Universidade do Minho
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Maria Beatriz Sanguedo Castro da Silva Oliveira

Extraction of bioactive compounds from the cork industry for the possible applications in the pharmaceutical, food and cosmetic industries

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Doutora Cláudia Botelho

Doutora Ana Novo Barros

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DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Resumo

Extração de compostos bioativos da indústria da cortiça para possíveis aplicações das indústrias farmacêutica, alimentar e cosmética

Este estudo é motivado pela necessidade ambiental de minimizar os resíduos produzidos pelas grandes indústrias. O objetivo é reaproveitar esses subprodutos industriais em vários setores, incluindo farmacêutico, alimentar e cosmético. Numa primeira fase a matéria-prima, cortiça, foi caracterizada quimicamente. Em seguida, obteve-se um extrato de cortiça considerando diferentes condições, como tempo, temperatura e solventes (água e etanol). Este extrato foi submetido a uma caracterização físico-química e biológica. O conteúdo fenólico dos extratos foi determinado pelo método Folin-Ciocalteu e a capacidade antioxidante foi avaliada pelos ensaios FRAP, DPPH e ABTS. O conteúdo fenólico total dos extratos variou de 11,81 ± 0,905 mg GAE/g amostra a 29,80 ± 2,694 mg GAE/g amostra, enquanto o teor de flavonóides entre 5,209 ± 0,049 mg CAT/g amostra e 36,96 ± 1,787 mg CAT/g amostra. Relativamente à capacidade antioxidante, obtiveram-se os valores mais elevados nas amostras Etanol 40%, 60 min e 80°C nos três ensaios, com valores de 0,309 ± 0,023 mmol TEAC/g amostra,0,299 ± 0,029 mmol TEAC/g amostra e 0,307 ± 0,024 mmol TEAC/g amostra, para as metodologias do FRAP, DPPH e ABTS, respetivamente. Com foco principal na pele, o estudo da atividade de inibição enzimática da tirosinase e da elastase assume um papel importante. Os extratos apresentaram atividade anti-tirosinase com percentagens de inibição entre 8,64% e 31,19% para os extratos de Água 60 min e Etanol 40% 60 min, respetivamente, tal sugere que estas amostras podem prevenir ou remover manchas na pele. Em relação à elastase, não se verificou inibição da enzima por parte de nenhum dos extratos analisados. Além disso, os extratos com as composições mais promissoras foram testados em linhagens celulares, HaCaT (queratinócitos humanos imortalizados), L929 (fibroblastos de ratinhos) e HEK (células renais embrionárias humanas). O ensaio celular demonstrou que os extratos podem aumentar a viabilidade celular nas amostras com menores concentrações de extrato. Nas células HaCaT os extratos foram aumentaram a atividade metabólica para 155,5 ± 7,049% de atividade metabólica %/controlo, nas células L929 os extratos aumentaram a viabilidade celular até 197,6 ± 21,23% de atividade metabólica %/controlo e, finalmente, para as células HEK a viabilidade celular atingiu 159,8 ± 17,29% de atividade metabólica %/controlo. Os resultados sugerem que os subprodutos da indústria da cortiça têm potencial como fonte de compostos bioativos, especialmente para a indústria farmacêutica. Esta abordagem alinha-se com a crescente procura social por recursos naturais em produtos relacionados com a pele.

Palavras-chave: cortiça, cicatrização cutânea, atividade antioxidante, compostos fenólicos

Abstract

Extraction of bioactive compounds from the cork industry for possible applications in the pharmaceutical, food and cosmetic industries

