere until confluence was reached. A scratch on the confluent cell layer was performed using a 10 µL pipette tip. The culture medium was then replaced with the different collagens dissolved in culture medium at a concentration of 0.5 mg/mL. Cell migration was monitored microscopically, and images were acquired at 0 h, 6 h and 24 h, in the same region (HaCaT cells), and at 0 h, 24 h and 48 h (BJ-5ta cells).

The area of the scratch was measured at different time points using Image J's plugin, MRI Wound Healing Tool.

3.1.2.8. Statistical Analysis

The data were analysed using IBM SPSS 29.0 statistical software through analysis of variance (ANOVA) and a multiple range test (Tukey's test) for a significance level set at $p < 0.05$.

The Pearson correlation was performed in Microsoft Excel 16.87 version.

3.1.3. Results and Discussion

3.1.3.1. Determination of phenolic content

The phenolic (TPC), ortho-diphenols (ODC), and flavonoid contents (FC) present in the extract obtained from the blend of grape stem varieties is described in Table 6.

Consistent with existing literature [26,27,31], the blend GS extract demonstrates a high content in phenolic compounds.

Table 6. Total phenolic, ortho-diphenols, and flavonoid content of the extract of mixed and single grape varieties.

TPC (mg GAE/g)	ODC (mg GAE/g)	FC (mg CAT/g)
78.55 ± 1.67	80.83 ± 2.69	66.77 ± 1.96

Results are presented as mean ± standard deviation (n=3).

Various isolated grape stem varieties have shown different values of TPC, ODC, and FC. Leal et al. [32] measured the TPC, ODC, and FC in methanolic extracts of various isolated grape stem varieties (Tinta Roriz, Touriga Nacional, Castelão, Syrah, Arinto, Fernão Pires). They reported TPC values ranging from 30.91 ± 0.73 to 96.12 ± 8.14 mg GAE/g dw, ODC values ranging from 32.17 ± 1.04 to 77.26 ± 5.31 mg GAE/g dw, and FC values ranging from 25.76 ± 1.14 to 65.14 ± 0.65 mg CAT/g dw. Similarly, Silva et al. [11] studied the TPC in ethanolic extracts of grape stems from two isolated varieties (Touriga Nacional e Preto Martinho), finding values between 45.9 ± 10.7 μg/mg and 226.8 ± 6.9 μg/mg.

In fact, combining grape stems from various varieties can result in chemical interactions between phenolic compounds, which may lead to either synergistic or antagonistic effects. For example, El-Belaga et al. [33] observed a synergistic effect in total phenolic and flavonoid contents when grape seed and green tea extracts were mixed in equal proportions. Specifically, they reported an increase of 22% to 26% in TPC and an increase of 53% to 60% in FC in the mixture of extracts.

Conversely, Giovanelli-Vicuña et al. [34] investigated the effect of mixing methanolic extracts from different fruits (grape, lemon, and blueberry) and identified an antagonistic effect. On average, the TPC in the mixture of extracts decreased by approximately 87%, while the FC decreased by around 63%.

3.1.3.2. Identification of Phenolic Compounds by HPLC-MS

Following the determination of TPC, ODC, and FC, phenolic compounds in the blend GS extract were identified using HPLC-MS. Detailed identification of these compounds is provided in Table 7.

Table 7. Compounds identified in GS extract and their retention times (Rt).

Identified Compounds	Rt (min)	m/z [M-H]⁻	Quantification (µg/g dw)
Proanthocyanidins and catechin derivatives			
Proanthocyanidin trimer (B-type) (Isomer 1)	3.75	865	605.13 ± 1.48 [a]
Proanthocyanidin trimer (B-type) (Isomer 2)	4.11	865	131.55 ± 10.68 [c]
Proanthocyanidin trimer (B-type) (Isomer 3)	6.16	865	229.67 ± 1.7 [b]
Proanthocyanidin trimer (B-type) (Isomer 4)	6.33	865	48.48 ± 1.27 [e]
Proanthocyanidin trimer monogallate isomer 1	7.11	1017	145.83 ± 3.56 [c]
Proanthocyanidin trimer monogallate isomer 2	9.95	1017	67.43 ± 0.27 [d]
Epicatechin-glucoside	11.35	449	42.38 ± 1.19 [e]
Total			1270.47 ± 2.88
Flavonols			
Rutin	10.10	609	9.82 ± 0.08 [c]
Quercetin-3-glucoside (isomer 1)	10.42	463	20.16 ± 0.29 [b]
Isorhamnetin-3-O-glucoside	10.64	301	66.38 ± 0.22 [a]
Kampferol-3-glucoside	11.42	447	12.01 ± 1.36 [c]
Total			108.36 ± 0.49
Stilbenes			
Resveratrol tetramer (Isomer 1)	14.75	905	14.41 ± 0.39
Total			14.41 ± 0.39

Based on the data from the table, it is clear that the blend GS extract is rich in flavonoids. It contains 1270.47 ± 2.88 µg/g dw of flavan-3-ols, including proanthocyanidins and catechin derivatives, and 108.36 ± 0.49 µg/g dw of flavonols. These findings are consistent with the results of the flavonoid content determination.

The ortho-diphenol content is confirmed by the identification of phenolic compounds such as catechin and proanthocyanidins. The predominant compounds in the blend GS extract include proanthocyanidin trimers (B-type) in isomers 1, 2, and 3, proanthocyanidin trimer monogallates in isomers 1 and 2, catechin derivatives.

In methanolic extracts from different isolated grape stem varieties (Tinta Roriz, Touriga Nacional, Castelão, Syrah, Arinto and Fernão Pires), Leal et al. [15] identified flavan-3-ols (catechin and epicatechin), flavonols (rutin), and stilbenes (resveratrol and viniferin) across all the varieties. Similarly, Silva et al. [11] identified flavan-3-ols (catechin, epicatechin, gallocatechin gallate, catechin gallate), flavonols (rutin, quercetin 3-O-galactoside, quercetin 3-O-glucoside, quercetin 3-O-rhamnoside), and stilbenes (*trans*-resveratrol) in ethanolic extracts of grape stems from isolated varieties (Touriga Nacional and Preto Martinho). The most predominant identified compound in isolated varieties were catechin, caftaric acid, and rutin.

In the blend GS extract, the formation of derivatives is particularly notable, with the identification of proanthocyanidin trimers and resveratrol tetramer. This difference can be attributed to interactions and the formation of complex compounds during the blending process. As a result, catechin, epicatechin and resveratrol were not identified in the blend GS extract, but their derivatives were, suggesting that they had complexed to form trimers and tetramer.

Additionally, Leal et al. [15] identified phenolic acids in their extracts from isolated grape stem varieties, such as gallic acid in Syrah, Arinto, and Fernão Pires varieties; protocatechuic acid in Touriga Nacional, Castelão, Arinto, and Fernão Pires varieties; and cinnamic and caftaric acids in all varieties. Similarly, Silva et al. [11] identified phenolic acids, including gallic acid, coumaric acids, vanillic acid, chlorogenic acids, protocatechuic acid, and ferulic acid, in their grape stem extracts from Touriga Nacional and Preto Martinho varieties.

However, no phenolic acids were identified in the blend GS extract. This discrepancy is likely due to chemical interactions or transformations that occurred during blending, which may have altered or degraded these compounds. El-Beltagi et al. [33] suggested that proanthocyanidins can combine with gallic acid to form gallate esters and glycosides. They identified these forms in a mixture of grape seed and green tea extracts. This interaction may explain the presence of proanthocyanidin derivatives, such as proanthocyanidin trimer monogallate, in the blend GS extract.

3.1.3.3. Determination of Antioxidant Capacity

In addition to assessing the phenolic compound content, the chemical antioxidant activity of the blend GS extract was determined by 3 distinct methods: ABTS, DPPH, and FRAP assays. Both ABTS and DPPH assays assess a sample's capacity to scavenge free radicals; however, while DPPH is more responsive to lipophilic compounds, ABTS is more versatile, measuring both hydrophilic and lipophilic antioxidants. The

FRAP assay, on the other hand, evaluates antioxidant potential by measuring the sample's ability to reduce ferric (Fe^{3+}) ions to ferrous (Fe^{2+}) ions.

The results of this analysis are presented in Table 8.

Table 8. Evaluation of antioxidant activities.

ABTS (mmol Trolox/g)	DPPH (mmol Trolox/g)	FRAP (mmol Trolox/g)
1.035 ± 0.04	0.574 ± 0.03	0.689 ± 0.02

Results are presented as mean ± standard deviation (n=3).

The blend GS extract demonstrated strong antioxidant activity, particularly in the ABTS assay, indicating a rich presence of hydrophilic phenolic compounds capable of scavenging free radicals, including glucosides. Several hydrophilic compounds were identified in the blend GS extract, such as rutin, quercetin-3-O-glucoside (isomer 1), isorhamnetin-3-O-glucoside, kaempferol-3-O-glucoside, and epicatechin-3-O-glucoside.

In comparison, antioxidant activity in grape stems from isolated varieties has been studied as well. Leal et al. [15] evaluated the antioxidant potential of various grape stem varieties (Tinta Roriz, Touriga Nacional, Castelão, Syrah, Arinto, and Fernão Pires), reporting a range of values across different assays: 0.35 ± 0.00 to 0.84 ± 0.06 mmol Trolox/g in the ABTS assay, 0.15 ± 0.01 to 0.64 ± 0.05 mmol Trolox/g in the DPPH assay, and 0.35 ± 0.02 to 1.03 ± 0.06 mmol Trolox/g in the FRAP assay. Costa et al. [26] conducted a similar study on other grape stem varieties (Arinto, Códega do Larinho, Esgana Cão, Folgasão, Fernão Pires, Moscatel, and others) and found comparable results using the ABTS, DPPH, and FRAP assays.

The difference in antioxidant activity between the blend GS extract and isolated grape varieties can be attributed to the complex interactions among the phenolic compounds present in different grape varieties. Phenolic compounds, such as proanthocyanidin and resveratrol, can form complex structures that either enhance or diminish antioxidant activity. For example, Kotov et al. [35] observed synergistic effects in combinations of plant extracts, such as bur-marigold and hawthorn, leading to a 110.6% increase in antioxidant activity. Similar results were found with three-component mixtures like bur-marigold, calendula, and hawthorn (112% increase). Aslam et al. [36] also reported enhanced antioxidant potential when combining plant extracts, particularly in mixtures of *Terminalia arjuna*, *Rauvolfia serpentina*, *Elettaria cardamomum*, and *Crataegus oxyacantha*.

However, not all combinations of extracts result in synergistic effects. Giovagnoli-Vicuña et al. [34] found antagonistic interactions in binary fruit mixture extracts (grape, lemon, and blueberry). Similarly, Peixoto

et al. [37] noted that grape seeds exhibited a higher concentration of phenolic compounds and greater antioxidant potential compared to pomace (a mixture of grape skins and seeds), indicating that combining these components could result in diminished antioxidant effectiveness.

Plumb et al. [38] explored how polymerization, galloylation, and glycosylation affect the antioxidant activity of catechins and proanthocyanidins. Using the TBARS assay for lipid-phase antioxidant activity and the ABTS assay for aqueous-phase activity, they discovered that as catechins polymerized from monomers to tetramers, their ability to prevent free radical damage in lipid systems decreased. For instance, monomeric catechins had an IC50 of 3.4 μM in the lipid peroxidation assay, while trimers had a higher IC50 of 6.5 μM, indicating reduced effectiveness. In contrast, antioxidant activity in the aqueous phase increased with polymerization, with monomers showing a TEAC value of 2.47 and trimers displaying a higher TEAC value of 4.87.

The findings of Plumb et al. [38] align with these results. The elevated antioxidant activity measured by ABTS, although uncommon in isolated varieties, is notably higher in the blend GS extract, likely due to the presence of proanthocyanidin trimers.

Additionally, these findings suggest that phenolic compounds exert their antioxidant effects through different mechanisms depending on the environment, and antioxidant effectiveness cannot be solely explained by the ability of phenolic hydroxyl groups to donate hydrogen. Antagonistic effects in mixtures may occur due to interactions between phenolic compounds, such as hydrogen bond formation among the hydroxyl groups of natural antioxidants. Additionally, as these mixtures oxidize, they can regenerate into free radicals, which may then accept electrons or hydrogen atoms from other antioxidants within the mixture, further influencing antioxidant activity.

3.1.3.4. Evaluation of anti-ageing and depigmenting activities

The anti-ageing and depigmenting activities of grape stem extract from a blend of grape varieties were explored through enzyme inhibition assays of elastase and tyrosinase, respectively.

The results of elastase and tyrosinase inhibition by the blend GS extract are presented in Table 9.

Table 9. Evaluation of anti-ageing and depigmenting activities.

% inhibition elastase	% inhibition tyrosinase
37.78 ± 2.26	45.94 ± 0.63

Results are presented as mean ± standard deviation (n=3) and are expressed in % inhibition.

Grape stems from isolated varieties have been evaluated for their inhibitory effects on elastase and tyrosinase. Leal et al. [15] reported that isolated grape varieties (Tinta Roriz, Touriga Nacional, Castelão, Syrah, Arinto, and Fernão Pires) achieved elastase inhibition ranging from 73% to 98% and tyrosinase inhibition ranging from 44% to 54%. In comparison, the blend GS extract showed lower elastase inhibition than isolated varieties studied by Leal et al. This suggests that isolated grape varieties might have more potent elastase enzyme inhibition activities compared to the blend GS extract.

This discrepancy can be attributed to the complex interactions among phenolic compounds in the grape varieties. Supporting this, Yu et al. [39] explored the inhibitory activities of isolated phenolic compounds (cinnamic acid, ferulic acid, quercetin, isorhamnetin, and gallic acid) and their combinations. Their findings indicate that different combinations of these compounds can produce varying effects: synergistic, additive, or subadditive. For example, the combination of quercetin (5 mM), cinnamic acid (5 mM), and ferulic acid (5 mM) exhibited a synergistic effect, enhancing enzyme inhibition. Conversely, the combination of quercetin (5 mM) and gallic acid (5 mM) showed a subadditive effect, and some mixtures involving ferulic acid (5 mM) demonstrated synergistic effects, although ferulic acid alone was found to activate tyrosinase activity.

These findings imply that phenolic compounds can interact in ways that either enhance or diminish their inhibitory activities. The lower elastase inhibition observed with the blend GS extract might be due to less optimal combinations or interactions of phenolic compounds compared to those in the isolated varieties.

3.1.3.5. Pearson Correlation

The Pearson correlation coefficient, also known as the product moment correlation coefficient, is dimensionless and ranges from -1 to +1. A positive value indicates a positive relationship, while a negative value indicates a negative relationship. A perfect correlation, where all data points on a scatter plot lie on a straight line, is represented by a coefficient of 1 (perfect positive) or -1 (perfect negative). A coefficient of zero indicates no linear relationship between the variables, meaning they are uncorrelated [40].

In order to understand what the different variable are correlated, the Pearson correlation was performed, and the result are presented in Table 10.

Table 10. Pearson correlation. (TPC, total phenolic content; ODC, ortho-diphenols content; FC, flavonoids content, FRAP assay; DPPH assay; ABTS assay; TYR, anti-tyrosinase assay; ELAS, anti-elastase assay).

	TPC	ODC	FC	FRAP	DPPH	ABTS	TYR	ELAS
TPC	1.00	0.89	0.86	0.54	-0.37	0.96	0.24	-0.96
ODC	0.89	1.00	1.00	0.09	-0.76	0.98	0.66	-0.72
FC	0.86	1.00	1.00	0.02	-0.80	0.96	0.71	-0.68

As observed in the table, TPC is positively correlated with ODC and FC, as indicated by Pearson coefficients close to 1. This is expected because ortho-diphenols and flavonoids are types of phenolic compounds; thus, an increase in TPC corresponds to increases in ODC and FC, and vice versa.

Phenolic content (TPC, ODC, and FC) appears to be positively correlated with antioxidant activity measured by the ABTS assay and moderately correlated with tyrosinase enzyme inhibition, mainly due to flavonoids. Conversely, there is a negative correlation between phenolic content, elastase enzyme inhibition, and antioxidant measured by DPPH assay.

Therefore, it can be concluded that the phenolic compounds present in the blend GS extract are primarily responsible for its antioxidant activity against radicals (ABTS) and its inhibitory effect on the tyrosinase enzyme.

The work of Ali et al. [41] supports these findings. They observed a Pearson coefficient close to 1 when correlating total phenolic content with flavonoid content in *Caragana brachyantha Rech.f* extract. Additionally, phenolic content showed a moderate correlation with tyrosinase inhibition. However, contrary to the results of this study, phenolic content appeared to be strongly correlated with antioxidant activity measured by the FRAP assay. Similarly, Teixeira et al. [42] reported significant correlations between TPC and ABTS (0.912) and FRAP (0.944), with a notable inverse correlation in DPPH assays (-0.848).

3.1.3.6. *In vitro* assays

Skin consists of diverse cell types, like keratinocytes and fibroblasts. Keratinocytes, found in the epidermis, are crucial for maintaining epithelial cohesion and providing protection from external factors. Fibroblasts, located in the dermis, play a key role in synthesizing structural proteins like collagen and elastin [43].

With aging, the epidermis can become 10 to 50% thinner due to keratinocyte atrophy. Aging also leads to the accumulation of senescent cells and increased apoptosis, which further reduces keratinocyte numbers [44–47]. In the dermis, fibroblasts decrease in number, size, and cytoplasmic extensions. These changes are linked to alterations in the endoplasmic reticulum, reduced proliferative and metabolic activity, and decreased expression of ECM components, particularly collagen and elastin [48–50].

In this context, several cellular assays were conducted using keratinocytes (HaCaT) and fibroblasts (BJ-5ta) to understand the effect of blend GS extract on human skin cells. This study also evaluated different cell densities to assess the potential effects of blend GS extract under varied cellular conditions.

3.1.3.6.1. Influence of GS extract in cells morphology

Keratinocytes exhibit a polygonal shape, appearing cuboidal in the basal layer and becoming increasingly flattened as they migrate to the surface. These cells contain a prominent nucleus that diminishes in size during maturation, reflecting their transition to a keratinized state [51,52].

Due to the importance of keratinocytes morphology in skin structure, the impact of the blend in this feature was observed [51,52]. The results are illustrated in Figure 13.

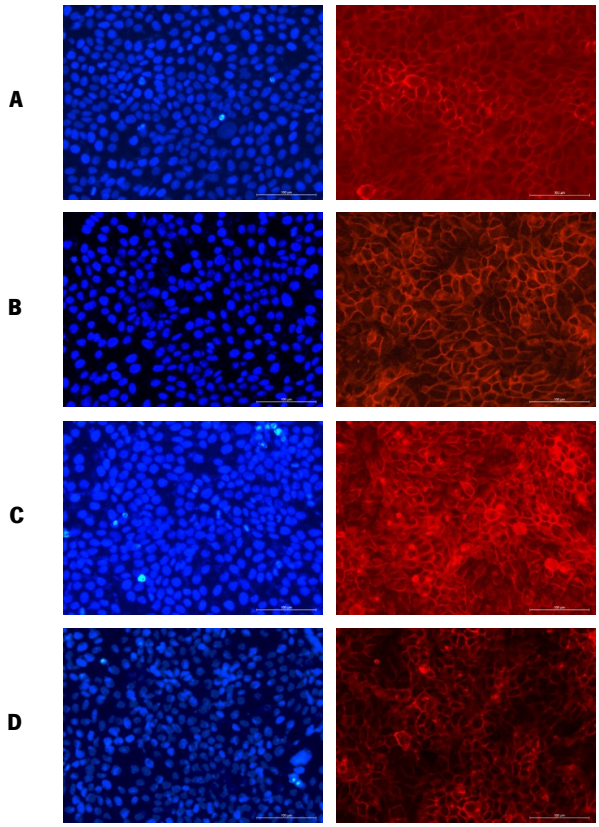

Figure 13. Influence of blend GS extract on HaCaT cells morphology. **(A).** DMSO Control. **(B).** 20.84 µg/mL GS extract. **(C).** 83.38 µg/mL GS extract. **(D).** 166.75 µg/mL GS extract.

In the control group, the cells appear flattened with a uniform distribution of their prominent nuclei (blue) and cytoplasmic proteins (red). The presence of blend GS extract at low concentration (20.84 µg/mL–83.38 µg/mL), seem to induce a disarrangement on cell orientation. At a higher concentration (166.75 µg/mL), the changes observed appear to be more pronounced.

Similarly, George et al. [53] examined the impact of luteolin (5–80 µM) on HaCaT cell morphology. Their study revealed that luteolin treatment led to a significant loss of cell-cell adhesion, causing the cells to lose their normal connections with each other. Additionally, they observed the development of membrane protrusions, distended areas on the cell surface, as well as the formation of fluid-filled spaces within the cells, which are indicative of cellular distress compared to the untreated control cells. Hsu et al. [54] also observed that keratinocytes treated with epigallocatechin-3-gallate (50 µM) showed morphological changes, including an increase in enlarged, flattened, squamous cells, which they attributed to keratinocyte differentiation. In contrast, D'Angelo et al. [55] reported morphological alterations including dissociation from the growth substrate, nuclear disintegration, and a decrease in cellular size in HaCaT cells treated with 250 µM and 500 µM of *Annurca* Apple polyphenols extract. These changes were linked to the extract's strong inhibitory effect on keratinocyte growth, highlighting its potential for treating psoriasis.

Fibroblasts are characterized by their plump, spindle-shaped or stellate forms and centrally located oval or round nucleus [56]. A similar study as previously described regarding fibroblasts morphology in the presence of the extract was performed. As it can be seen in Figure 14.

In Figure 14, the blend GS extract appears to cause a disruption in fibroblast morphology, similar to the effects observed in keratinocytes. Czemplik et al. [57] also reported changes in normal human dermal fibroblasts (NHDF) morphology when treating these cells with *Linum usitatissimum* extract (140,4 µg TPC). The cells exhibited a rounded shape instead of their typical elongated form. However, the nuclei remained similar in size and shape to those in the control group, consistent with observations in our study. The authors attributed these morphological alterations to the toxicity caused by the high concentrations of phenolic compounds affecting the cells. Kikowska et al. [58] also observed that human skin fibroblasts treated with a callus extract at lower concentrations (12.5 µg/mL extract) maintained a proper, elongated shape and had large, round nuclei. In contrast, fibroblasts exposed to higher

concentrations (100 µg/mL extract) of the extract exhibited abnormal morphology, lacking the typical elongations.

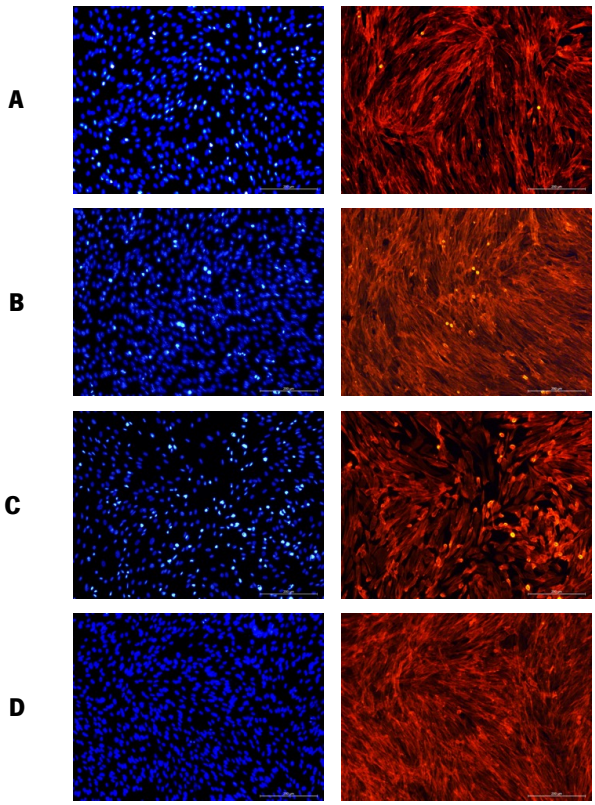

Figure 14. Influence of GS extract on BJ-5ta cells morphology. **(A).** DMSO Control. **(B).** 20.84 µg/mL GS extract. **(C).** 83.38 µg/mL GS extract. **(D).** 166.75 µg/mL GS extract.

In conclusion, while the GS extract blend does not seem to have a significant effect on cell morphology, it does cause noticeable changes in the organization of the cell monolayer.

3.1.3.6.2. Influence of GS extract in cells metabolic activity

To understand the influence of blend GS extract on cells, the metabolic activity of HaCaT keratinocytes and BJ-5ta fibroblasts was evaluated following treatment with the extract.

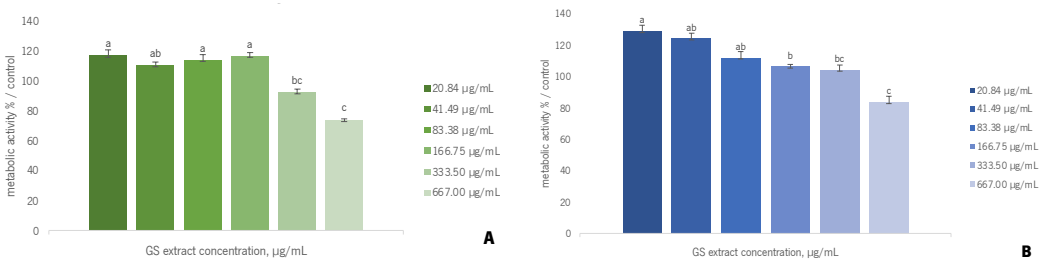

Figure 15. Influence of GS extract on cell metabolic activity of HaCaT keratinocytes. **(A).** High cell density. **(B).** Low cell density. Results are presented as mean ± standard error (n=3). Significant differences between samples are denoted by different lowercase letters ($p < 0.05$).

The findings depicted in Figure 15 demonstrate that the metabolic activity of HaCaT cells at high cell density increases (≈115%) when treated with GS extract up to concentrations of 166.75 µg/mL. Beyond this concentration, there is a decrease in metabolic activity, but not lower than 70%. This indicates that higher concentrations than 166.75 µg/mL, GS extract can decrease keratinocytes metabolic activity.

As aging occurs, the number of skin cells diminishes. Thus, it was important to explore the effects of GS extract under conditions of low cell density. Under these conditions, metabolic activity in HaCaT cells increases up to concentrations of 333.50 µg/mL (≈129%–104%). Beyond this, metabolic activity decreases but does not drop below 90%. This suggests that metabolic activity is less compromised at low cell density than at high cell density. This may be because, at low-density cell cultures tend to be more resistant to toxic compounds than high-density cultures. This is primarily due to greater nutrient availability and more space in low-density cultures, which promotes better cell health and metabolic function, enabling cells to handle more effectively with toxins [59]. In contrast, high-density cultures often face limited nutrients and the buildup of waste products, leading to increased cellular stress and a heightened sensitivity to toxic substances [60].

The close proximity of cells in high-density cultures can also amplify stress signalling, while low-density cultures are better equipped to activate protective mechanisms. Differences in gene expression, metabolism, and stress response pathways further explain why low-density cultures have a higher tolerance for slightly toxic compounds [61].

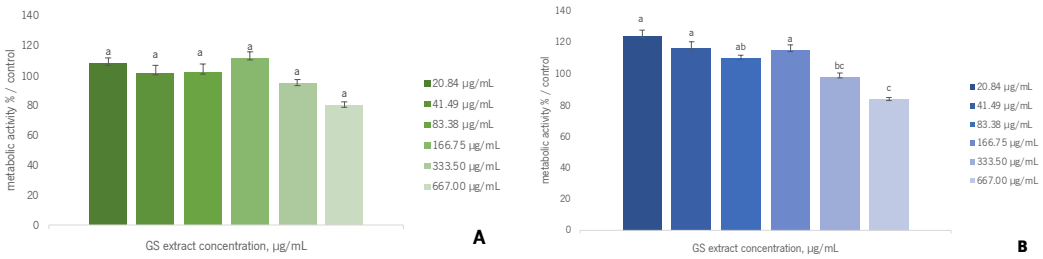

Figure 16. Influence of GS extract on cell metabolic activity of BJ-5ta fibroblasts. **(A).** High cell density. **(B).** Low cell density. Results are presented as mean ± standard error (n=3). Significant differences between samples are denoted by different lowercase letters ($p < 0.05$).

As shown in Figure 16, the metabolic activity of BJ-5ta cells remains statistically unchanged when treated with blend GS extract up to 667.00 µg/mL if in the presence of high cell density conditions.

Under low cell density conditions, the metabolic activity of BJ-5ta cells increases (≈115%) up to 166.75 µg/mL. Beyond this concentration, metabolic activity starts to decrease, reaching ≈80% at 667.00 µg/mL GS extract.

The metabolic activity of both cell types follows the same trend: it is higher under low cell density conditions. Therefore, it appears that blend GS extract is less toxic at higher concentrations in aged skin (low cell density) compared to young skin, where cell density is typically higher

This observation is supported by Domínguez-Perles et al. [62], who investigated the cytotoxic effects on HaCaT keratinocytes (high cell density) treated with extracts from various grape stem varieties (Tinto Cão, Tinta Barroca, Moscatel Branco, Malvasia Fina) across a range of concentrations (0.04–30.60 ng GAE/mL). Their research revealed that these cells maintained their viability, with metabolic activity even surpassing 97.5%. Similarly, Sangiovanni et al. [63] investigated cell cytotoxicity using extracts derived from leaves of the Teinturiers variety in HaCaT cells. Their study revealed no signs of cytotoxicity in cells exposed to the extract within a concentration range of 5 to 500 µg/mL.

The metabolic activity of BJ-5ta human fibroblasts when treated with phenolic compounds was also investigated by Dong et al [64]. Their study involving latifolin, a natural flavonoid, showed cytotoxic effects, with metabolic activity decreasing to approximately 80%, only at a concentration of 40 µg/mL. Positive effects similar to those observed with grape stem extract were also noted with *Annurca* apple and açai extracts on BJ-5ta human fibroblasts. Even at increased concentrations, 100 µM for *Annurca* apple extract and 35.50 µg GAE/g for açai extract, cell viability was not impaired. In fact, a slight increase in viability was observed [65,66].

Overall, the results suggest that blend GS extract is generally compatible with various cell lines, such as HaCaT keratinocytes and BJ-5ta fibroblasts. However, depending on cell density, the concentration of GS extract should be carefully selected to avoid compromising the metabolic activity of the cells.

3.1.3.6.3. Evaluation of anti-inflammatory activity of blend GS extract in macrophages

Inflammation is the body's defence mechanism against harmful agents. Chronic inflammation, marked by excessive production of pro-inflammatory mediators, is a key factor in many inflammatory diseases. Therefore, reducing inflammation, especially using natural products without adverse effects, is crucial [67,68].

Firstly, the metabolic activity of macrophages treated with various concentrations of blend GS extract was analysed. As shown in Figure 17, the blend GS extract did not affect the metabolic viability of macrophages under normal and inflammatory conditions, remaining above 100% (approximately 103%–110%). These findings are consistent with Asadi's study, which demonstrated that macrophages remained viable under inflammatory conditions (LPS 10 µg/mL) when treated with Matricaria chamomilla extract at concentrations ranging from 25 to 1600 µg/mL [69].

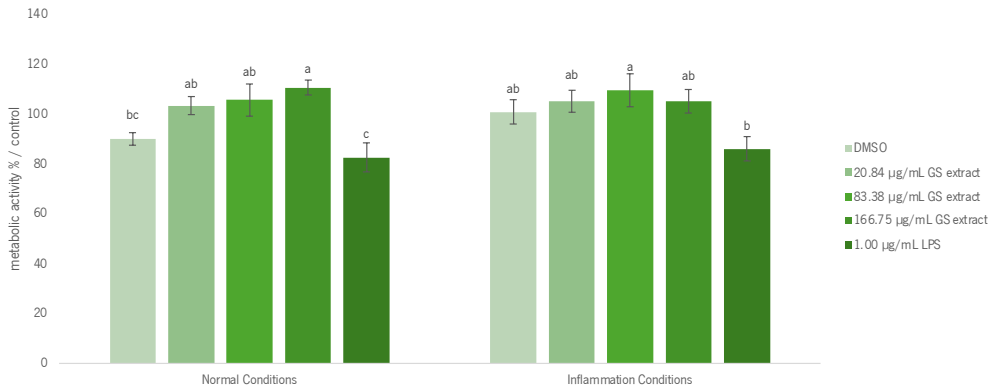

Figure 17. Influence of GS extract in macrophages cell metabolism. Results are presented as mean ± standard error (n=5). Significant differences between samples at normal conditions and samples at inflammation conditions are denoted by different lowercase letters ($p < 0.05$).

To evaluate the anti-inflammatory potential of blend GS extract, it was analysed the production of pro-inflammatory (TNF-α, IL-1β, IL-6) and anti-inflammatory cytokines (IL-10, TGF-β1) by macrophages. Notably, IL-6 has both pro-inflammatory and anti-inflammatory roles [70,71]. DMSO was used as the

negative control (GS extract dilution), while LPS-treated cells served as the positive control to induce inflammatory cytokine production [72,73].

The findings are presented in Figure 18 and Figure 19.

In the absence of inflammation (Figure 18), the levels of most cytokines (IL-1β, IL-6, TGF-β1) are similar to those of the negative control (DMSO), indicating that the blend GS extract does not induce inflammation.

However, TNF-α levels in the blend GS extract are higher than in the negative control and increase with higher concentrations of the blend GS extract, reaching levels comparable to the positive control (LPS). Regarding IL-10 levels, there is an increase when cells are treated with 20.84 µg/mL of the blend GS extract. This can be explained by the fact that these cytokines are involved in the innate immune response, a defence mechanism that combats microorganisms and other harmful agents, constantly preventing the invasion and establishment of infections [74].

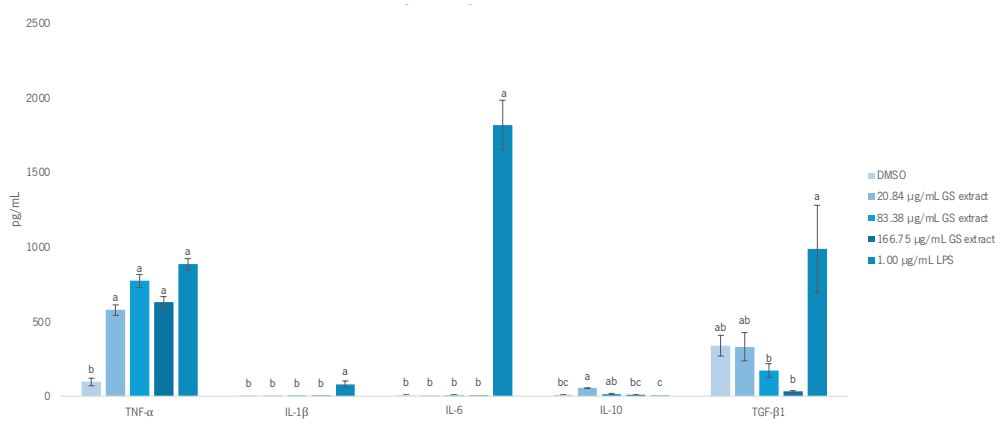

Figure 18. Evaluation of anti-inflammatory activity of GS extract (normal conditions). Results are presented as mean ± standard error (n=5). Significant differences between samples for each cytokine assay are denoted by different lowercase letters ($p < 0.05$).

Under inflammatory conditions (Figure 19), cells treated with the blend GS extract do not exhibit significant differences in cytokine levels compared to the negative control (DMSO), indicating that the GS extract does not possess notable anti-inflammatory activity. The only observed differences are an increase in TNF-α levels in cells treated with 166.75 µg/mL blend GS extract and a decrease in TGF-β1 levels in cells treated with the blend GS extract. The increase in TNF-α levels may be linked to a defence mechanism of the innate immune response.

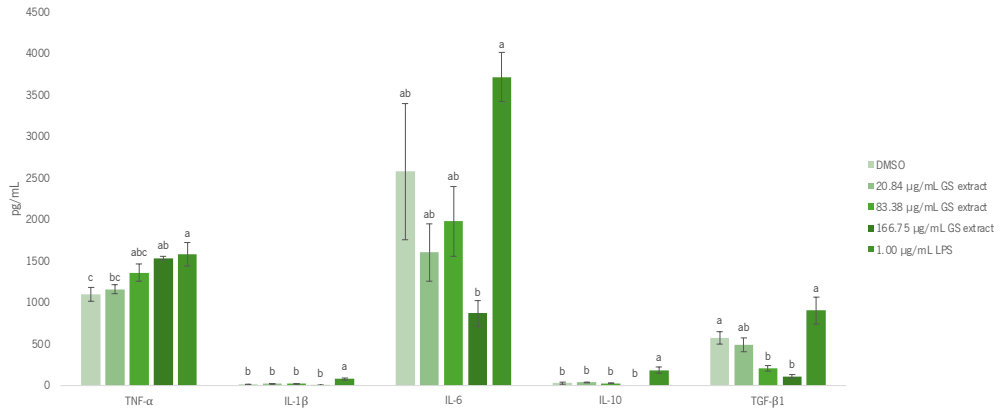

Figure 19. Evaluation of anti-inflammatory activity of GS extract (inflammation conditions). Results are presented as mean ± standard error (n=5). Significant differences between samples for each cytokine assay are denoted by different lowercase letters ($p < 0.05$).

TGF-β1 is a key cytokine in skin cell function, playing a crucial role in promoting fibrosis, stimulating fibroblast proliferation and differentiation, and regulating collagen expression. It is essential for tissue homeostasis by enhancing extracellular matrix synthesis, regulating immune and inflammatory responses, and supporting angiogenesis. Consequently, TGF-β1 is critical in scar formation [75–77].

By reducing TGF-β1 levels, blend GS extract could potentially mitigate fibrosis, regulate collagen deposition, and influence angiogenesis. These effects make it a promising candidate for addressing pathological skin scarring and fibrotic skin diseases [78], such as hypertrophic scars [77] and keloids [79].

Proanthocyanidins (0, 2.5, 5, 10, and 20 µg/µL) have been investigated for their effects on wound healing in Hs27 human dermal fibroblasts. These compounds were found to inhibit procollagen secretion in fibroblasts when collagen overproduction was induced by TGF-β1. This suggests that proanthocyanidins may help manage conditions characterized by excessive collagen production, such as abnormal scarring. Although the study did not examine cytokine expression or the specific mechanisms of inhibition, it highlights the crucial role of proanthocyanidins in controlling collagen production, which is essential for managing scar formation [80].

Similarly, blend GS extract, which is rich in proanthocyanidins, showed a reduction in TGF-β1 levels, indicating decreased collagen deposition. This supports the potential of blend GS extract to modulate scar formation, as also supported by Kim et al [80].

Additionally, various natural extracts have been explored in keloid fibroblasts as potential treatments for keloids, including *Aneilema keisak* extract [81], Roselle flower extract [82], Xanthium stramarium and

Psoralea corylifolia extracts [83], Astragalus membranaceus and *Salvia miltiorrhiza* extracts [84]. These extracts have demonstrated the ability to reduce TGF-β1 expression in keloid fibroblasts, thereby exerting an anti-keloid effect. Similarly, isolated phenolic compounds such as resveratrol [85], loureirin A [86], and quercetin [87,88] have shown potential in treating keloids by exhibiting similar effects.