This study is primarily motivated by the increasing environmental necessity to minimize waste produced by major industries. The aim is to repurpose these industrial by-products in multiple sectors, including pharmaceuticals, food, and cosmetics. As a first step the raw material, cork, was chemically characterized. Following this, a cork extract was obtained under various conditions, considering factors like time, temperature, and solvents (water and ethanol). This cork extract was then subjected to physical-chemical and biological characterization. The phenolic content in each cork extract was determined by the Folin-Ciocalteu method and the antioxidant capacity was evaluated by the FRAP, DPPH and ABTS assays. The extracts total phenolic content ranged from 11.81 ± 0.905 mg GAE/g sample to 29.80 ± 2.694 mg GAE/g sample, while the flavonoids content stayed between 5.209 ± 0.049 mg CAT/g sample and 36.96 ± 1.787 mg CAT/g sample. The antioxidant activity had the higher values on the Ethanol 40%, 60 min and 80°C samples in all three assays with 0.309 ± 0.023 mmol TEAC/g sample, 0.299 ± 0.029 mmol TEAC/g sample and 0.307 ± 0.024 mmol TEAC/g sample for FRAP, DPPH and ABTS assays, respectively. Setting our focus on the skin, the study of enzyme inhibition activity for tyrosinase and elastase pose as an important role. The extracts showed an anti-tyrosinase activity with percentages between 8.64% and 31.19% for the extracts water 60 min and ethanol 40% 60 min, respectively, which suggests that these samples can prevent or remove skin blemishes. Regarding elastase, no extract was able to inhibit the enzyme. Furthermore, the extracts with the most promising compositions were tested on various cell lines, including HaCaT (human immortalized keratinocytes), L929 (mouse fibroblasts), and HEK (human embryonic kidney cells). The cellular assay demonstrated that the extracts, can increase cell viability, especially the samples with lower extract concentrations. On HaCaT cells, the extracts were able to increase the metabolic activity to 155.5 ± 7.049% metabolic activity %/control, on L929 cells the extracts increased the cells viability up to 197.6 ± 21.23% metabolic activity %/control and, finally, for the HEK cells the cells viability reached 159.8 ± 17.29% metabolic activity %/control. The results suggest that subproducts from the cork industry hold potential as a source of bioactive compounds, especially for the pharmaceutical industry. This approach aligns with the growing societal demand for natural resources in skin-related products.

Keywords: cork, skin healing, antioxidant activity, phenolic compounds

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List of Abbreviations

ABTS – 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt

DMSO – dimethyl sulfoxide

DPPH – 2,2-diphenyl-1-picrylhydrazyl

DW – dry weight

FC - flavonoid content

FRAP – ferric reducing antioxidant power

HEK – human embryonic cells

HPLC – high-performance liquid chromatography

IL-6 – interleukin 6

iNOS – inducible nitric oxide synthase

MMP – metalloproteinase

NO – nitric oxide

PBS – phosphate-buffered saline

ROS – reactive oxygen species

TNF – tumor necrosis factor

TPC - total phenolic content

TPTZ – 2,4,6-Tris(2-pyridyl)-s-triazine

UV – ultraviolet

1. State-of-art

1.1. Cork Industry

The majority of the world's cork is primarily produced in the European Union, with a significant focus on the southern Mediterranean region. Approximately 80% of the world's cork extraction and processing is attributed to Portugal, a country that boasts around 60% of the global cork trees population and serves as a leading cork exporter worldwide.[1–3] Despite its prevalence, cork remains underexploited, yet its diverse physical, chemical, and mechanical properties can lead to its usage in different areas such as in the winery industry for bottle stoppers and for insulation corkboards.[4] Cork's properties are not homogeneous, varying according to factors such as regional influences, soil and climate conditions, tree genetic properties, as well as tree dimension, growth conditions and age.[4,5]

The cork oak specie *Quercus suber* L. produces cork as its subereous coating, also known as bark or suberose parenchyma. The *Q. suber* L. cork shields the tree cells from environmental aggressions such as woodland fires, due to its thickness, porous, and fissured characteristics. Cork oak is involved on the hydrological cycle, prevention of soil erosion and desertification, and decreases CO_2 releases to the atmosphere, preserving the biodiversity in its immediate surroundings. [1,2]

1.1.1. Cork waste

The global winery industry produces nearly 300 million hectoliters of wine annually.[6] Remarkably, nineteen percent of bottled wine are sealed with cork stoppers, underscoring the widespread presence of the cork industry on a global scale. Inevitably, cork production generates waste (as depicted in **Figure 1**), which can amount to as much as 25% of the raw material, equating to approximately 50,000 tons of waste requiring disposal. It is feasible to distinguish cork waste based on various characteristics such as density, moisture content, size, ash content, granulometry, and tannic concentration.[1,3,7,8]

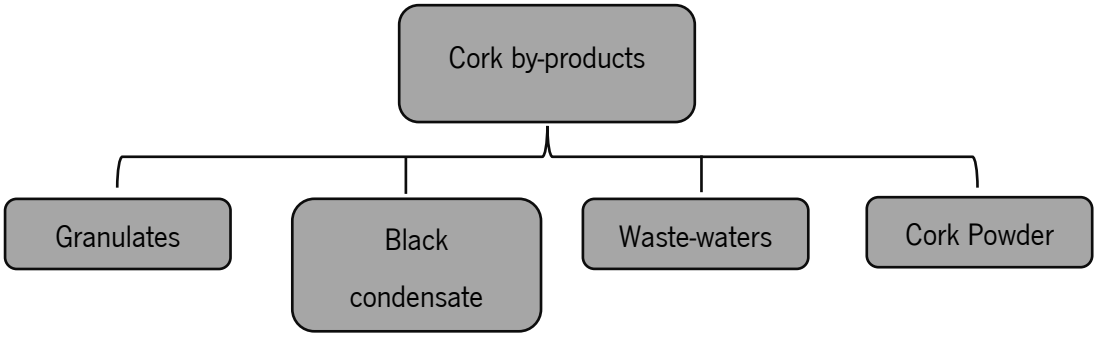

Figure 1. Cork Industry by-products.