3.1.3.6.4. Influence of blend GS extract in HaCaT and BJ-5ta cells cycle

The cell cycle is the process by which cells grow, duplicate their DNA, and divide into two daughter cells. This cycle is essential for the growth, development, and repair of multicellular organisms. It consists of several distinct phases that ensure precise replication and segregation of genetic material, as well as proper cell growth [89].

The Sub G0-G1 phase typically indicates the presence of apoptotic or dead cells, characterized by reduced DNA content due to fragmentation. In contrast, the G0-G1 phase includes cells that are either resting or at the initial stage of the cell cycle. During the S phase, cells engage in active DNA replication, reflecting a heightened level of proliferative activity as they prepare for division. Subsequently, in the G2-M phase, cells finalize DNA synthesis and prepare for mitosis, the actual process of cell division [90].

Understanding these phases is crucial for comprehending the dynamics of cell proliferation and the cellular response to various treatments. In this context, the impact of blend GS extract on the HaCaT cell cycle was studied using flow cytometry. The gate was set to the keratinocyte region, and single cells were selected to prevent interference from doublets in the assay (Figure 20). The results are presented in the figures below (Figure 21).

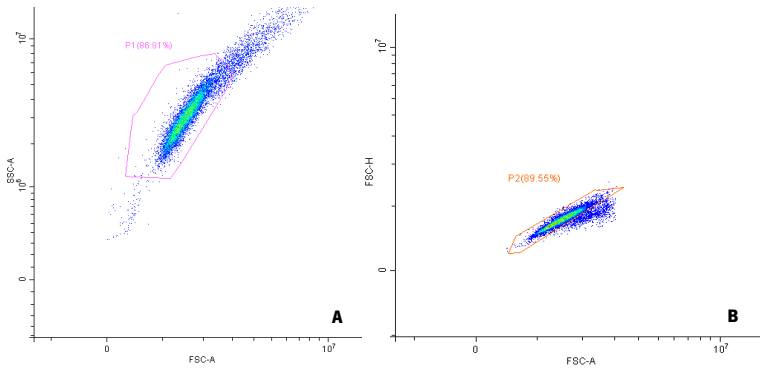

Figure 20. (A). Dot plot of Size vs. Granularity with Gate in keratinocytes region. **(B).** Forward Scatter Height (FSC-H) vs. Forward Scatter Area (FSC-A) density plot to exclude doublets cells.

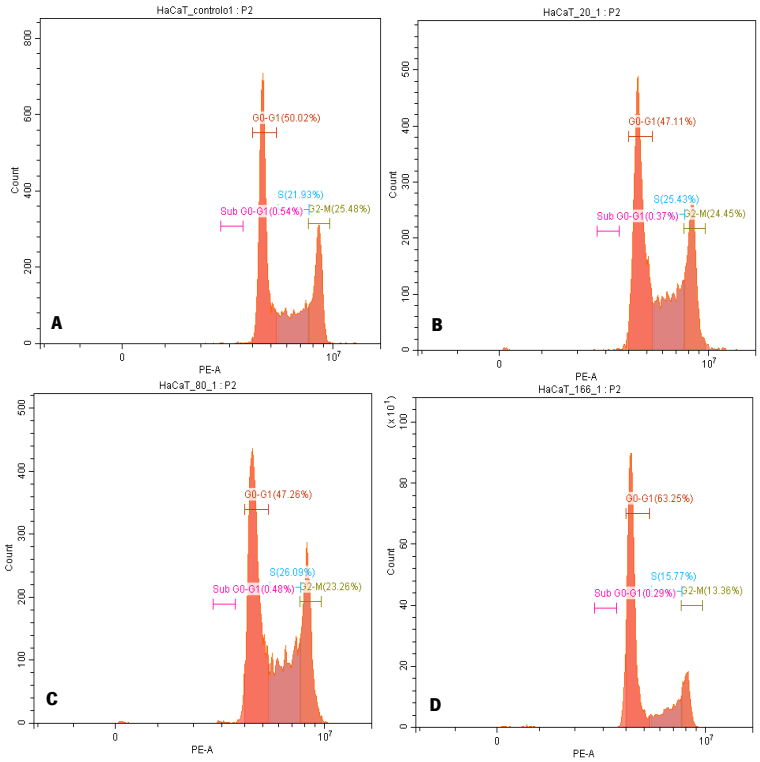

Figure 21. HaCaT cell cycle evaluation by flow cytometry. Fluorescence intensity is proportional to the amount of DNA, defining cells in different phases of the cell cycle. Apoptotic cells exhibit less marked DNA than viable cells in the G0/G1 phase. **(A).** Control. Untreated HaCaT cells; **(B).** GS extract treated cells (20.84 µg/mL); **(C).** GS extract treated cells (83.38 µg/mL); **(D).** GS extract treated cells (166.75 µg/mL).

Based on the interpretation of figures, it can be concluded that blend GS extract does not affect the HaCaT cell cycle at lower concentrations (20.84 and 83.38 µg/mL), as there are no significant differences compared to the control (untreated cells). However, at a higher concentration of 166.75 µg/mL, GS extract causes cells to be arrested in the G0-G1 phase, resulting in a decrease in the number of cells in the S phase. This indicates that, at this higher concentration, GS extract inhibits cell proliferation by preventing cells from progressing to the S phase, thereby blocking DNA replication and subsequent cell division. In fact, when cells were treated with concentrations of the blend GS extract higher than 166.75 µg/mL, there was a significant reduction in metabolic activity (≈70%–100%).

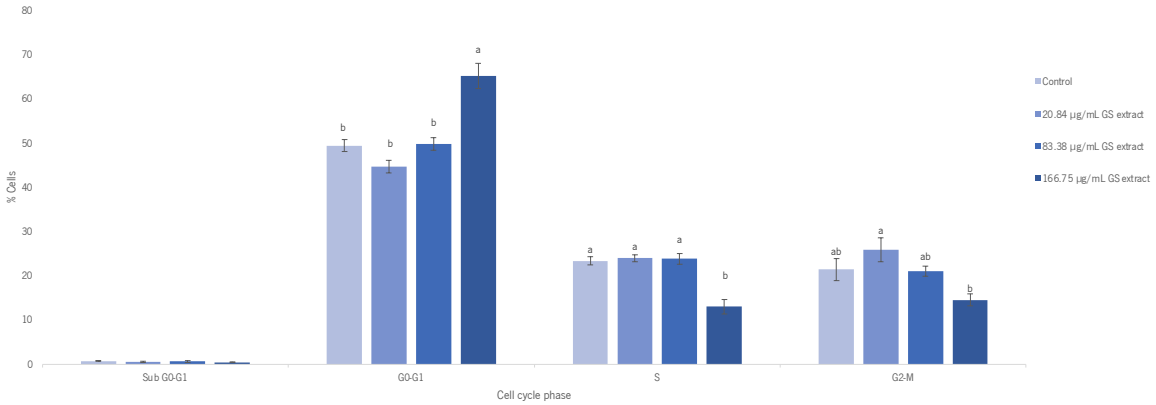

Figure 22. Influence of GS extract in HaCaT keratinocytes cell cycle. Results are presented as mean ± standard error (n=3). Significant differences between samples for each cell cycle phase are denoted by different lowercase letters ($p < 0.05$).

The cell cycle of BJ-5ta fibroblast cells was also evaluated using flow cytometry, with the fibroblast gate selected in the same mode. The results are presented below.

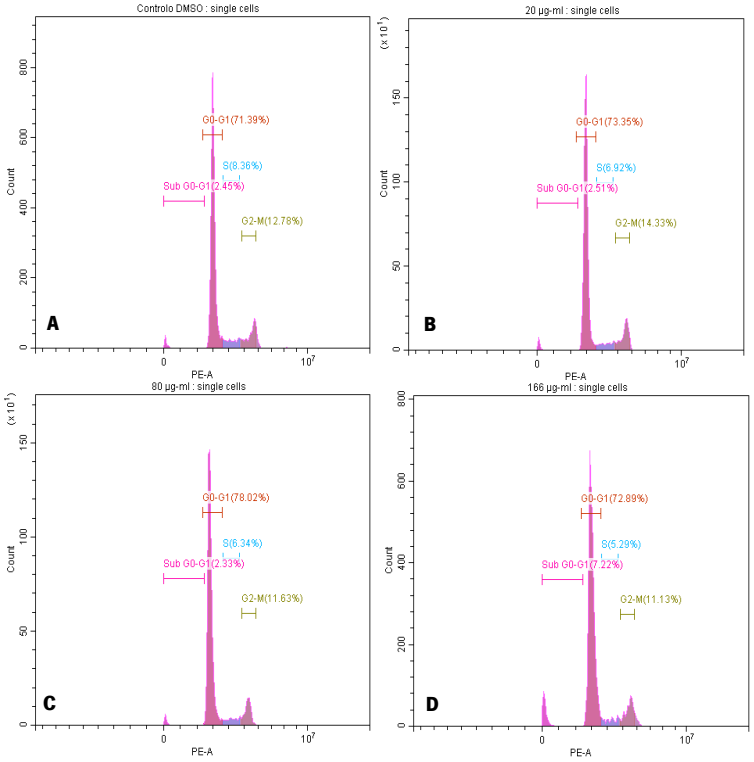

Figure 23. BJ-5ta cell cycle evaluation by flow cytometry. Fluorescence intensity is proportional to the amount of DNA, defining cells in different phases of the cell cycle. Apoptotic cells exhibit less marked DNA than viable cells in the G0/G1 phase. **(A).** Control. Untreated BJ-5ta cells; **(B).** GS extract treated cells (20.84 µg/mL); **(C).** GS extract treated cells (83.38 µg/mL); **(D).** GS extract treated cells (166.75 µg/mL).

Regarding the BJ-5ta cell cycle, blend GS extract does not appear to significantly influence it. However, at higher concentrations (83.38 and 166.75 µg/mL), the GS extract arrests cells in the Sub G0-G1 phase, which may indicate apoptosis.

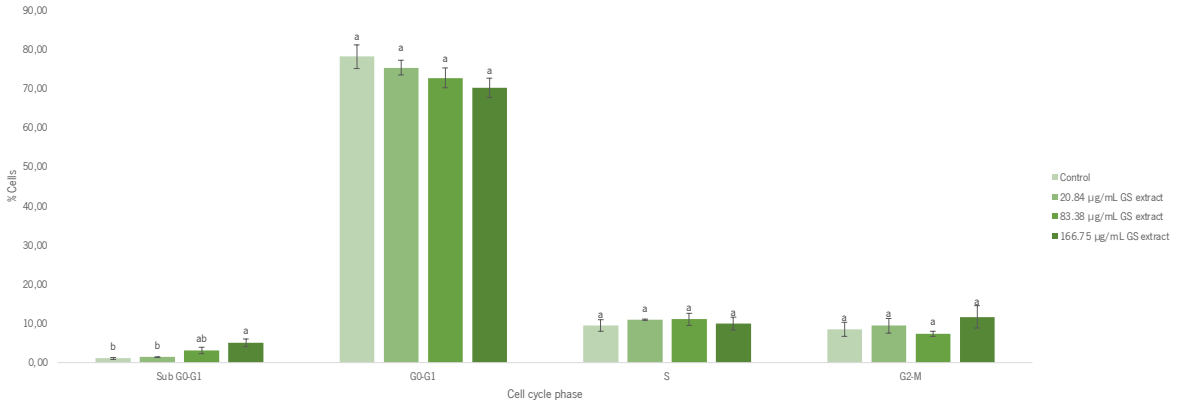

Figure 24. Influence of GS extract in BJ-5ta fibroblasts cell cycle. Results are presented as mean ± standard error (n=3). Significant differences between samples for each cell cycle phase are denoted by different lowercase letters ($p < 0.05$).

These findings support the potential of GS extract in managing scar formation. GS extract arrests keratinocytes and fibroblasts in the G0-G1 and Sub G0-G1 phases, impeding proliferation and promoting apoptosis. This is crucial for managing keloids by reducing excessive fibrotic responses [91–93]. Similarly, treating hypertrophic scars involves inhibiting fibroblast proliferation and inducing apoptosis [94].

Overall, these findings highlight the therapeutic potential of GS extract in managing scars formation by regulating cell cycle and promoting apoptosis.

3.1.3.6.5. Effect of GS extract on cell migration of HaCaT keratinocytes and BJ-5ta fibroblasts

Cell migration is a critical process in skin wound healing. When the skin is damaged, keratinocytes migrate to cover the wound, stimulated by the release of cytokines and growth factors, leading to the formation of a new epithelial layer (re-epithelialization). Additionally, fibroblasts are activated and migrate into the wound to lay down collagen, facilitating the remodelling of the epidermis [95,96].

To understand the influence of blend GS extract on the response of HaCaT keratinocytes and BJ-5ta fibroblasts during wound healing, the evaluation of cell migration was performed. The achieved results are shown in Figure 25 and Figure 26.

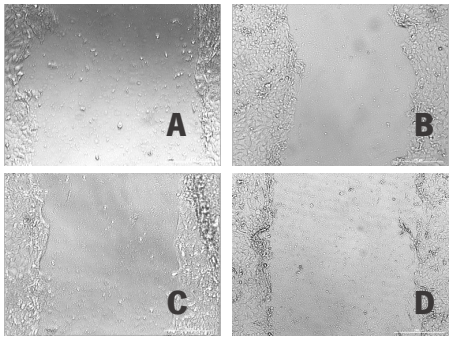

Figure 25. Analysis of HaCaT cells migration. **(A).** 0h; **(B).** 24h (control, untreated cells); **(C).** 24h (cells treated with 20.84 µg/mL GS extract); **(D).** 24h (cells treated with 166.75 µg/mL GS extract).

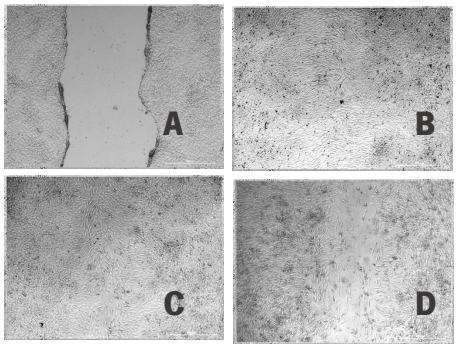

Figure 26. Analysis of BJ-5ta cells migration. **(A).** 0h; **(B).** 48h (control, untreated cells); **(C).** 48h (cells treated with 20.84 µg/mL GS extract); **(D).** 48h (cells treated with 166.75 µg/mL GS extract).

As shown in the figures, the blend GS extract appears to have no effect on cell migration in BJ-5ta cells and may even delay it in HaCaT cells. The results of the statistical analysis are provided in the figures below.

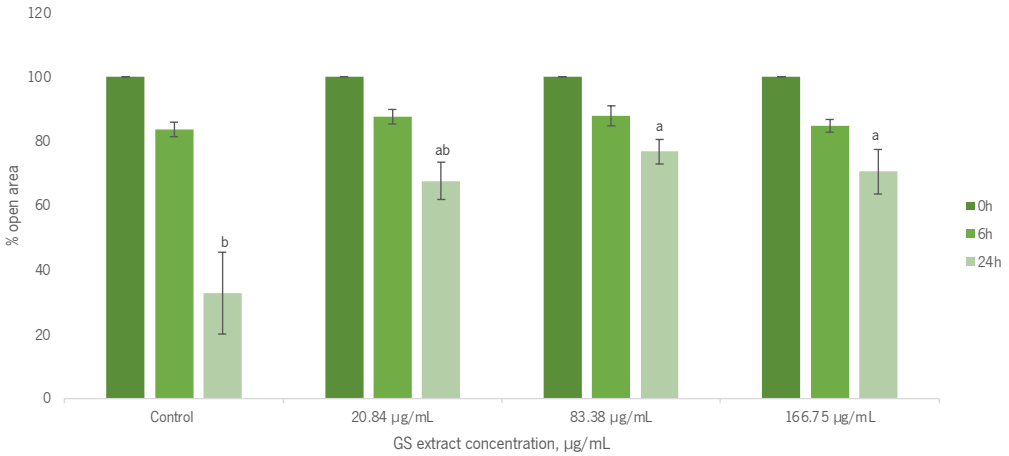

Figure 27. Influence of GS extract in HaCaT cell migration. Results are presented as mean ± standard error (n=3). Significant differences between samples at 24h are denoted by different lowercase letters ($p < 0.05$).

As shown in Figure 27, blend GS extract does not induce cell migration in HaCaT cells. At a lower concentration (20.84 µg/mL), cell migration is similar to the control (cells without treatment). At higher concentrations (83.38–166.75 µg/mL), HaCaT cell migration is inhibited. Thus, it can be concluded that blend GS extract is not effective in inducing wound healing and may even retard or inhibit the wound healing process at high concentrations.

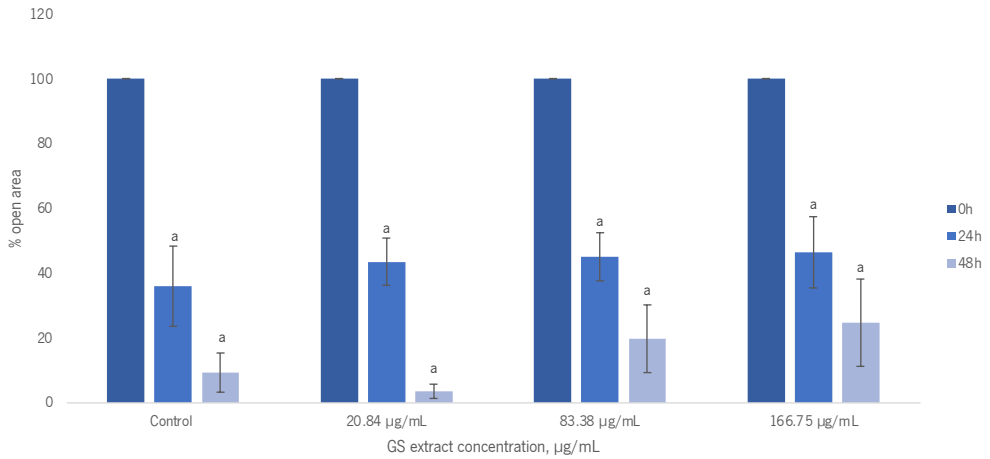

Figure 28. Influence of GS extract in BJ-5ta cell migration. Results are presented as mean ± standard error (n=3). Significant differences between samples at 24h and samples at 48h are denoted by different lowercase letters ($p < 0.05$).

As shown in the figure above, the blend GS extract does not affect BJ-5ta cell migration. The percentage of open area is similar among cells treated with various concentrations of GS extract and the untreated cells. Therefore, blend GS extract does not appear to have significant potential for inducing wound healing Research by Loggenberg et al. [97] on *Greyia radlkoferi Szyszyl* leaf extract (at 50 and 100 µg/mL) revealed significant inhibition of wound closure in HaCaT cells, reducing it by 50% to 60% compared to the control (0.25% DMSO). This effect was attributed to the suppression of PDGF-AA (platelet-derived growth factor), a key factor in regulation of cell growth and proliferation in human keratinocytes, and also have an important role in angiogenesis.

Scar formation is closely linked to angiogenesis, the process through which new blood vessels are formed [98–100]. Studies have shown that hypertrophic scars typically have a higher density of blood vessels than normal skin, while keloids, a more severe form of scarring, are also characterized by increased angiogenesis. This suggests that reducing angiogenesis may help limit scar formation and prevent severe scarring, such as keloids and hypertrophic scars [101–103].

In light of this, the inhibition of HaCaT cell migration observed with blend GS extract treatment may indicate that the extract has anti-angiogenic properties. These properties could be useful in controlling scar formation and reducing the likelihood of keloid and hypertrophic scar development.

Overall, although blend GS extract does not stimulate cell migration in HaCaT and BJ-5ta cells, its inhibitory effects are consistent with previous findings related to anti-inflammatory activity and cell cycle regulation. This suggests the extract has potential therapeutic applications, especially in managing keloids and hypertrophic scars.

3.1.4. Conclusion

This study thoroughly examined the GS extract from a blend of varieties, focusing on its phenolic content, including total phenolics, flavonoids, and ortho-diphenols. The extract was found to have high levels of phenolic compounds such as catechin, procyanidin trimers, rutin, quercetin, and resveratrol tetramers, with proanthocyanidin trimers being the most abundant. These findings were confirmed by phenolic content analysis, which showed elevated levels of flavonoids and ortho-diphenols.

The blend GS extract exhibited significant antioxidant activity, particularly against radical cations, as measured by the ABTS assay. This high antioxidant activity is likely due to the presence of proanthocyanidin trimers, complex compounds that are more prominent in the blend than in isolated grape varieties [11,15]. The strong positive correlation between total phenolic content and antioxidant

activity further supports this, as well as similar trends observed with ortho-diphenol and flavonoid contents.

In *in vitro* assays with HaCaT keratinocytes and BJ-5ta fibroblasts, the blend GS extract was safe, showing no significant effects on cell structure or function. The extract also reduced levels of TGF-β1, a key molecule in scar formation that promotes fibrosis and new blood vessel growth, which can lead to excessive scarring in conditions such as keloids and hypertrophic scars [75–79]. Additionally, the blend GS extract inhibited cell proliferation and promoted cell death in specific phases of the cell cycle (G0-G1 and Sub G0-G1 phases), which could help manage abnormal scar formation [91–94]. It also inhibited HaCaT cell migration, likely due to its anti-angiogenic properties affecting new blood vessel formation—an important factor in scar development [97,101–103].

Overall, these results suggest that the blend GS extract, with its unique profile of bioactive compounds, may be effective in managing and reducing problematic scar formation, including severe types like keloids and hypertrophic scars. The extract's potential to regulate scar formation is likely attributed to the high levels of proanthocyanidins, the most prominent compounds identified.

Given these promising findings, further research is underway to explore the molecular mechanisms behind these effects and to fully understand the extract's potential in dermatological applications.

3.1.5. References

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3.2. Grape Stems Phenolic Compounds as Active Ingredients for the Cosmetic Industry

3.2.1. Introduction

The European cosmetic industry, valued at EUR 88 billion in 2022, stands as a dynamic and competitive sector for personal care needs. In the face of intense competition, companies within this industry attempt to stand out through innovative product by offering elevated quality products and competitive pricing strategies [1–3]. Nowadays, the awareness of society for the use of natural product as led to a change in the overall industry, including the cosmetic industry. Therefore, the growing consumer preference for natural and organic products, due to the heightened awareness of environmental responsibility and health-conscious choices is driven the industry to develop new products using natural and organic products as active ingredients. [4–6]. But not only, the increase incidence of adverse reactions to synthetic ingredients, encompassing conditions like allergic contact dermatitis, irritant dermatitis, phototoxicity, and photoallergic reactions, as led industry to focus on innovation. The use of natural products, even as a cosmetic has been used for centuries, their attractive skin benefits and rapid absorption rate contributes to their increased usage [7].

Despite the favoured integration of natural compounds, prioritizing resource sustainability is imperative. An environmentally conscious approach involves the use of industrial by-products as sources of natural compounds [8].

In this context, grape stems, discarded during the winemaking process, has gained attention due to their rich content in special compounds like resveratrol, catechins, quercetin, and other phenolic compounds [9–11]. These compounds are known for their several exhibit diverse effects on the skin, inhibiting melanin production, acting as free radical scavengers, and showing anti-inflammatory properties, thereby protecting against environmental damage, including UV radiation [5,12–15].

The safety validation of 24 grape-derived cosmetic ingredients by the CIR (Cosmetic Ingredient Review) confirms their effectiveness, highlighting the industry's commitment to natural skincare aligned with environmental and transparent manufacturing trends [16].

In terms of regulation, the European Union ensures the safety and quality of cosmetic products through adherence to standards outlined in Cosmetics Regulation (EC) No 1223/2009. This regulation mandates stringent safety and quality criteria, including thorough toxicity assessments and efficacy demonstrations. Rigorous evaluations, including stability assessments, underscore the industry's unwavering commitment to consumer safety [17].

Adhering to ISO 22716 [18], a Good Manufacturing Practices guideline, is crucial for maintaining production consistency, and guiding manufacturers in quality control, hygiene, and documentation. On the other hand, ISO/TR 18811 (Cosmetics – Guidelines on the stability testing of cosmetic products) and "Cosmetics Europe: Guidelines on the stability testing of cosmetic products" provide a standardized approach to testing cosmetic products, ensuring fair and reliable assessments. These reports establish guidelines that offer a comprehensive framework for evaluating the efficacy, safety, and performance of cosmetic products. Rigorous testing is essential for cosmetic products, considering factors like chemical composition, stability under varying environmental conditions, and potential interactions with packaging materials. Stability holds particular importance for product quality, especially considering the diverse environmental challenges during transportation, storage, and usage. The guidelines provide a standardized approach to assess cosmetic product stability under stress factors, including temperature variations, humidity, and light exposure. This meticulous testing supports manufacturers in designing products that maintain integrity and efficacy throughout their intended shelf life [19,20].

Therefore, the primary goal of this study is to examine the integration of phenolic compounds derived from grape stems into cosmetic formulations. In the initial phase, various concentrations of phenolic compounds were incorporated into a water gel cream base, and the resulting formulations were characterized. This involved the assessment of key parameters crucial for cosmetic formulation development, such as pH, viscosity, and spreadability, among others. Following this, a study was undertaken to verify the stability of cosmetic formulations containing phenolic compounds.

This research propels the cosmetic industry towards a future where innovation, safety, and efficacy converge impeccably in cosmetic formulations enriched by the natural benefits of phenolic compounds from grape stems. This research aims to ensure consumer safety and product quality and evaluate the effectiveness of these compounds in cosmetic applications.

3.2.2. Materials and Methods

3.2.2.1. Chemicals and Reagents

Gallic acid (3,4,5-trihydroxybenzoic acid) and acetic acid (all extra pure >99%) were obtained from Panreac (Panreac Química S.L.U., Barcelona, Spain). Sodium hydroxide (98%), sodium nitrite, aluminum chloride (purity levels >99%), and ethanol were acquired from Merck (Merck, Darmstadt, Germany). Additionally, catechin (98%), TROLOX (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, purity ≥98.0%), $ABTS^{\cdot+}$ ((2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid) diammonium salt, purity

≥98.0%), potassium persulfate (purity ≥99.0%), TPTZ (2,4,6-Tripyridyl-s-Triazine, purity ≥98.0%), iron (III) chloride (purity ≥99.9%), tyrosinase and elastase enzymes, and the reagents employed in all enzymatic activities were sourced from Sigma Aldrich (Steinheim, Germany), while hydrochloric acid (≈37%) was obtained from Honeywell, Fluka, (Germany). Potassium ferricyanide was obtained from Acros Organics. Polyacrylamide, C13-14 isoparaffin, and Laureth-7 were purchased from Quimica Masso. Methylchloroisothiazolinone and methylisothiazolinone were acquired from Escuder. Parfum was sourced from Inarom. Distilled water was generated using a Millipore water purification system (Millipore, Bedford, MA, USA).

3.2.2.2. Incorporation of phenolic compounds in cosmetic formulation

The initial stage in incorporating phenolic compounds into cosmetic formulations involved extracting these compounds from grape stems. Subsequently, a cosmetic formulation was prepared by incorporating the phenolic compounds through mixing.

3.2.2.2.1. Preparation of Stem Extracts

The extraction of phenolic compounds followed the methodology defined by Costa et al. [21] with certain modifications.

Ground samples weighed at 18 g, were homogenized and stirred in 198 mL of extraction solvent (Ethanol:Water, 70:30) for 30 minutes at 300 rpm. Post-homogenization, the mixture was subjected to centrifugation (ScanSpeed 1580R) at 4000 rpm for 15 minutes, a process repeated three times.

The resulting supernatant volumes from the repeated centrifugations were adjusted to 500 mL in a volumetric flask with extraction solvent. After, ethanol was removed via rotary evaporation (Stuart), and the remaining volume was freeze-dried (VirTis Benchtop Pro Freeze-drier with OmnitronicsTM, SP Industries, Inc, Warminster, PA, USA).

3.2.2.2.2. Preparation of cosmetic formulations

Lyophilized powder from GS extract at various concentrations was integrated into a water gel cream base using gentle mixing, as outlined in Table 11. The composition details of the water gel cream base can be found in Table 12.

Table 11. Concentration of GS extract in water gel cream base.

Sample	g GS powder / 30 g cream base	% GS
CRT	0.000	0
I	0.100	0.33
II	0.250	0.83
III	0.300	0.99
IV	0.500	1.64
V	1.000	3.23
VI	1.200	3.85
VII	2.000	6.25

Table 12. Water gel cream base composition.

Function	Ingredient	%
Thickening, stabilizer, texturizing agent	Polyacrylamide, C13-14 Isoparaffin, Laureth-7	91.5
Preservative	Methylchloroisothiazolinone, methylisothiazolinone	3.4
Fragrance	Parfum	3.5
Solvent	Water	1.6

3.2.2.3. Cosmetic Formulation Characterization and Accelerated Stability Evaluation

Cosmetic formulations based on grape stem (GS) extract were characterized following ISO/TR 18811 and "Guidelines on the stability testing of cosmetic products" [19,20]. The stability metrics assessed included organoleptic characteristics, pH, texture, and rheological properties. The formulation's phenolic compounds were evaluated, including total phenolic and flavonoid contents. Antioxidant activity was assessed through ABTS and FRAP assays, while antiaging and depigmenting activities were determined using elastase and tyrosinase inhibition assays.

3.2.2.3.1. Organoleptic characteristics and pH measurement

The organoleptic features, including colour, appearance, and odour, were subjected to visual evaluation. The pH values were directly determined at room temperature using a pH meter (pH MesuLab PHS-3E).

3.2.2.3.2. Evaluation of texture and rheological properties

Texture parameters, including firmness and adhesiveness, were evaluated using a texturometer, specifically the Stable Micro Systems TA-HD plus (Haslemere, UK). The evaluation encompassed the following test conditions: compression mode, cylinder probe (10 mm, P/10), penetration distance of 5 mm, test speed of 3 mm/s, trigger force of 0.049 N, and a loading cell of 5000 g.

Rheological measurements were performed at 25 °C in a Discovery HR-1 rheometer equipped with a Peltier plate (TA Instruments). Flow curves were obtained using a 40 mm plate and performing a two steps program (up-down) using a continuous ramp and shear rate range between 0.1 and 500 s^{-1}. The two steps program was carried out in order to access the thixotropy of the formulations.

3.2.2.3.3. Biological contamination monitorization

Evaluation of biological contamination was made by spreading uniformly 1 gram of each cream formulation using a sterile swab across the agar surfaces of two distinct media: Trypticase Soy Agar (TSA) for quantifying mesophilic aerobic bacteria and Sabouraud Dextrose Agar – Chloramphenicol (SDA-C) for quantifying yeast and fungi. Incubation of the plates took place at 30–35 °C for 5 days (TSA) and at 20–25 °C for 7 days (SDA-C) in incubators (VWR). The results are presented in colony-forming units per gram of the sample (CFU/g).

3.2.2.3.4. Extract preparation from creams

In order to assess the phenolic content, antioxidant, antiaging, and depigmenting activities of the cosmetic formulation containing grape stem extract (CF-GS), the phenolic compounds — bioactive ingredients in the formulation — were extracted from the cream base. After the extraction process, both the phenolic content and the biological activities were determined.

The preparation of the cream extract followed the procedure delineated by Mapoung et al [22] with certain modifications. The process began by weighing 1 g of cream, followed by freezing it at -20 °C for 15 to 20 minutes. Subsequently, 9 mL of ethanol was added, and the mixture suffered vortexing and centrifugation at 15000 rpm for 15 minutes. The resulting supernatant was saved, and subsequent dilutions were prepared for conducting the tests.

3.2.2.3.5. Quantification of Phenolic Compounds

The quantification of phenolic content in CF-GS utilized modified spectrophotometric methods specifically designed for 96-well microplates (PrimeSurface MS-9096MZ, Frilabo, Maia, Portugal), as outlined by Costa et al [21]. Absorbances were subsequently determined using microplate readers (Multiskan GO Microplate Photometer, Thermo Fisher Scientific, Vantaa, Finland).

3.2.2.3.5.1. Determination of total phenolic content

The determination of the total phenolic content (TPC) in CF-GS was conducted using the Prussian blue assay, a method involving the formation of a blue-coloured complex resulting from a reaction between different components. The procedure started with the addition of 100 µL of a 0.5 mmol/L ferric chloride hexahydrate solution and 100 µL of a diluted sample to a microplate well, where they were allowed to react for 2 minutes. Following this, 100 µL of a 0.5 mmol/L potassium ferricyanide solution was introduced. After shaking the microplate for 20 seconds, the absorbance was measured at 725 nm after 15 minutes. To construct the analytical curve, gallic acid was utilized at various concentrations. The total phenolic content was quantified and expressed as milligrams of gallic acid equivalent per 100 grams of the sample (mg GAE/ 100 g sample).

3.2.2.3.5.2. Determination of Flavonoids Content

The methodology utilized to determine flavonoid content (FC) involved the creation of a flavonoid-aluminum complex. In this procedure, 24 µL of the diluted sample was introduced into a microplate well, followed by the addition of 28 µL of a 50 g/L sodium nitrite solution. After a 5-minute reaction period, 28 µL of a 100 g/L aluminum chloride solution was added. Following a 6-minute reaction period, 120 µL of a 1M sodium hydroxide solution was introduced into the well. The microplate was then stirred for 30 seconds, and the absorbance was measured at 510 nm. For the construction of the calibration curve, various concentrations of catechin were employed. The results were expressed as milligrams of catechin per 100 grams of sample (mg CAT/100 g sample).

3.2.2.3.6. Antioxidant, antiaging, and depigmenting activities

Biological activities were assessed using adapted spectrophotometric techniques designed for 96-well microplates (PrimeSurface MS-9096MZ, Frilabo, Maia, Portugal), following the protocols described by Costa et al [21] and Taghouti et al [23]. Absorbance readings were then recorded using microplate readers (Multiskan GO Microplate Photometer, Thermo Fisher Scientific, Vantaa, Finland).

3.2.2.3.6.1. ABTS assay

The initial step in this assay involved the oxidation of the ABTS salt, achieved by introducing a 148 mM potassium persulfate solution (88 µL) to a 7 mM ABTS solution (5 mL). The mixture was allowed to rest for 12 to 16 hours at room temperature, protected from light, to attain its most stable oxidative state. Subsequently, the $ABTS^{\cdot+}$ working solution was prepared by diluting the cationic $ABTS^{\cdot+}$ solution in a 20 mM sodium acetate buffer (pH 4.5) until an absorbance of 0.700 ± 0.020 at 734 nm was achieved. Following this, the evaluation of the sample's antioxidant capacity involved adding 188 µL of the $ABTS^{\cdot+}$ working solution to each microplate well, followed by the introduction of 12 µL of the diluted sample and blank. After a 30-minute incubation period at room temperature, shielded from light, the absorbance was measured at 734 nm [Abs (734 nm)]. TROLOX at various concentrations served as the standard for constructing the analytical curve, with water acting as the blank.

The ABTS assay estimated the scavenging capacity of $ABTS^{\cdot+}$ by the samples, and the inhibition percentage was calculated using the formula:

$$\%\ \text{inhibition}_{\text{Sample}} = \frac{\text{Abs (734 nm)}_{\text{Blank}} - \text{Abs (734 nm)}_{\text{Sample}}}{\text{Abs (734 nm)}_{\text{Blank}}} \times 100$$

The results were presented in TROLOX Equivalent Antioxidant Capacity (TEAC) and expressed in millimoles of TROLOX per 100 grams of sample (mmol TROLOX/100 g sample).

3.2.2.3.6.2. FRAP assay

The FRAP method for assessing antioxidant activity is initiated with the preparation of the FRAP working solution. This involved combining 10 volumes of 220 mM acetate buffer (pH 3.6), 1 volume of a 40 mM TPTZ (2,4,6-Tripyridyl-s-Triazine) solution dissolved in 40 mM HCl, and 1 volume of 20 mM ferric chloride. The freshly prepared FRAP working solution was preheated to 37°C 10 minutes before use.

Following this preparation, 20 µL of the sample was added to a microplate well, followed by the introduction of 280 µL of the preheated FRAP working solution. The microplate was then shaken and placed in darkness, incubating at 37°C for 30 minutes, after which the absorbance was measured at 593 nm. The calibration curve was constructed using various concentrations of TROLOX. The reported results were in millimoles of TROLOX per 100 grams of sample (mmol TROLOX/100 g DW).

3.2.2.3.6.3. Tyrosinase inhibition assay

The tyrosinase inhibition assay measured the percentage of tyrosinase inhibition following a specified procedure. Initially, 20 µL of 1000 U/mL tyrosinase and 170 µL of a mixture (containing 1 mM L-tyrosine solution, 50 mM phosphate buffer at pH 6.5, and water in a 10:10:9 ratio) were added to the sample. Subsequently, the microplate experienced incubation at 37 °C for 10 minutes, and the absorbance was read at 490 nm. Positive and negative controls, using 1 mg/mL Kojic acid and 10% DMSO, respectively, were included. The percentage of inhibition was calculated using the formula:

$$\%\ \text{inhibition}_{\text{Sample}} = \frac{\text{Abs (490 nm)}_{\text{Negative Control}} - \text{Abs (490 nm)}_{\text{Sample}}}{\text{Abs (490 nm)}_{\text{Negative Control}}} \times 100$$

3.2.2.3.6.4. Elastase inhibition assay

The elastase inhibition assay was designed to determine the percentage of elastase inhibition. In this assay, 50 µL of the sample was combined with 160 µL of 0.20 mM Tris-HCl buffer (pH 8) and 20 µL of 0.80 mM N-Succinyl-Ala-Ala-Ala-p-nitroanilide substrate (in Tris-HCl buffer). After a 10-minute incubation at room temperature, 20 µL of 1 U/L elastase enzyme (in Tris-HCl buffer) was added, and the microplate underwent an additional 20-minute incubation at room temperature. The absorbance was then measured at 410 nm, with Tris-HCl buffer serving as the negative control. The percentage of inhibition was calculated using the formula:

$$\%\ \text{inhibition}_{\text{Sample}} = \frac{\text{Abs (410 nm)}_{\text{Negative Control}} - \text{Abs (410 nm)}_{\text{Sample}}}{\text{Abs (410 nm)}_{\text{Negative Control}}} \times 100$$

3.2.2.4. Accelerated stability study

The cream's physicochemical stability was assessed through accelerated stability testing, involving storage at elevated temperature (40 ± 2 °C) and relative humidity (75 ± 5%) for 3 months within a climatic chamber (Shjianheng SDJ-150GSD, Jianheng Instrument Co., Shanghai, China). The analysis included organoleptic characteristics, pH, rheological behaviour, total microbiological contamination, as well as

parameters related to phenolic and flavonoid content, and antioxidant, antiaging, and depigmenting activities. This comprehensive evaluation aimed to elucidate the cream's stability under accelerated conditions and its temporal performance.