The primary by-product of the cork industry is cork powder, which is typically burned in boilers to produce energy and it can also be used as filler to improve the quality of cork stoppers.[2,9] New studies suggest that this by-product may play a role on the production of biomass, producing activated carbon, which can be used to filter out contaminants like heavy metals and pharmaceuticals from water.[10,11] Similarly, to cork powder, the black condensate can be used for energy production through combustion, however it can also be applied as a protective coating.[12] Cork´s waste has also been used in agriculture as plant growth media contributing for plants´ diseases suppression.[13] Moreover, due to its absorption properties water, cork´s waste can be used to in retain water´s contaminants.[14,15]

1.1.2. Cork Composition

Presently, there is a reasonably good understanding of the chemical composition of cork, although variations can be observed. The key feature in the cork´s chemical composition is the predominant presence of suberin, with reported contents ranging from 33% to 50%. Additionally, cork possesses a substantial quantity of extractives, such as waxes, which are integrated into the suberin complex and make up around 5% of the cork composition. Furthermore, tannins and other phenolic compounds constitute roughly 7% of the cork material. The research about lignin and polysaccharides, which are also integral structural components of the cork, have been comparatively limited (**Figure 2**).[4,16,17]

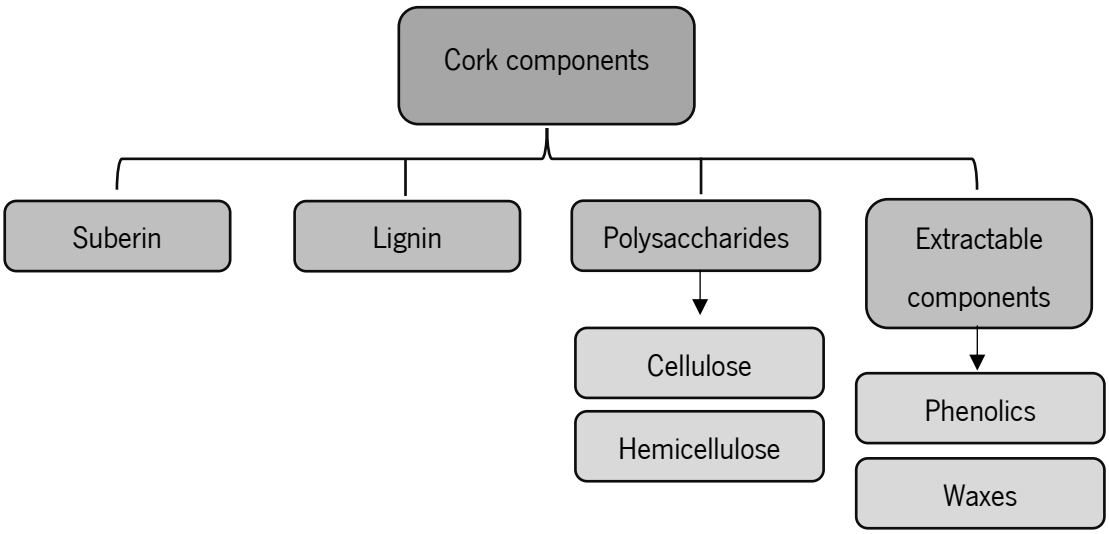

Figure 2. Cork components.

The composition of cork is indeed influenced by various factors, including climate, geographical location, the age of the cork oak tree, and the specific tree segment from which it is harvested. These

variables can contribute to the unique characteristics and properties of cork, making it a material with distinct qualities based on its origin and source.