In the context of accelerated stability testing, the Q rule (Q10 rule) is commonly employed to estimate the rate of reaction changes with temperature fluctuations. Within the cosmetic industry's Accelerated Stability Model, the conservative assumption of Q = 2 is often adopted, suggesting that the reaction rate doubles for every 10 °C increase in storage temperature [24]. This cautious approach was implemented to provide a prudent estimate of the impact of temperature variations on the reaction rate in cosmetic formulations, allowing for a careful prediction of potential degradation or alterations in the cosmetic product over time. Consequently, Table 13 illustrates the correspondence, based on the Q rule (Q = 2), between the storage time of samples under accelerated stability conditions and the storage time under normal conditions.

Table 13. Correspondence, based on the Q rule (Q = 2), between the storage times of samples under accelerated stability conditions and normal conditions.

Storage Time (Months) - Accelerated Conditions	Storage Time (Months) - Normal Conditions
1	4
2	8
3	12

3.2.2.5. Statistical Analysis

The data were analysed using IBM SPSS 29.0 statistical software through analysis of variance (ANOVA) and a multiple range test (Tukey's test) for a significance level set at $p < 0.05$.

The results are expressed as mean values ± standard deviation and were conducted in triplicate (n = 3).

3.2.3. Results and Discussion

3.2.3.1. Cosmetic Formulation Characterization and Accelerated Stability Evaluation of Developed Cosmetic Formulations

The phenolic compounds extracted from grape stems were incorporated into a cream base, resulting in a cosmetic formulation. The cosmetic formulation with distinct concentrations of phenolics compounds underwent a thorough characterization following the guidelines outlined in ISO/TR 18811 and "Cosmetics Europe: Guidelines on the stability testing of cosmetic products". This comprehensive assessment

encompassed the evaluation of various organoleptic properties, including colour, appearance, and odour, in addition to the measurement of pH and viscosity [19,20]. The firmness, adhesiveness, and thixotropy were also assessed. Since the developed formulations incorporate phenolic compounds obtained from grape stems, the total phenolic and flavonoid content was quantified. Furthermore, the potential biological effect of phenolic compounds, such as antioxidant, antiaging, and depigmenting activities, were examined.

Considering the stability of cosmetics is crucial for product development, ensuring safety, effectiveness, and quality. Stability studies play a pivotal role in assessing how cosmetic formulations tolerate changes over time in factors like colour, texture, composition, and active ingredient concentration.

The EU Cosmetic Regulation requires cosmetic products to demonstrate formulation stability before entering the market [17]. Although the regulation highlights long-term stability assessment, it lacks specific protocols for data acquisition. Guidelines provided in ISO 18811:2018 [19] and Cosmetics Europe [20] involve stress testing under elevated temperature and humidity, using the Q rule to estimate shelf lives.

In alignment with recommended stress conditions, the stability testing of developed samples in this study involved storing samples at elevated temperatures (40 °C) and humidity (75%) for three months, which is equivalent to 12 months under normal conditions.

This study provides a comprehensive exploration of cosmetic formulation stability, employing a range of metrics. The pivotal role of these stability studies extends beyond optimizing product performance; it ensures adherence to regulatory standards, contributing significantly to the constant advancement of cosmetic science.

3.2.3.1.1. Evaluation of organoleptic features and their stability

Evaluating organoleptic properties in cosmetic formulations is essential for consumer satisfaction, product differentiation, and quality assurance. Changes in these parameters during stability studies may indicate chemical alterations or microbiological processes [19].

Table 14 presents the detailed results of the organoleptic evaluation conducted immediately after the development of the formulations (time 0). As it can be seen the variations in colour, appearance, and odour are evident, correlating with the concentration of GS extract within the cream base. The control sample (cream base) exhibits a bright white colour. The increase in GS extract concentration leads to a gradual alteration towards a browner tone.

Table 14. Presentation of the organoleptic characteristics of the developed cosmetic formulations (time 0).

Sample (% GS extract)	Colour	Appearance	Odor
CRT (0%)		▪ Glossy and shiny ▪ Creamy and soft texture	▪ Parfum
I (0.33%)		▪ Glossy and shiny ▪ Creamy and soft texture	▪ Parfum
II (0.83%)		▪ Glossy and shiny ▪ Creamy and soft texture	▪ Parfum ▪ Woody
III (0.99%)		▪ Glossy and shiny ▪ Creamy and soft texture	▪ Parfum ▪ Woody
IV (1.64%)		▪ Glossy and shiny ▪ Creamy and soft texture	▪ Parfum ▪ Woody
V (3.23%)		▪ Glossy and shiny ▪ Thinner texture	▪ Parfum ▪ Woody
VI (3.85%)		▪ Glossy and shiny ▪ Thinner texture	▪ Woody
VII (6.25%)		▪ Glossy and shiny ▪ Thinner texture	▪ Woody

In a safety assessment, Fiume et al. [16] classified ingredients derived from grapes as colouring agents. This observation is accordance with the results obtained in this work, where the colour of the cosmetic formulations is influenced by the concentration of GS extract.

Similarly, in a parallel study, Hübner et al. [25] demonstrated that the changes in colour were correlated with the increment in grape pomace extract content (0–10%) into topical creams, with its tone moving towards a purple hue. Kawarkhe et al. [26] also reported a similar result upon the incorporation of grape by-products into a face cream, which presented a faint brown colour. Interestingly, the cosmetic

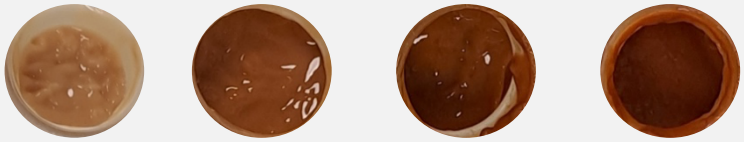
formulation with 1.64% grape stem extract presented a colour similar to the formulation prepared in this study containing 2% grape seed extract.
Thus, the incorporation of GS extracts not only induces a colour variation, but also confers a differentiation feature through its unique and natural colouring properties.

In terms of visual appearance, all samples initially display a shiny and soft creaminess. However, the samples with a higher concentration of GS extract (between 0.99% and 6.25% GS extract) demonstrate a decrease in creaminess, leading to a thinner formulation. This texture becomes more pronounced with increasing GS extract concentration.
Supriadi et al. [27], in their investigation, examined the apparent texture of formulations featuring 5% of grape seed oil, observing a gentle texture in their preparations. This observation reinforces the results obtained in this work, as the developed cosmetic formulations demonstrated a creamy and soft texture.
The decrease in visual apparent viscosity as the concentration of GS extract increases implies a notable influence on the internal structure of the emulsion, resulting in a thinner consistency. Therefore, it is hypothesized that the changes observed in the rheological properties are due to the incorporation of GS extract. It is believed that the GS extract alters the overall structure of the cosmetic formulation. To validate the hypothesis raised, further rheological studies were performed and discussed in section *3.2.3.1.3 Evaluation of rheological and texture* properties.

The olfactory profile also changes with GS extract concentration. The control sample and those with lower GS extract concentrations emit the characteristic perfume odour present in the formulation. However, as the GS extract concentration rises, a transition to a woody aroma becomes apparent. Notably, formulations with a higher GS extract concentration (3.85% and 6.25%) exclusively exhibit this woody fragrance, surpassing the initial aroma of the perfume.
This woody odour aligns with the findings of an olfactometric study on derived grape extracts, specifically GS extract, conducted by María J. Ruiz-Moreno et al [28]. They characterized the odorant profile of grape stem extract (Syrah variety) and identified different odour perceptions such as woody, almond, sweet, fruity, paper, toasty, caramel, metallic, vanilla, among others. Hübner et al. [25] also observed a characteristic odour of grape extract in their preparations based on grape pomace extract .
The assessment of odour variation is subjective, as perceptions may differ based on individual preferences. While some individuals may find the woody fragrance appealing, associating it with natural and botanical scents, others may prefer the initial perfume odour.

During the stability study, changes in these feature were observed. Table 15 presents the variations in the visual aspects of the developed samples, specifically focusing on macroscopic colour and appearance. The presented results indicate that the colour of the samples intensified over time, reaching a darker shade by the end of the third month in the stability chamber, equivalent to up to 12 months in real storage conditions. This transformation seems to be more pronounced in samples with a higher GS extract concentration, specifically from 3.23% GS extract.

Table 15. Visual colour and appearance variations in developed samples over 3 months.

	months in a stability chamber			
Sample (% GS extract)	**0**	**1**	**2**	**3**
CRT (0%)				
I (0.33%)				
II (0.83%)				
III (0.99%)				
IV (1.64%)				
V (3.23%)				
VI (3.85%)				
VII (6.25%)				

This finding aligns with the study by Cefali et al. [29], where they investigated the stability of formulations containing Benitaka grape peel extract for 90 days under various storage conditions, including room temperature, refrigerator, freezer, and oven. They reported observing a colour change in the samples stored in the oven as early as the 13th day, indicating the susceptibility of the formulations to environmental factors. Furthermore, Yarovaya et al. [30] observed similar colour changes in stability studies of formulations containing 3% grape seed extract. They found that the colour shifted from light brown to brown and eventually dark brown when the samples were stored for three months at 30 °C and 50 °C, respectively. This colour change was attributed to the degradation of catechins at elevated temperatures.

The observed alterations in colour suggest a potential correlation between the concentration of the extract and the extent of colour change in the samples. These changes are often linked to chemical destabilization processes occurring within the formulation, such as oxidation, hydrolysis, transesterification, and interactions among ingredients. These processes can degrade compounds, break chemical bonds, and lead to the formation of new chemical species, ultimately contributing to changes in the colour of the formulations. [19].

Concerning appearance, samples with up to 0.83% GS extract retained their texture during the storage period in a stability chamber. However, the sample containing 0.99% GS extract in the cream base maintained its texture until the second month of storage in accelerated conditions. Subsequently, samples with 1.64% and 3.23% GS extract retained their soft texture only for the initial month of storage, after which they exhibited increased lumpiness. Notably, the more concentrated samples exhibited increased instability, becoming lumpier and more viscous after the first month of storage in a stability chamber.

These findings align with the results of Hernani et al.'s study [31], where formulations with higher concentrations of Gambier leaf extract (2% and 3%) became coarser after 6 weeks in accelerated conditions. Additionally, Cefali et al. [29] reported a slight increase in viscosity in grape peel extract-based samples when subjected to storage in an oven.

The increased lumpiness observed in the more concentrated samples during storage in the stability chamber can be attributed to various factors. The complex composition of the extract, combined with the cream base, may have contributed to increased interactions between particles or droplets in the more concentrated samples. This could lead to coalescence, a process where smaller droplets or particles

combine to form larger entities, resulting in the observed lumpiness and changes in viscosity. Coalescence is an irreversible process initiated by thermal or mechanical energies and can lead to significant alterations in texture, appearance, and viscosity in cosmetic formulations [32].

Throughout the storage period in the stability chamber, all developed samples maintained their odour, transitioning from a parfum fragrance to a woody odour following the concentration of GS extract incorporated into the samples. These results are consistent with the findings of Rodrigues et al. [33] and Hernani et al. [31], who similarly observed no changes in odour during their study.

In summary, the concentration of GS extract in cosmetic formulations affects colour, appearance, and odour. It is important to mention that colour changes are acceptable, as they do not indicate structural impacts. These changes are only characteristic of the raw material (GS extract). Olfactory alterations from perfume to a woody aroma align with studies on grape extracts. These results are step forward on the understanding of GS extract's influence on cosmetic attributes.
Additionally, the stability study demonstrates that colour intensification occurs over time in samples with higher concentrations of GS extract. Appearance changes, such as increased lumpiness and viscosity, are prominent in more concentrated samples due to particle interactions. However, the odour remains consistent, transitioning from a parfum fragrance to a woody odour.

3.2.3.1.2. pH Measurement, its stability analysis, and biological contamination monitoring

The pH of cosmetic formulations is a critical parameter that significantly influences both product stability and its interaction with the skin. The pH level, indicating the acidity or alkalinity of a solution, plays a pivotal role in determining the overall effectiveness and user experience. Therefore, a meticulous evaluation of pH is essential to ensure that cosmetic formulations meet the desired specifications for optimal performance and user safety [34,35].
In the context of this study, the pH of the formulated samples was assessed. As it can be seen in Table 16 there is a clear trend on pH variation. A decrease in pH from 6.34 to 3.93 was observed with the increase in the concentration of grape stem extract within the formulations (time 0). This rise in acidity is justified by the phenolic composition of the GS extract, specially proanthocyanidin trimer monogallates isomers.

The table also presents the pH variations of samples during storage under accelerated conditions. As evident from the data, there are no significant differences in pH over time, with the variance being very close to 0. The samples consistently maintained their pH between 5.99 and 3.84, corresponding to the incorporated GS extract percentage (0%–6.25%).

Table 16. Variations in pH of developed samples during storage in stability chamber.

	months in a stability chamber				
Sample (% GS extract)	**0**	**1**	**2**	**3**	***Variance***
CRT (0%)	6.34	6.08	6.05	5.99	*0.018*
I (0.33%)	4.50	4.49	4.69	4.69	*0.010*
II (0.83%)	4.21	4.20	4.32	4.24	*0.002*
III (0.99%)	4.18	4.16	4.32	4.18	*0.004*
IV (1.64%)	4.01	4.08	4.21	4.05	*0.006*
V (3.23%)	4.02	3.99	4.05	3.92	*0.002*
VI (3.85%)	3.97	3.95	4.11	3.88	*0.007*
VII (6.25%)	3.93	3.94	3.96	3.84	*0.002*

These results corroborate the findings of Salem et al. [36], who developed cosmetic formulations with different concentrations of grape seed extract from Marselan variety (0%–5%), observing a decrease in pH (from 5.33 to 5.26) with the increase in the concentration of the incorporated extract. In their study, the pH of creams remained stable, averaging around 5.3, throughout the four-month evaluation period.

Similarly, Rodrigues et al. [37] reported a decrease in pH (from 9.65 to 9.39) in a facial mask when 5% of olive pomace extract, an extract also rich in phenolic compounds, was incorporated. The results obtained by Gomes et al. [38] and Stojiljkovic et al. [39] support similar conclusions. In their studies, when incorporating kiwi peel extract, the pH decreased from 6.04 to 4.11. Likewise, the introduction of wild apple fruit extract led to a decrease in pH from 6.61 to 5.69 in a placebo cream sample.

Additionally, Rafique et al. [40] and Ferreira et al. [41] reported similar findings, with the pH values of grape extract-based emulsions remaining within the acceptable range for skin compatibility over a study period under accelerated conditions.

The observed decrease in the formulation's pH (4.50–3.93), compared to the cream base (6.34), indicates an adjustment towards a pH similar to skin's natural acidity, typically ranging between 4.00 and 4.50. This alignment with the physiological characteristics of the skin is crucial, as the skin's acidic pH promotes an environment beneficial to the resident microflora, fostering a balance that is favourable for overall skin health [35]. Considering their proximity in pH to the skin's natural acidity, the developed

cosmetic formulations are expected to enhance the product's effectiveness in promoting skin well-being, making them compatible with the skin.

Regarding microbial contamination, the developed samples remained safe for use, with microorganism levels consistently below the acceptable limit for cosmetics (≤ 100 CFU/g) [42]. Rodrigues et al. [33] did not observe any microbial contamination during 180 days of storage in accelerated conditions when natural extract was added to cosmetic formulations. This finding is consistent with the results reported by Gomes et al. [38].

In summary, the presented results illustrate the consistent pH stability of the developed cosmetic formulations over time, reflecting their compatibility with the skin. Furthermore, the absence of microbial contamination further reinforces the safety of the studied formulations.

3.2.3.1.3. Evaluation of rheological and texture properties and their stability analysis

An ideal fluid conforms to Newtonian flow is characterized by a linear relationship between shear stress and shear velocity, with viscosity as the constant of proportionality. However, numerous fluids deviate from this linearity, demonstrating non-Newtonian behaviour. The rheological characteristics of the developed formulations were assessed using a flow curve representing shear stress or viscosity against shear rate (Figure 29) [43,44].

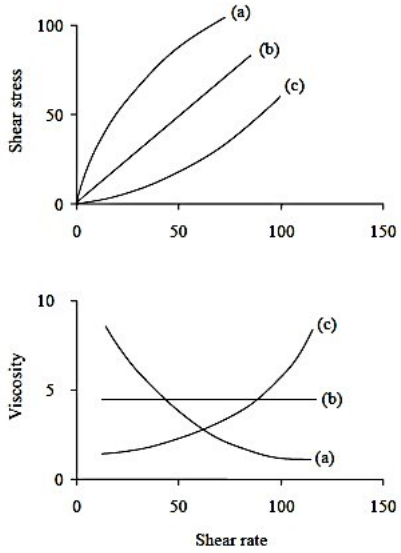


Figure 29. Flow characteristics of fluids: (a) fluid exhibiting shear-thinning behaviour or pseudoplastic fluid, (b) Newtonian fluid, (c) fluid demonstrating shear-thickening behaviour or dilatant fluid. Adapted from [45].

The rheograms of developed samples with various GS extract concentrations are depicted in Figure 30. Examination of the curves indicates that all formulations demonstrate a pseudoplastic or shear-thinning behaviour. Pseudoplastic fluids, such as cosmetic emulsions, show a decrease in viscosity as the shear rate increases [46].

The power-law model is commonly used to describe shear-thinning characteristics, expressing the relationship between shear stress (σ) and shear rate (γ) as follows:

$$\sigma = K.\gamma^{n}$$

in which n denotes the flow behaviour index, and K represents the flow consistency index. For shear-thinning fluids, the flow behaviour index is between 0 and 1, while it equals 1 for Newtonian fluids and exceeds 1 for dilatant fluids. The degree of shear-thinning increases as the value of n decreases for pseudoplastic fluids [43,47].

Application of the power-law model to the flow curves of the samples confirms their pseudoplastic behaviour, particularly shear-thinning behaviour, as indicated by the flow behaviour index (n) being less than 1. This confirmation is achieved by analysing the obtained equation of the tendency potency line of the downward flow curve (in order to avoid deviations caused by the destructuring that occurs initially) for each sample presented in Figure 30.

The sample with the most pronounced shear-thinning behaviour is the most concentrated sample containing 6.25% GS extract (n = 0.1858), followed by CRT with 0% GS extract (n = 0.1878), while the samples with the least shear-thinning behaviour (0.2603 and 0.2365) are samples II and V (with 0.83% and 3.23% GS extract, respectively).

Another parameter that can be analysed using this model is K, the flow consistency index, which represents the necessary force to flow the formulation. This index ranges between 46.697 and 161.19 in the developed formulations, with the samples with the lower consistency index being CRT with 0% GS extract (K = 46.697), followed by V (K = 94.043) and VI (K = 90.967) with 3.23% and 3.85% GS extract, respectively. The samples with the higher consistency index are II (K = 150.41) and III (K = 161.19) with 0.83% and 0.99% GS extract, respectively.

These values of flow consistency index (K) and flow behaviour index (n) are consistent with the values found in other cosmetic products, such as sunscreen lotions (75, 0.28) and mascara (200, 0.24) [47]. Dănilă et al. [48] also used the power-law model to analyse the flow curves of rheograms for antiaging creams. They found flow behaviour index values ranging from 0.508 to 0.606 and consistency index values ranging from 4.414 to 44.794. Additionally, their findings indicated that emulsions with lower consistency index values exhibited greater fluidity.

Petkova-Parlapanska et al. [49] investigated the rheological properties of cosmetic compositions containing extracts of rosemary and grapefruit pulp as well as seeds in two distinct cream base. Interestingly, each formulation exhibited a different response upon the addition of grapefruit-based extract. In one formulation, the consistency index increased (from 3.325 to 15.465) while the flow behaviour index decreased (from 0.496 to 0.273). Conversely, in the other formulation, with a different cream base but the same concentration of grapefruit-based extract, the opposite trend was observed. The authors attributed this variation to differences in the formulation composition and pH.

The rheological properties were evaluated at the end of a 3-month storage period under accelerated conditions to assess the samples' ability to maintain their rheological behaviour and viscosity. The rheograms at time 3, depicted in Figure 30, confirm the presence of pseudoplastic behaviour.

After reapplication of the power-law model to the downward flow curves at time 3, flow behaviour index (n) values between 0 and 1 were observed in all samples, confirming their pseudoplastic behaviour, particularly shear-thinning behaviour. However, the flow behaviour index (n) and consistency (K) values varied from time 0 to time 3, as depicted in Table 17.

Table 17. Variation of flow behaviour and consistency indexes from time 0 (before storage) to time 3 (at the end of storage in accelerated conditions)

	n, flow behaviour index		K, consistency index	
Sample (% GS extract)	**Time 0**	**Time 3**	**Time 0**	**Time 3**
CRT (0%)	0.1878	0.2362	46.697	97.904
I (0.33%)	0.2225	0.2851	137.170	122.020
II (0.83%)	0.2603	0.3030	150.410	78.248
III (0.99%)	0.2240	0.2760	161.190	146.220
IV (1.64%)	0.2237	0.3276	139.810	49.434
V (3.23%)	0.2365	0.2873	94.043	78.453
VI (3.85%)	0.2208	0.2665	90.967	106.270
VII (6.25%)	0.1858	0.2826	129.480	88.792

The observed changes in rheological properties over time are significant indicators of the evolving behaviour of cosmetic formulations. The flow behaviour index values increased from time 0 to time 3 across all samples, indicating a reduction in shear-thinning behaviour. This reduction was particularly noticeable in samples IV and VII (1.64% and 6.25% GS extract, respectively). Conversely, the consistency index values decreased, except for samples CRT (0% GS extract) and VI (3.85% GS extract). Consequently, the force required to flow the samples decreased compared to the initial time, with the most significant differences observed in samples II (0.83% GS extract) and IV (1.64% GS extract).

Thixotropy

Thixotropy, another parameter that can be characterized in a rheograms analysis, describes the phenomenon where the viscosity decreases over time when a sample at rest experiences flow, and then viscosity recovers when the flow discontinues [50]. This parameter provides insights into how the cosmetic formulation returns to its original structure after the applied stress is removed. Moreover, topical formulations with thixotropic properties are desirable as they become more fluid during the application, facilitating the spreading, and recuperating of initial characteristics, and preventing the product from running off the skin [46] [51].

Thixotropy can be assessed by examining the hysteresis area in the rheograms, which represents the region between the upward and downward curves. A larger hysteresis area is indicative of a more pronounced time-dependent behaviour, which is typical of thixotropic materials. Consequently, such

materials exhibit more significant variations in viscosity over time during both flow and recovery phases [46,50]. When both curves coincide, it indicates the absence of thixotropy [46].

Figure 30 illustrates the thixotropic behaviour of the samples. Thixotropic behaviour correlates with the concentration of GS extract in the samples. Those with concentrations between 0.33% and 1.64% of GS extracts display relatively low thixotropy, with hysteresis values ranging from 777 to 3086 Pa/s. In contrast, samples V (3.23% GS extract), VI (3.85% GS extract), and VII (6.25% GS extract) exhibit more pronounced thixotropic behaviour, as indicated by their significantly larger hysteresis area values of 8163, 10931, and 17487 Pa/s, respectively. This suggests an enhanced capacity for structural recovery. Remarkably, the control sample demonstrates the highest capacity for structural recovery, with a hysteresis area of 62121 Pa/s.

Similarly to this study, in which the thixotropy increased in more concentrated samples, Yarovaya et al. [52] investigated the thixotropy of samples containing 3% grape seed extract and observed an increase in thixotropy (from 1868 ± 32 to 3244 ± 78 Pa/s) with the addition of the extract to the sample. Pinto et al. [53] also examined the thixotropic behaviour of formulations containing *Castanea sativa* shells extract. They observed a 15% increase in thixotropy compared to the control sample.

During stability study, the rheograms provide compelling evidence of the evolving thixotropic behaviour of the samples. Both the control sample and sample I (0.33% GS extract) exhibit a loss of thixotropic capacity, as indicated by the nearly overlapping upward and downward curves. Conversely, the remaining samples (Samples II, III, IV, V, VI, and VII) demonstrate an increase in hysteresis area in the rheograms, suggesting an enhancement in their thixotropic behaviour.

This enhancement is particularly pronounced in samples III (from 2507 to 10673 Pa/s), V (from 8163 to 62725 Pa/s), VI (from 10931 to 90501 Pa/s), and VII (from 17487 to 64352 Pa/s). It enables these samples to return to their initial structure after stress application, highlighting their time-dependent behaviour.

Moraes et al. [54] examined the stability of formulations containing 0.4% rutin succinate. They evaluated thixotropy after storage in accelerated conditions for 90 days and noted a 38.32% increase in the hysteresis area, a finding consistent with our study. Specifically, the thixotropy of sample II (0.83% GS extract) increased by 46%.

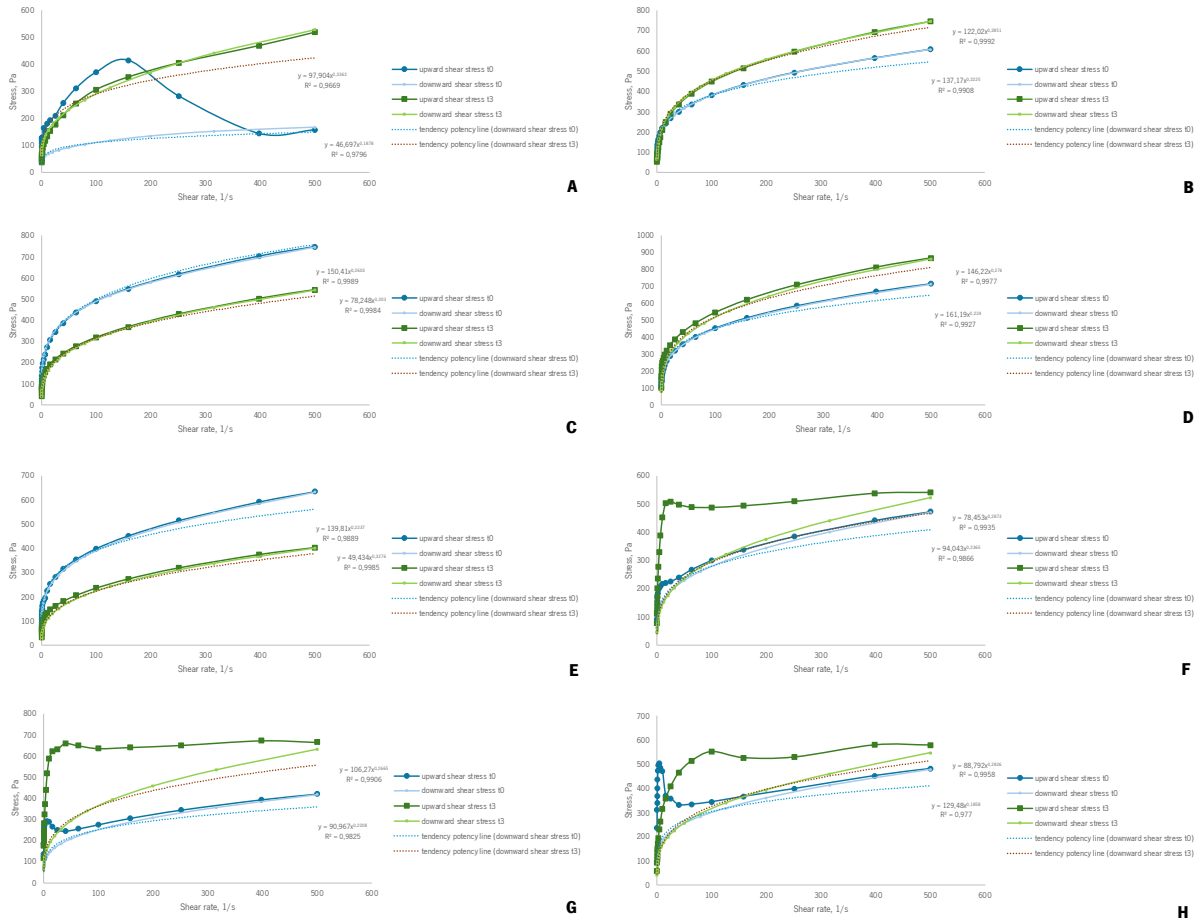


Figure 30. Rheograms of developed samples before (time 0) and at the end (time 3) of storage under accelerated conditions. (**A**). Sample CRT (0% GS extract). (**B**). Sample I (0.33% GS extract). (**C**). Sample II (0.83% GS extract). (**D**). Sample III (0.99% GS extract). (**E**). Sample IV (1.64% GS extract). (**F**). Sample V (3.23% GS extract). (**G**). Sample VI (3.85% GS extract). (**H**). Sample VII (6.25% GS extract).

Viscosity

Transitioning to another relevant aspect, viscosity emerges as another critical parameter in rheological analysis. Higher viscosity indicates greater resistance to change in shape, providing insights into the resistance of fluids to flow, which is crucial for understanding cosmetic formulation behaviour. The viscosity values of the developed samples are outlined in Figure 31, corresponding to a shear rate of 500 s^{-1}. This shear rate is selected as it is within the range typically encountered during the application of cosmetics onto the skin (100 – 10000 s^{-1}), representing the highest shear rate employed in the experiment [55].

As evident from the analysis of Figure 31, the addition of GS extract to the control sample resulted in an overall increase in viscosity. However, the viscosity increased until the concentration of 0.83% GS extract

was reached (1.49532 Pa.s), upon which a decreased with additional increases in GS extract concentration was observed.

Similar viscosity changes were noted by Nunes et al. [56] when incorporating 5% extracts from olive oil industry by-products (also rich in phenolic compounds) into the control formulation. These modifications may arise from complex interactions between the extract components and the emulsion matrix, potential dilution effects, or structural adjustments within the cosmetic product.

Additionally, Yarovaya et al. [52] observed an increase in viscosity (at 200 s^{-1}) of the formulation from 0.44 to 0.66 when 3% of grape seed extract was added to it, mirroring the findings of this study. García-Villegas et al. [57] also observed that viscosity increased in line with the concentration of Mangifera indica L. peel extract in their formulations.

The viscosity values also experienced changes from time 0 (before storage) to time 3 (after 3 months under accelerated conditions). As depicted in Figure 31, the viscosity of the developed samples generally increased under accelerated conditions, except for samples II (0.83% GS extract) and IV (1.64% GS extract), where a decrease in viscosity was observed. Among the samples, the one with the least difference in viscosity values between time 0 and 3 was sample V (3.23% GS extract), which showed a modest increase of 14%. Following closely were samples I, III, and VII, with viscosity increases ranging between 20% and 23%. In contrast, sample IV exhibited more significant differences, with viscosity decreases of 58%.

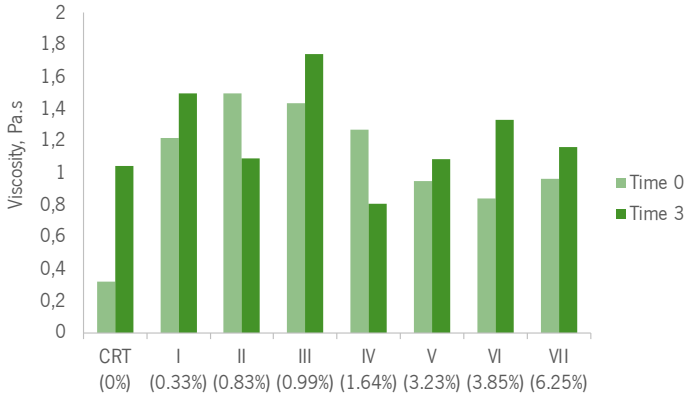


Figure 31. Variation of viscosity (at a shear stress of 500 s^{-1}).

The change in viscosity over the storage period under accelerated conditions does not exhibit a consistent trend. Generally, there was an increase in viscosity from the initial time (before storage) to the final time

(after 3 months of storage), except for samples II and IV. However, this increase does not show a clear pattern; it is not correlated with the concentration of extract in the samples.

The lack of a discernible trend in viscosity variation could be attributed to the presence of visible lumps in the samples after storage. As previously mentioned, a common physical destabilization phenomenon in such products is coalescence, where smaller particles combine to form larger ones. Visual observation and rheological analysis data suggest that this phenomenon likely occurred in our samples.

Authors have extensively explored the stability of formulations concerning their rheological properties, particularly viscosity. The results of these investigations have been varied and diverse. García-Villegas et al. [57] noted a decrease in viscosity in formulations containing various concentrations of Mangifera indica L. peel extract (0.5%, 1%, 2%), with the maximum variation being 1.9 Pa.s. In our study, the maximum variation was 0.72 Pa.s, occurring in the control sample without GS extract. In their study, Anggraini et al. [58] observed a decrease in viscosity of 0.66%, 1.33%, and 0.89% in formulations containing 0%, 5%, and 10% of macassar kernels (Rhus javanica) stem extract, respectively, after storage under accelerated conditions for 12 weeks. Similarly, Mahmood et al. [59] reached similar conclusions. Their formulation, which contained 5% green tea extract, experienced a decrease in viscosity from 18.01 cP to 2.62 cP after being stored under accelerated conditions for 30 days. They proposed two potential explanations for this phenomenon: either the migration of water molecules from the internal to the external aqueous phase or the rupture of multiple globules due to osmotic pressure.

Contrary to these findings, Moraes et al. [54] explored the changes in viscosity of formulations containing rutin succinate. After storing the samples for 90 days under accelerated conditions, they observed a 7.89% increase in viscosity.

Firmness and Adhesiveness

The increase in viscosity observed with the addition of GS extract to the control sample (time 0) aligns with the understanding that textural properties like firmness and adhesiveness in cosmetic formulations are influenced by various factors, such formulation's composition. These properties are crucial for evaluating the performance and user experience of cosmetic products.

Firmness refers to the resistance of a formulation to deformation under applied force, indicating its solidity and structural integrity. Adhesiveness measures the product's ability to adhere to the skin or other surfaces upon application, ensuring effective adherence and longevity.

Firmness impacts the spreadability and manipulability of creams and lotions, whereas adhesiveness is pivotal for prolonged adherence and efficacy of a cosmetic product, preserving its intended appearance

or functionality over an extended duration post-application. [60]. In addition, these properties help define the type of primary packaging for the formulation (such as a tube or jar) by determining the mechanical stability of the formulation.

Texture properties were measured to understand the impact of grape stem extract on the firmness and adhesiveness of cosmetic formulations. Figure 32 displays texturometer curves of samples with different concentrations of GS extract: CRT (0%), II (0.83%), III (0.99%), IV (1.64%), and VI (3.85%). By analysing the data of the curve, it is possible to determine the firmness corresponding to the highest positive peak and the adhesiveness corresponding to the area under the negative curve, respectively.

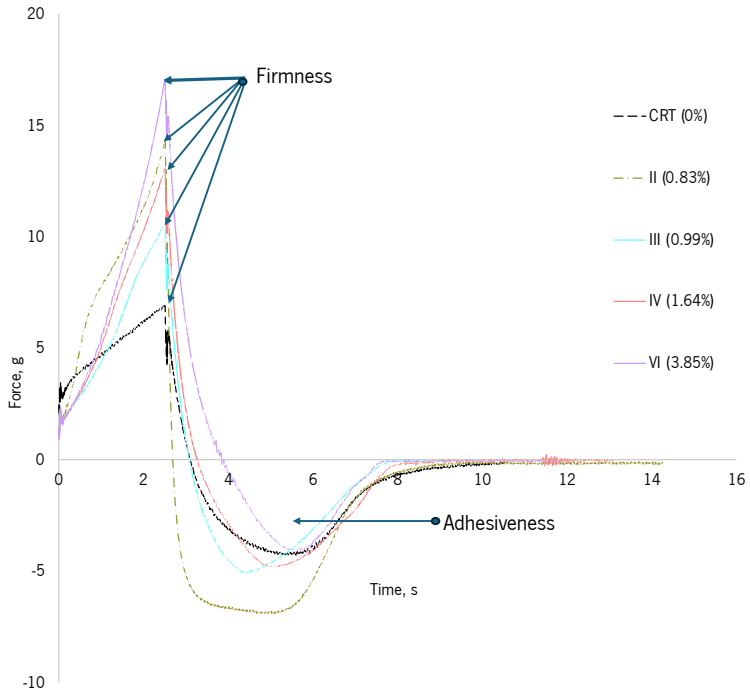


Figure 32. Texturometer Curves of samples CRT (0%), II (0.83%), III (0.99%), IV (1.64%), and VI (3.85% GS extract).

Firmness is closely linked to the hardness of a formulation, an essential attribute in cosmetic products due to their non-liquid nature, which naturally resists to an applied force. However, excessive firmness can impede the spreadability of the product on the skin. Studies by Tai et al. [61] and Yaovaya et al. [52] clarify the firmness values of various commercial cosmetic products, ranging from 11.20 g to 15.90 g for shampoos, hair conditioner, and sunscreen. Additionally, Huynh et al. [62] explored firmness values in different emulsions, reporting a wide range between 4.2 g and 40.5 g. Furthermore, McMullen et al. [60] analysed the texture properties of commercial cosmetic creams and lotions, revealing firmness values spanning from 9.10 g to 87.85 g.

As shown in Figure 33, it is evident that the addition of GS extract to the cream base generally increased the firmness of the formulations. Samples I (0.33% GS extract) and III (0.99% GS extract) showed firmness levels (10.60 g and 10.63 g, respectively) similar to CRT (6.95 g), whereas sample VI displayed the highest firmness value at 17.13 g.

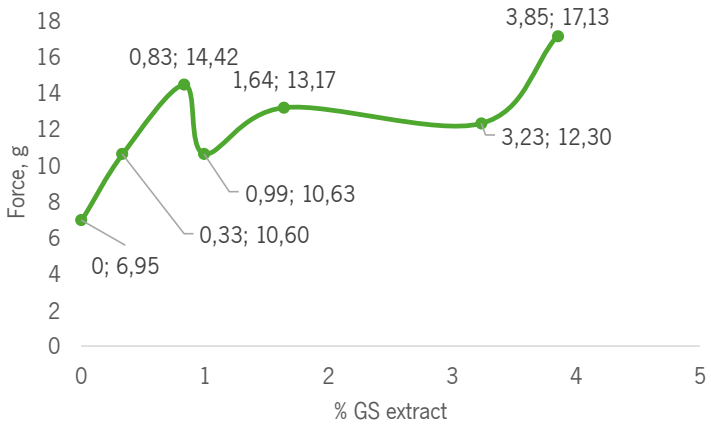


Figure 33. Evaluation of texture property: Firmness.

The general increase in firmness values in the developed samples can be attributed to the incorporation of GS extract, which consists of solid components, into the formulation. This addition can contribute to the structural integrity of the formulation, resulting in increased firmness. However, the relationship between extract concentration and firmness does not appear to follow a linear trend. This inconsistency could be explained by the disruption of balance between the GS extract concentration and other ingredients in the formulation.

The results obtained in this study seem to contradict findings from another research. For instance, Yarovaya et al. [52] investigated the addition of 3% grape seed extract to a sunscreen formulation and found no significant differences in firmness. Additionally, Pinto et al. [63] observed a decrease in firmness values (from 0.554 to 0.288 N) with an increase in the concentration of natural extracts rich in phenolic compounds in their samples.