Food, agricultural, cosmetic, or pharmaceutical sectors are interested on the development of new formulations through the incorporation of bioactive molecules, particularly aromatics compounds. These compounds can be found in different industrial by-products. It is widely understood that phenolic compounds present in plants are linked to distinct biological activities, which include antioxidative, anticarcinogenic, and antitumor properties. [8,18–20]

Molecules extracted from *Quercus suber* L. have been linked to health benefits, such as hydrolysable tannins, coumarins, flavonoids, low molecular weight phenolic compounds, such as phenolic acids and aldehydes and high molecular weight polyphenols, like ellagitannins and gallotannins. Given the wide range of applications for these compounds in cosmetic, pharmaceutical, and food industries, it is envisaged that cork residues can be used raw materials for the development of new formulations for these industries. [8,19,21]

1.1.2.1. Aromatic compounds

There is still little research about the aromatic compounds present in cork, however studies showed a total of 23 aromatic compounds present in cork, including vanillins and derivates, volatile phenols, aldehydes, alcohols, terpenes, lactones, fatty acids, and furans. These compounds are described to be a natural bioactive molecule with an important antioxidant activity.[5,8,22–31]

Vanillin serves as the principal component of vanilla and is considered a secondary metabolite in plants. It is commonly present in plant oils, processed foods, beverages, perfumes, and pharmaceutical products. This compound boasts not only antioxidant potential but also exhibits antitumor properties.[32–34]

The aromas produced by the aldehydes, lactones, terpenols, alcohols, and fatty acids are strong and appealing, making them suitable for the food and cosmetic sectors. Additionally, the alcohols have antimicrobial properties. [5,8,22–31]

Terpenols or terpenoids have the capability to act as elastase inhibitors, effectively halting the degradation of elastin fibers within the dermal matrix. These compounds are derived from terpenes and constitute the foundational elements of essential oils, which are found in various plants. Terpenols have exhibited several pharmacological activities, such as neutrophil elastase inhibition, anti-inflammatory effects, as well as antiviral and antibacterial properties.[35,36]

1.1.2.2. Suberin

Suberin has been described as the main component of cork cells, reaching up to 40% of the cork composition. Due to its role in the impermeability of plants, suberin can serve as a barrier to shield plants from external stimuli. The deposition of suberin takes place through two mechanisms: first as a part of regular growth in the endodermis and epidermis of roots, bark, seed coats, and specific organs like tubers, and second in reaction to stress caused by injury. Suberin is a unique cell wall-altering polymer made up of phenolic and aliphatic compounds originating from the phenylpropanoid and fatty acid pathways, however suberin structure is yet to be fully understood (**Figure 3**). It has been hypothesized that the polyester structure of suberin is made up of long-chain fatty acids, hydroxy fatty acids, and phenolic acids connected by ester groups. [4,5,8,37–39]

Since cork powder alone generates more than 16.000 tons of suberin annually, natural cork and cork powder are potential sources for suberin.[8]

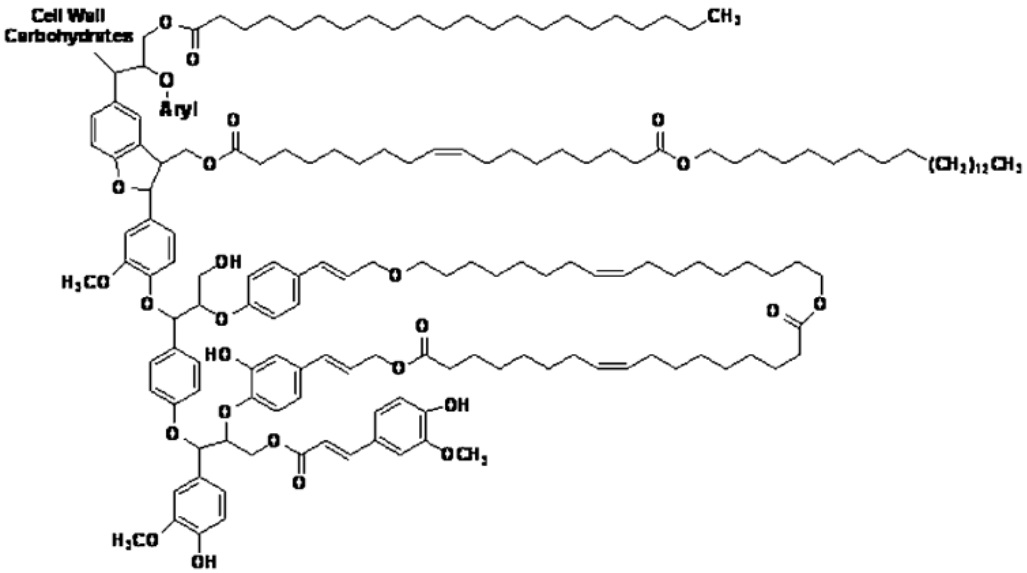

Figure 3. Proposed suberin structure. [5]