Regarding adhesiveness, it refers to the ability of the formulation to adhere to the skin. The formulation needs to adhere adequately to the skin to achieve the desired effect. However, excessively low or high adhesiveness can lead to undesirable outcomes. A formulation with low adhesiveness may not adhere well to the skin, affecting its efficacy, while a formulation with high adhesiveness may result in a sticky and tacky feeling, impeding the spreadability of the product on the skin.

Huynh et al. [62] explored adhesiveness values across various emulsions, reporting a range of -6.00 to -23.6 g.s. Similarly, Yarovaya et al. [52] assessed adhesiveness in commercial sunscreen products, with values averaging -22.5 g.s.

In Figure 34, it is evident that there is no clear correlation between the adhesiveness and the concentration of GS extract incorporated in the samples from 0.83% GS extract (sample II). Initially, as the GS extract concentration in the formulation increases to 0.83%, adhesiveness rises from -6.13 g.s to -13.59 g.s. However, thereafter, adhesiveness values decrease to -4.49 g.s (0.99% GS extract, sample III) before a slight increase to -6.63 g.s (3.23% GS extract, sample V).

Samples III (-4.34 g.s), IV (-5.14 g.s), and V (-6.63 g.s) demonstrate adhesiveness values closest to the control sample (-6.13 g.s). In contrast, samples I (0.33% GS extract) and II (0.83% GS extract) display significantly different adhesiveness values, measuring -11.05 g.s and -13.59 g.s, respectively.

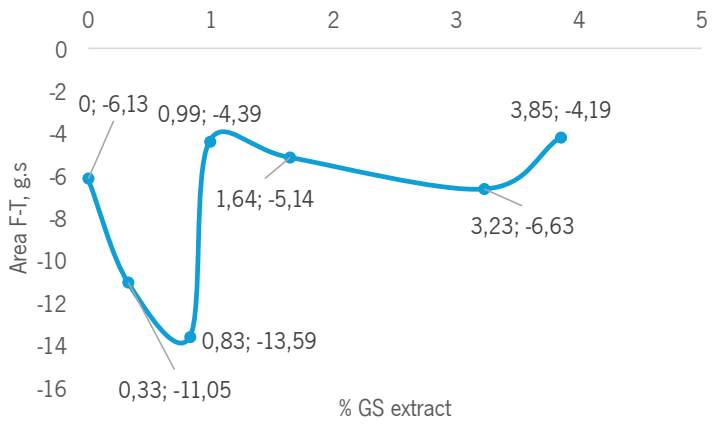


Figure 34. Evaluation of texture property: Adhesiveness.

Interestingly, Pinto et al. [63] verified a decrease in adhesiveness values (from -1.48 to -0.707 N.mm) when the concentration of natural extracts rich in phenolic compounds increased in their samples. Additionally, Yarovaya et al. [52] also observed a marginal decrease in adhesiveness from -13.2 to -12.1 g.s upon incorporating 3% grape seed extract into the formulation. Remarkably, in our study, sample V (3.23% GS extract) displayed adhesiveness values (-6.63 g.s) similar to those of the control sample (-6.13 g.s). They attribute this change to the astringent properties of polyphenols in grape seed extracts. The astringent properties of polyphenols in GSE result in the removal of excess oil and create a sensation of tightness on the skin, contrasting with the sticky and smooth sensation.

The textural properties of the samples were analysed only at the initial time to achieve a comprehensive characterization. However, according to the research by Jimtaisong et al. [64], formulation texture varies when viscosity changes by 30% or more. Therefore, among the samples, IV and VI likely experienced texture variation, with viscosity changes of 37% and 58%, respectively.

In conclusion, comprehensive analysis reveals a discernible pattern in the behaviour of cosmetic formulations containing GS extract (time 0). Up to approximately 1% GS extract concentration, the parameters consistently exhibit an upward trend, including viscosity, consistency index, firmness, adhesiveness, and flow behaviour index. However, beyond this critical percentage, the correlation between parameter variations and GS extract concentration diminishes, indicating a disruption in formulation balance likely due to increased dilution of the thickening agent.

Furthermore, the ease of spreadability and thixotropic behaviour directly influence viscosity reduction during application onto the skin.

Consequently, samples with higher concentrations of GS extract, notably those with 3.85% and 6.25%, tend to exhibit greater spreadability. However, this heightened spreadability may lead to product runoff and decreased adhesion to the skin, potentially impacting the absorption of bioactive compounds. Although adhesiveness was not directly assessed in the more concentrated samples. The sample containing 3.85% GS extract demonstrated the lowest adhesiveness. This suggests a trade-off between spreadability and adhesion.

Intriguingly, samples demonstrating greater adhesion to the skin, such as the one with a value of -13.59 g.s for adhesiveness, exhibited higher flow behaviour index values (0.2603) and lower thixotropic behaviour (1656 Pa/s), particularly the sample containing 0.83% GS extract. Consequently, formulations with increased skin adhesion may face challenges in spreading.

Overall, while certain formulations may exhibit superior spreadability or adhesion, all samples meet acceptable rheological and textural standards for cosmetic formulations, as outlined in the literature.

Additionally, when correlating and analysing all rheological data collectively after stability study (time 3), it becomes evident that sample IV (containing 1.64% GS extract) exhibited the highest level of instability. It was one of the samples that displayed the most significant variations (ranging from 37% to 65%) across all parameters, except thixotropy. In contrast, sample III (containing 0.99% GS extract) demonstrated the greatest stability, although with observed variations in all parameters, despite being less pronounced

compared to other samples. Specifically, the consistency index decreased by 9%, while the flow behaviour index and viscosity increased by 23% and 21%, respectively.

3.2.3.1.4. Quantification of phenolic compounds and long-term assessment

Quantification of total phenolic compounds (TPC) and flavonoid (FC) content was conducted after incorporating GS extract into the samples. Given that GS extract is rich in phenolic compounds, with flavonoids being one of its subclasses, the assessment of total phenolic and flavonoid content is crucial for characterizing the samples. The results of this evaluation are presented in Table 18.

In a previous chapter, the GS extract was characterized concerning its total phenolic and flavonoid contents. The analysis revealed values of 78.55 ± 1.67 mg GAE/g dw (dry weight) extract for TPC and 66.77 ± 1.96 mg CAT/g dw extract for FC. When comparing these values with those of sample III (0.99% GS extract), which contains approximately 1% GS extract, a decrease of approximately 52% in TPC is evident when the extract is diluted in a cream base. However, regarding FC, flavonoids were able to maintain their content when incorporated into a cream base.

This observation indicates a decrease in the total phenolic content when incorporating GS extract into a cream base, presumably due to the insolubility of phenolic compounds in the formulation. However, it appears that these insoluble phenolic compounds are not part of the flavonoid subclass, as evidenced by the relatively unchanged FC values in the formulation compared to the extract. This suggests that flavonoids, in general, are soluble within the formulation.

Censi et al. [65] also observed a 28% decrease in total phenolic content when comparing the TPC values of açai extract with those of an emulsion containing 1% açai extract. Namngam et al. [66] also noted a significant decrease in total phenolic content (TPC) in the formulation when 1% natural extract was incorporated, as compared to the TPC in the extract itself.

Regarding the variation of TPC and FC in formulations, the observed increase in both total phenolic compounds (from 9.44 ± 0.79 to 232.65 ± 14.11 mg GAE/100 g cream) and flavonoids (from 44.04 ± 4.07 to 274.60 ± 6.68 mg CAT/100 g cream) with the addition of GS extract (0–6.25%) to the control sample cream aligns with expectations.

Similar results were reported by Censi et al. [65] and Namngam et al. [66], who both noted an increase in total phenolic content when incorporating natural extracts into emulsions at higher concentrations. In

Censi et al.'s study, the addition of açai extract (0–2%) to a cream base control increased TPC (from 2.3 ± 0.2 to 29.2 ± 0.7 µg GAE/g), and a similar effect was observed by Namngam et al. when incorporating mango seed kernel extracts (0–3%), resulting in an increase in TPC (from 0 to 4.92 ± 0.12 mg GAE/ml). However, the increase in phenolic content (TPC and FC) is not proportional to the increase in GS extract concentration in samples. The rise in phenolic compounds (34.30 ± 2.99 mg GAE/100 g cream) is only noticeable for the sample with 0.83% GS extract, while a significant increase in flavonoids content (130.93 ± 14.06 mg CAT/100 g cream) becomes notable from the sample with 3.23% GS extract. The disproportionate increase in phenolic compounds and flavonoids with varying concentrations of GS extract could be attributed to the complex composition of the extract. Different phenolic compounds, including various subclasses like flavonoids, may have distinct solubilities, stabilities, or extraction efficiencies [67–69].

The total phenolic and flavonoid contents of the developed samples were monitored over time. As shown in Table 18, a significant decrease in total phenolic content was observed in most of the samples after just one month of storage under accelerated conditions. However, sample IV maintained its total phenolic content (44.40 mg GAE/100 g of cream) until the end of the first month (43.54 mg GAE/100 g of cream), demonstrating the greatest stability along with sample III. These formulations experienced only a 16% decrease in TPC over the 3-month storage period. In comparison, the sample with 3.85% GS extract (sample VI) showed a 20% decrease, while the sample with 0.33% GS extract (sample I) showed a 29% decrease.

From one month to three months, there was no significant decrease in most of the samples, except for the most concentrated sample VII (6.25% GS extract), which showed a significant variation of 44%, matched by sample II, which showed a variation of 47%.

The results for flavonoid content are also presented in Table 18. It is noticeable that less concentrated samples can retain more flavonoids in their composition, making them more stable. Certain samples, namely samples I, III, and V, maintained their flavonoid content consistently throughout the accelerated storage period, with fluctuations in values being statistically insignificant between time 0 and time 3. Conversely, sample IV retained its flavonoid content only until the first month in the stability chamber, experiencing an 18% decrease in FC from the initial time to the final time of the study.

In contract, the most concentrated sample was unable to retain its flavonoid content, experiencing a 53% decrease.

Table 18. Assessment of total phenolic and flavonoid content over time.

	Total Phenolics (mg GAE/100 g cream)			Flavonoids (mg CAT/100 g cream)		
	months in a stability chamber			months in a stability chamber		
Sample (% GS extract)	**0**	**1**	**3**	**0**	**1**	**3**
GS extract	78.55 ± 1.67*	-	-	66.77 ± 1.96**	-	-
CRT (0%)	9.44 ± 0.79 ^{aE}	5.72 ± 0.40 ^{b}	5.39 ± 0.52 ^{bF}	44.04 ± 4.07 ^{aD}	50.06 ± 3.22 ^{a}	24.65 ± 1.41 ^{bD}
I (0.33%)	16.35 ± 1.91 ^{aE}	11.04 ± 1.22 ^{b}	11.58 ± 0.18 ^{bEF}	45.10 ± 3.71 ^{aD}	53.58 ± 5.20 ^{a}	47.36 ± 2.41 ^{aC}
II (0.83%)	34.30 ± 2.99 ^{aD}	15.96 ± 1.01 ^{b}	18.17 ± 0.12 ^{bE}	62.88 ± 0.71 ^{aD}	56.91 ± 2.82 ^{b}	46.58 ± 0.98 ^{cC}
III (0.99%)	37.50 ± 3.39 ^{aD}	29.51 ± 1.15 ^{b}	31.65 ± 0.33 ^{bD}	66.38 ± 3.32 ^{aD}	63.37 ± 4.34 ^{a}	61.08 ± 1.17 ^{aC}
IV (1.64%)	44.40 ± 0.97 ^{aD}	43.54 ± 2.71 ^{ab}	37.42 ± 3.80 ^{bD}	64.94 ± 3.89 ^{aD}	67.76 ± 2.50 ^{a}	53.34 ± 1.97 ^{bC}
V (3.23%)	102.38 ± 3.92 ^{aC}	64.85 ± 1.88 ^{b}	65.45 ± 4.84 ^{bC}	130.93 ± 14.06 ^{aC}	117.05 ± 4.21 ^{a}	107.78 ± 12.89 ^{aB}
VI (3.85%)	127.70 ± 6.29 ^{aB}	97.95 ± 1.21 ^{b}	101.70 ± 3.22 ^{bB}	162.48 ± 17.32 ^{aB}	102.87 ± 2.70 ^{b}	117.36 ± 13.91 ^{bAB}
VII (6.25%)	232.65 ± 14.11 ^{aA}	175.75 ± 3.1 ^{b}	129.37 ± 2.56 ^{cA}	274.60 ± 6.68 ^{aA}	211.09 ± 13.45 ^{b}	128.91 ± 5.08 ^{cA}

Results are presented as mean ± standard deviation (n=3). *Results are presented as mean ± standard deviation (n=3) and are expressed in mg GAE/g dw (dry weight). **Results are presented as mean ± standard deviation (n=3) and are expressed in mg CAT/g dw.
Significant differences between samples in the same row are denoted by different lowercase letters, while significant differences between samples in the same column are indicated by different capital letters ($p < 0.05$).

The decrease in phenolic and flavonoid contents over time can be attributed to chemical reactions occurring within the formulation. Oxidation, hydrolysis, and transesterification processes may lead to the degradation of these compounds, resulting in a reduction in their concentrations. Additionally, the formation of new chemical species as a result of these reactions may contribute to the observed decrease, as these new compounds may not be detected by the analytical methods used to quantify total phenolic and flavonoid contents.

The findings presented are supported by studies conducted by Salem et al. [36], where a decrease in total phenolic content (TPC) was observed in creams containing grape seed extract at various concentrations (0–5%) for four months. Significant differences were noticeable as early as the first month at 50°C, with the most pronounced decrease observed in the sample containing 5% grape seed extract. Additionally, Rodrigues et al. [33] also reported a decrease (from 4192.95 ± 50.25 to 2918.71 ± 90.42 µg GAE/mL) in TPC in creams containing natural extracts at the end of 180 days under accelerated conditions. Similar findings were reported by Maisuthisakul et al. [70], indicating a decrease in total phenolic content (TPC) in natural extract-based formulations over time under accelerated storage conditions. Esparza et al. [71] also studied the phenolic content stability in grape stem extracts under accelerated conditions for 6 months, observing a decrease in TPC from 0.50 to 0.33 mmol GAE/g extract.

3.2.3.1.5. Extended Evaluation of Antioxidant, Antiaging, and Depigmenting Activities

The evaluation of various biological activities, including antioxidant, antiaging, and depigmenting activities, was conducted to explore the specific cosmetic applications of the developed samples.

Antioxidant Activity

The antioxidant activity of samples was evaluated through ABTS and FRAP assays, which are widely recognized methods for assessing the ability of compounds to scavenge free radicals and reduce ferric ions, respectively. Phenolic compounds are widely acknowledged for their antioxidant properties, as documented in various studies [72–75].

In previous chapter, the antioxidant activity of GS extract was evaluated, showing values of 1.035 ± 0.04 mmol TROLOX/g dw extract (ABTS) and 0.689 ± 0.02 mmol TROLOX/g dw extract (FRAP). Comparing these values with those of sample III, containing approximately 1% GS extract, reveals a decrease of approximately 50% (ABTS assay) and 25% (FRAP assay) in antioxidant activity when the extract is incorporated into the formulation.

These findings are consistent with the research conducted by Censi et al. [65], where the antioxidant activity decreased by approximately 65% (ABTS) and 60% (FRAP) when transitioning from açai extract to a formulation containing 1% extract.

This alteration in antioxidant activity could be attributed to the presence of insoluble and/or unstable phenolic compounds within the formulation. It appears that phenolic compounds possessing ferric ion-reducing capability exhibit greater solubility and/or stability within the formulation relative to those with free radical-reducing properties.

Consequently, the incorporation of GS extract, rich in phenolic compounds, into the samples is expected to reveal antioxidant properties. The results in Table 19 confirm this expectation, revealing a consistent increase in antioxidant activity with the escalating concentration of GS extract (ranging from 0 to 6.25%) for both the ABTS (from 0.01 ± 0 to 4.14 ± 0.33 mmol TROLOX/100 g cream) and FRAP methods (from 0.02 ± 0 to 3.10 ± 0.02 mmol TROLOX/100 g cream).

Intriguingly, similar to the quantification of total phenolics and flavonoids, the escalation in antioxidant activity with GS concentration does not follow a proportional pattern. Notably, a significant increase is observed only from 0.83% GS extract in the ABTS method (0.49 ± 0.01 mmol TROLOX/100 g cream). Conversely, in the FRAP method, the antioxidant activity exhibits a notable increase, starting as early as the 0.33% GS extract concentration (from 0.02 ± 0 to 0.22 ± 0.02 mmol TROLOX/100 g cream).

This disparity between methods may be attributed to the distinct mechanisms assessed by the two methods. Consequently, certain phenolic compounds might demonstrate the capacity to reduce free radicals (evaluated by ABTS), while others may be adept at reducing ions (evaluated by FRAP).

The non-proportional increase in antioxidant activity with the concentration of GS extract containing phenolic compounds could be attributed to several factors. Phenolic compounds encompass a diverse range, including various subclasses such as flavonoids and phenolic acids, each with distinct antioxidant properties [76,77]. Additionally, the solubility, stability, and extraction efficiency of different phenolic compounds may vary, influencing their contribution to overall antioxidant activity.

This study is supported by Censi et al. [65], who obtained similar results. They observed that, as the concentration of açai extract increased (from 0 to 2%) in the cream samples, the antioxidant activity also increased in most samples (from 1.2 ± 0.1 to 60.7 ± 1.5 µmol TROLOX/g), although not in proportion. The antioxidant potential measured by the FRAP assay for samples 0.5% and 1% was 52.8 ± 1.2 and 55.2 ± 1.2 µmol TROLOX/g, respectively. Similarly, Bernatoniene et al. [78] noted a non-proportional increase in antioxidant potential when incorporating calendula extract into the cream base. At a concentration of 0.3%, there was no significant increase in antioxidant activity compared to the cream base, with a significant increase observed only between concentrations of

0.6% (102 ± 7 µmol quercetin equivalents/100 mL) and 0.9% (163 ± 9 µmol quercetin equivalents/100 mL). They measured the antioxidant activity using the DPPH method.

The antioxidant properties of the developed samples were monitored during the accelerated stability assessment. Table 19 presents the results of the accelerated stability evaluation using the ABTS and FRAP methods.

It is evident that all samples experienced a decline in antioxidant capacities against free radicals. However, the samples III and IV stood out by maintaining its antioxidant properties until the end of the first month of storage, demonstrating the most stability with a 58% and 61% decrease in antioxidant properties. Inversely, the less concentrated samples II and III (0.33% and 0.83% GS extract) exhibited greater instability, experiencing an 80% decrease in antioxidant activity.

The antioxidant properties, evaluated through the reduction of ferric ions using the FRAP assay, exhibited a decline in most developed samples during the accelerated stability evaluation. The pattern observed in the results from the FRAP assay mirrors that of the ABTS assay.

The samples III and IV demonstrated the highest stability in retaining its ability to reduce ferric ions species, with a decrease of 30% and 32%, respectively. Following this, samples containing 3.23% and 6.25% GS extract experienced reductions of 39% and 40%, respectively. In contrast, the sample containing 0.33% GS extract showed the least stability in maintaining its antioxidant capacity.

Table 19. Evaluation of antioxidant activity by ABTS and FRAP assays.

	ABTS			FRAP		
	months in a stability chamber			months in a stability chamber		
Sample (% GS extract)	**0**	**1**	**3**	**0**	**1**	**3**
GS extract	1.035 ± 0.04*	-	-	0.689 ± 0.02*	-	-
CRT (0%)	0.01 ± 0 ^{bE}	0.03 ± 0 ^{a}	0 ± 0 ^{cE}	0.02 ± 0 ^{bF}	0.05 ± 0.01 ^{a}	0.02 ± 0 ^{bF}
I (0.33%)	0.17 ± 0.02 ^{aDE}	0.13 ± 0.01 ^{b}	0.03 ± 0 ^{cDE}	0.22 ± 0.02 ^{aE}	0.24 ± 0.02 ^{a}	0.06 ± 0 ^{bF}
II (0.83%)	0.49 ± 0.01 ^{aCD}	0.41 ± 0.03 ^{b}	0.10 ± 0 ^{cD}	0.55 ± 0.05 ^{aD}	0.46 ± 0.01 ^{b}	0.20 ± 0 ^{cE}
III (0.99%)	0.50 ± 0.05 ^{aCD}	0.51 ± 0.05 ^{a}	0.21 ± 0.01 ^{bC}	0.52 ± 0.03 ^{aD}	0.43 ± 0.02 ^{b}	0.36 ± 0.01 ^{cD}
IV (1.64%)	0.66 ± 0.05 ^{aC}	0.79 ± 0.07 ^{a}	0.26 ± 0.02 ^{bC}	0.71 ± 0.03 ^{aC}	0.62 ± 0.02 ^{b}	0.48 ± 0.02 ^{cC}
V (3.23%)	1.83 ± 0.15 ^{aB}	1.46 ± 0.02 ^{b}	0.72 ± 0.02 ^{cB}	1.57 ± 0.09 ^{aB}	1.32 ± 0.05 ^{b}	0.96 ± 0.04 ^{cB}
VII (6.25%)	4.14 ± 0.33 ^{aA}	3.51 ± 0.11 ^{b}	1.50 ± 0.05 ^{cA}	3.10 ± 0.02 ^{aA}	2.56 ± 0.04 ^{b}	1.86 ± 0.02 ^{cA}

Results are presented as mean ± standard deviation (n=3) and are expressed in mmol TROLOX/100 g cream. *Results are presented as mean ± standard deviation (n=3) and are expressed in mmol TROLOX/g dw.
Significant differences between samples in the same row are denoted by different lowercase letters, while significant differences between samples in the same column are indicated by different capital letters ($p < 0.05$).

Similar findings were reported by Salem et al. [36], who observed a decrease in antioxidant activity following a decline in total phenolic content (TPC) in samples stored at 50°C over four months. Their samples with 5% grape seed extract from the Marselan variety, had a decrease of 40% in antioxidant activity (evaluated by DPPH). Esparza et al. [71] studied the variation of antioxidant activity by DPPH assay in grape stem extracts and observed a decrease from 0.45 to 0.29 mmol TROLOX/g extract (36%) at the end of 6 months under accelerated conditions.

In conclusion, adding grape stem extract to cosmetic samples markedly increased their antioxidant activity. These results, in line with similar studies, underscore the role of phenolic compounds in optimizing cosmetic formulations and enhancing their antioxidant properties.

Antioxidants are essential in combating oxidative stress, a significant contributor to skin aging. ROS accumulation leads to cellular damage and senescence, driving skin aging processes [79,80]. Phenolic compounds, well-known for their antioxidant properties, confer antiaging benefits by neutralizing free radicals and preventing oxidative damage. Formulations enriched with antioxidants like GS extract are expected to exhibit antiaging effects. Therefore, assessing antioxidant activity provides valuable insights into the potential antiaging efficacy of such cosmetic formulations.

Furthermore, the stability study reveals a decline in the antioxidant capacity of the samples, both in scavenging free radicals and reducing ferric ions. This decline correlates with the reduction in phenolic content observed over time, as discussed earlier. Consequently, as the samples exhibit lower levels of phenolic compounds, their antioxidant activity, primarily attributed to these compounds, diminishes accordingly. Additionally, interactions between phenolic compounds and other components of the formulation, such as emulsifiers or preservatives, may also affect their stability and antioxidant efficacy. These factors collectively contribute to the observed decline in antioxidant activity of the samples over time.

Antiaging and Depigmenting Activities

The antiaging property of cosmetic formulations was assessed by evaluating the inhibition of elastase. Elastase, also known as human macrophage metalloelastase, is overexpressed in ageing and photoaging, leading to the degradation of elastin. Elastin, a protein, imparts elasticity to various human tissues and organs, including the skin, lungs, and blood vessels. The degradation or lower production of elastin is associated with the aging process, resulting in the appearance of wrinkles and sagging skin. Therefore, inhibiting elastase is a key principle in reducing the signs of skin aging, such as wrinkles and sagging skin [81,82].

Regarding the depigmenting property, phenolic compounds have also demonstrated the capacity to inhibit the tyrosinase enzyme [83,84]. Tyrosinase is responsible for converting tyrosine into L-DOPA, which is involved in melanogenesis, leading to skin pigmentation. In skin aging, the level of tyrosinase increases, which can result in dark spots on the skin. Thus, inhibiting tyrosinase is a crucial factor in preventing or reducing skin pigmentation [85,86].

In previous work chapter, the GS extract showed an elastase inhibition of 37.78 ± 2.26%. This inhibition increased when the GS extract was incorporated into a formulation, resulting in a range of 40.05 ± 1.67% to 52.83 ± 2.50% (Table 20). This increase may be due to interactions with the ingredients of the formulation, as the CRT sample (0% GS extract) exhibited 22.98 ± 9.13% inhibition. It appears that the inhibition is not dose-dependent, since samples I (0.33% GS extract) and VII (6.25% GS extract) showed statistically similar elastase inhibition percentages: 53.22 ± 0.89% and 52.83 ± 2.50%, respectively.

The activity of GS extract in inhibiting the tyrosinase enzyme was also explored in previous chapter, showing 45.94 ± 0.63% tyrosinase inhibition. This activity decreased when the extract was incorporated into a formulation, as shown in Table 20. Only formulations with higher GS extract concentrations (3.23%, 3.85%, 6.25%) exhibited significant tyrosinase inhibition, specifically samples V, VI, and VII. The tyrosinase inhibition ranged between 11.64 ± 6.50% and 29.44 ± 4.02%, increasing with higher GS extract concentrations.

Some studies support these findings. In the investigation by Hong et al. [87], a formulation containing 5% green tea extract was found to inhibit elastase by 24.79%. In this context, GS extract appears to be a more effective elastase inhibitor when incorporated into a formulation. On the other hand, the same green tea extract formulation was more effective in inhibiting tyrosinase than the GS extract formulations, presenting 35.38% inhibition.

Moreover, Anggraini et al. [58] verified that the stem extract of macassar kernels (*Rhus javanica*) exhibited significant elastase inhibition. When incorporated into a formulation at a concentration of 10%, it increased the skin elasticity levels of 29 volunteers. Similarly, our findings demonstrate that the GS extract, when incorporated into formulations, enhances elastase inhibition, achieving a range between 40.05 ± 1.67% and 52.83 ± 2.50%. This suggests that GS extract may be a good option for improving skin elasticity.

Additionally, Taofiq et al. [88] explored the inhibition of tyrosinase by different extracts (*Agaricus bisporus*, *Pleurotus ostreatus*, and *Lentinula edodes*) and by formulations containing these extracts. They found that the ability to inhibit the enzyme decreased from extracts to formulations by between 92% and 95%. Similarly, in our study, the ability to inhibit the enzyme decreased from extract to formulations. However, the decrease in our study was less pronounced, ranging between 36% and 74%.

These properties of the samples were evaluated over time, revealing that less concentrated formulations exhibited greater stability in inhibiting elastase enzyme. Sample II (0.83% GS extract) maintained its ability to inhibit elastase until the third month under stability conditions, making it the most stable. This was

followed by samples I and IV (0.33% and 1.64% GS extract, respectively), which showed a decrease in their elastase inhibition by 5% and 20%.

In contrast, the most concentrated samples (V, VI, VII) were the least stable in retaining their ability to inhibit elastase.

Regarding tyrosinase inhibition, only sample V maintained its inhibitory ability until the third month under accelerated conditions.

Table 20. Evaluation of elastase and tyrosinase inhibition.

	Elastase inhibition assay			Tyrosinase inhibition assay		
	months in a stability chamber			months in a stability chamber		
Sample (% GS extract)	**0**	**1**	**3**	**0**	**1**	**3**
GS extract	37.78 ± 2.26	-	-	45.94 ± 0.63	-	-
CRT (0%)	22.98 ± 9.13 ^{aC}	31.45 ± 3.41 ^{a}	19.40 ± 4.24 ^{aC}	0 * ^{CD}	-	-
I (0.33%)	53.22 ± 0.89 ^{bA}	56.96 ± 0.51 ^{a}	50.22 ± 0.45 ^{cA}	0 * ^{E}	-	-
II (0.83%)	52.19 ± 3.57 ^{aA}	53.62 ± 1.33 ^{a}	52.47 ± 1.62 ^{aA}	0 * ^{DE}	-	-
III (0.99%)	47.61 ± 5.40 ^{aAB}	44.33 ± 2.54 ^{a}	29.62 ± 2.11 ^{bB}	0 * ^{C}	-	-
IV (1.64%)	40.05 ± 1.67 ^{bB}	48.06 ± 2.67 ^{a}	32.06 ± 1.56 ^{cB}	0 * ^{CD}	-	-
V (3.23%)	5.40 ± 1.01 ^{aD}	4.72 ± 5.53 ^{a}	9.39 ± 1.19 ^{aD}	11.64 ± 6.50 ^{aB}	10.68 ± 1.90 ^{a}	10.04 ± 3.10 ^{aA}
VI (3.85%)	44.15 ± 1.31 ^{aAB}	0 * ^{c}	7.00 ± 7.11 ^{bD}	22.69 ± 9.25 ^{aAB}	13.99 ± 2.62 ^{a}	0 * ^{bB}
VII (6.25%)	52.83 ± 2.50 ^{aA}	43.90 ± 2.94 ^{b}	12.74 ± 2.74 ^{cCD}	29.44 ± 4.02 ^{aA}	21.36 ± 1.74 ^{a}	0 * ^{bB}

Results are presented as mean ± standard deviation (n=3) and are expressed in % inhibition.
Significant differences between samples in the same row are denoted by different lowercase letters, while significant differences between samples in the same column are indicated by different capital letters ($p < 0.05$).
* Not determined (below detection limit).

3.2.4. Conclusion

The study successfully incorporated GS extract into a cream formulation, leading to an increase in total phenolic and flavonoid content. However, a decrease of approximately 52% in total phenolic content was observed in the cosmetic formulations compared to the isolated GS extract. This suggests that not all phenolic compounds from the GS extract are soluble in the cream, with flavonoid compounds being the most soluble and maintaining their content within the formulations.

Despite the successful incorporation of GS extract into the cream, some physical and chemical changes were observed, such as alterations in colour, odour, pH, viscosity and spreadability.

However, these changes remained acceptable. Changes in colour (to browner tone) and odour (to woody) are characteristic of the raw material, as these are natural properties of the GS extract. Regarding pH, although the addition of GS extract caused changes, these were also acceptable as the formulations were adjusted to the skin's natural acidity (4.00–4.50).

In terms of rheological and textural properties, the addition of GS extract followed a noticeable pattern. The formulations became more viscous as GS extract was added, reaching a peak between 0.83% and 1.64%. Beyond these concentrations, the viscosity began to fluctuate, showing irregular increases and decreases unrelated to the amount of extract added. This pattern was evident in the consistency index (K), viscosity, and firmness; however, the formulations still maintained the expected structural characteristics of a cosmetic formulation.

Regarding spreadability, it decreased as the concentration of GS extract increased, particularly within the range of 0.83% to 1.64%, after which the pattern became irregular. This behaviour was reflected in the flow behaviour index (n), thixotropy, and adhesiveness. Nevertheless, all formulations remained within the acceptable range for cosmetic products. They maintained their shear-thinning behaviour, meaning they became more fluid when a force was applied to facilitate spreadability. Once the force was removed, the formulations returned to their original viscosity, allowing them to adhere to the skin and be absorbed effectively.

In terms of biological activities, the cream formulations gained beneficial properties from the GS extract, including antioxidant activity. However, the ability to reduce free radicals and ions decreased by 50% in the ABTS assay and 25% in the FRAP assay compared to the pure GS extract. This reduction is likely due to the poor solubility of some phenolic compounds in the cream.

The ability to inhibit the elastase enzyme was mostly preserved, as the cream base already had some inhibition (≈23%). However, the formulations did not fully retain the GS extract's ability to inhibit the

tyrosinase enzyme, except in higher concentrations. This suggests that the compounds responsible for this activity may not dissolve well in the cream.

Stability tests conducted over three months under controlled conditions (40 ± 2 °C and 75 ± 5% relative humidity) revealed significant changes in almost all properties of the cosmetic formulations. The most concentrated samples (starting from 3.23% GS extract) were generally the least stable, showing darker colours and becoming lumpier due to coalescence.

In terms of rheological properties, there were no clear trends in viscosity and consistency, likely due to the lumpiness that developed. However, the most stable formulations in terms of consistency index were samples II and IV, while samples III and IV were the most stable regarding viscosity. Notably, both thixotropy and the flow behaviour index increased, indicating that the formulations became more resistant to spreading but could return to their original thickness once the force was removed. Sample IV was particularly stable in terms of flow behaviour index.

Despite these changes, all formulations remained safe, with stable pH levels and microbial content consistently below the acceptable limit for cosmetics (≤ 100 CFU/g).

Regarding phenolic content, there was an overall decrease over time. Samples III and IV were the most effective at retaining total phenolic content, while samples I to IV were greatest at maintaining flavonoid content. For biological properties, samples II and III remained stable and continued to reduce free radicals, as measured by the ABTS assay. Samples III and IV retained their ability to reduce ions, and sample II continued to inhibit the elastase enzyme over time.

By analysing the parameters from all assays, it is concluded that the least stable samples were the most concentrated ones. Conversely, the most stable samples over time under accelerated conditions were those with lower concentrations of GS extract (samples II–IV), specifically between 0.83% and 1.64%.

In conclusion, incorporating phenolic compounds from grape stems into a cream formulation enhances its functional properties, such as antioxidant and anti-ageing benefits, while ensuring stability over time. This study supports the potential use of grape stem extract as valuable ingredients in creams, with promising commercial applications.

However, further studies are needed to optimize the formulation. It is essential to investigate a broader range of GS extract concentrations, particularly between 0.5% and 1.5%, to identify the formulation with the optimal stability and efficacy.

Testing encapsulation methods for the GS extract could also improve formulation stability and control the release of active compounds. Additionally, evaluating the incorporation of GS extract into various cream bases will help determine which base offers the best stability and bioactivity.

Moreover, conducting long-term *in vivo* studies is crucial to assess the effects of the GS extract-based cream on skin health. These studies should focus on parameters such as elasticity, hydration, and wrinkle reduction to provide a comprehensive understanding of the cream's benefits.

By focusing on these areas, future research can improve the formulation of GS extract cream, ensuring its effectiveness and supporting its successful commercialization.

3.2.5. References

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4. Conclusions

4.1. Final Conclusion

The cosmetic industry is increasingly recognizing the importance of sustainability and the principles of a circular economy. As consumers become more environmentally conscious, there is a growing demand for products that deliver effective results while minimizing ecological impact. This shift emphasizes the need for natural compounds derived from renewable resources, enhancing product efficacy while supporting environmental management. This research aligns with these trends by focusing on GS extract, a natural ingredient rich in bioactive compounds.

This study thoroughly examined the potential of blend GS extract as an ingredient in cosmetic formulations, highlighting its significant phenolic content and biological activities. The extract showed high levels of compounds such as catechin, procyanidin trimers, rutin, quercetin, and resveratrol tetramers, with proanthocyanidin trimers being the most abundant. These compounds contribute to the extract's strong antioxidant activity, particularly against radicals, as demonstrated by the ABTS assay. *In vitro* assays with HaCaT keratinocytes and BJ-5ta fibroblasts confirmed the blend GS extract's safety, showing no adverse effects on cell structure or function. Notably, the extract reduced TGF-β1 levels, a key molecule in scar formation, and inhibited cell proliferation while promoting apoptosis in specific phases of the cell cycle. This suggests potential benefits in managing abnormal scar formation, including keloids and hypertrophic scars.

Incorporating blend GS extract into cream formulations was successful, leading to increased total phenolic and flavonoid content. However, a notable decrease of approximately 52% in total phenolic content was observed compared to the pure GS extract, indicating that not all phenolic compounds are soluble in the cream base, with flavonoids showing better solubility.

Despite physical and chemical changes (such as alterations in colour to a browner tone, a woody odour, and increased viscosity) the formulations retained acceptable characteristics. The values for flow behaviour index, thixotropy, and adhesiveness indicate that the formulations maintained shear-thinning behaviour, allowing them to spread easily and absorb into the skin. Additionally, the consistency index, viscosity, and firmness indicated that the formulations maintained the structural characteristics typical of a cream.

Moreover, the developed formulations demonstrated notable antioxidant activity; however, there was a significant reduction in their capacity compared to the pure blend GS extract. This decline is likely due to the limited solubility of certain phenolic compounds within the cream. The formulations largely preserved their ability to inhibit elastase, partly due to inherent inhibition from the cream base, although the capacity to inhibit tyrosinase was not fully retained except at higher concentrations.

Lastly, stability testing revealed that the most concentrated samples (above 3.23% GS extract) were generally the least stable, exhibiting changes such as darkening and lumpiness due to coalescence. Conversely, formulations with lower concentrations of GS extract (specifically between 0.83% and 1.64%) demonstrated greater stability, maintaining consistent rheological properties and acceptable thixotropic behaviour. Over time, the phenolic content decreased in all samples, with samples III (0.99% GS extract) and IV (1.64% GS extract) being the most effective at retaining total phenolic content. In addition, samples I (0.33% GS extract), II (0.83% GS extract), III (0.99% GS extract), and IV (1.64% GS extract) were more effective at maintaining flavonoid content.

In terms of biological properties, samples II (0.83% GS extract) and III (0.99% GS extract) remained stable and effective in reducing free radicals, while samples III (0.99% GS extract) and IV (1.64% GS extract) retained their ability to reduce ions, and sample II (0.83% GS extract) continued to inhibit elastase over time.

In conclusion, the findings support the potential of grape stem extract as a valuable active ingredient in cosmetic formulations. Its ability to manage scars, including keloids and hypertrophic scars, while maintaining formulation characteristics such as shear-thinning behaviour and effective spreadability, highlights its suitability for cosmetic applications. The extract’s capability to retain phenolic compounds over time further enhances its application in the skincare market, aligning with the industry's movement toward sustainable practices and natural ingredients. Further research is needed to optimize formulations and explore encapsulation methods to enhance stability and bioactivity.

4.2. Final Note

This doctoral thesis was conducted within an industrial context in collaboration with Mesosystem, a company dedicated to the development and commercialization of solutions aimed at improving various skin conditions, such as skin aging, hyperpigmentation, and the prevention and reduction of scars, among others.

The initial objective of this research was to optimize the microbial production of hyaluronic acid using alternative culture media. However, this goal remained unachieved due to several challenges, including difficulties in obtaining the correct bacterial strain essential for production. In response to these obstacles, it was also explored the production of polyglutamate, a compound with similarities to hyaluronic acid.

Given the extensive time requirements of the thesis, a strategic decision was made midway through to pivot towards the development of formulations with properties suitable for skin application. This direction aligns closely with the interests of the associated company and involves the use of natural matrices.

The work presented in this thesis is thus not only relevant to the academic field but also holds significant industrial value for the company's ongoing efforts to innovate in skin care solutions.