1.1.2.3. Lignin

Lignin stands as a significant secondary metabolite, produced through the phenylalanine/tyrosine metabolic pathway within plant cells and along with its related metabolic processes, holds pivotal functions in the growth and development of plants. This complex phenolic polymer bolsters the rigidity of plant cell walls, imparts hydrophobic characteristics, and facilitates the transport of minerals via the plant's vascular bundles. Furthermore, lignin serves as a vital protective barrier against pests, pathogens, and environmental stresses. Moreover, lignin itself presents valuable potential as a resource in the fields of energy and the pharmaceutical industry.[40–44]

Another highly noticeable substance present in cork is lignin, as it takes about 20 to 25% of cell walls. It guarantees cork cell walls' mechanical support and stiffness. According to studies, lignin is the major aromatic component in cork's and has a structure that is strikingly identical to the aromatic domain of suberin. Lignin´s structure can vary depending on its origin, nonetheless it is a polymer with phenylpropane monomers, such as coniferyl, sinapyl and pcoumaryl alcohols and aromatic groups derived from the mentioned monomers (**Figure 4**). Vanillin and vanillin acid results from lignin alkaline oxidation.[5,45,46]

Most lignin is found in cork powder. The concentration of lignin in cork powder is often higher than that of the original cork, making it an improved supply of this aromatic.[8,47]

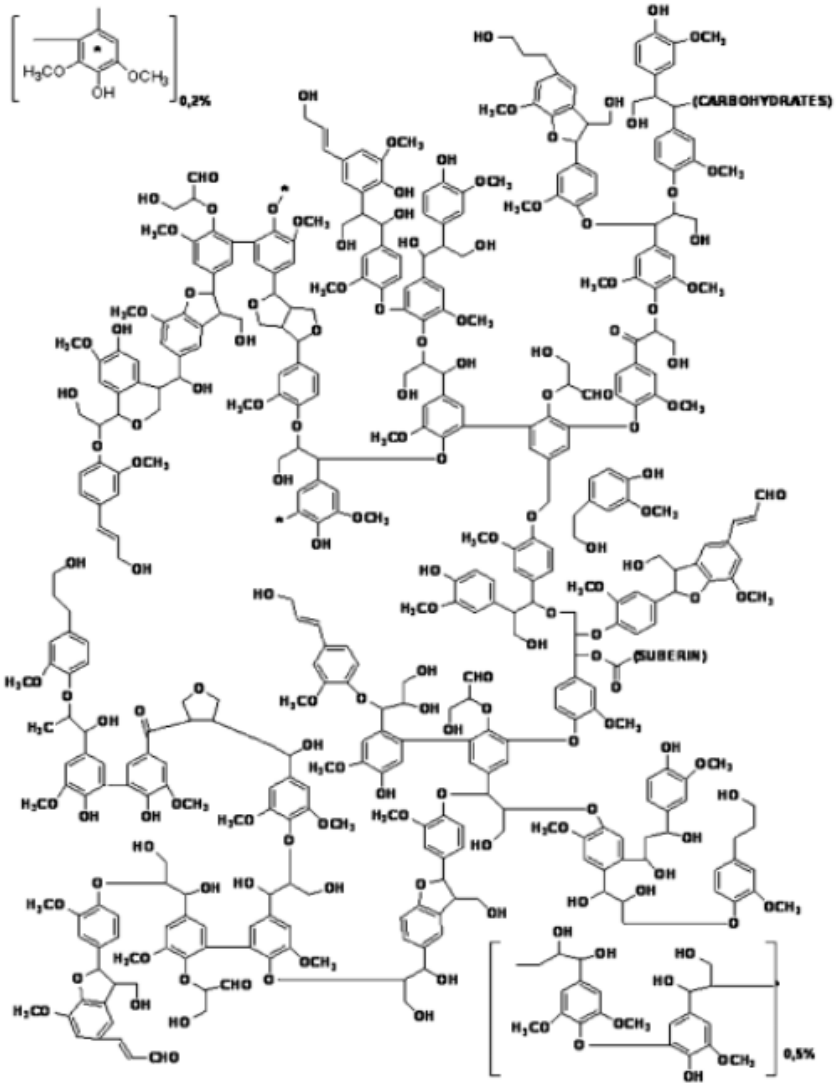

Figure 4. Lignin structure. [5]

1.1.2.4. Polysaccharides

Hemicellulose and cellulose (**Figure 5**) are hydrophilic carbohydrates found in cork. In cork, these structural elements, while present, are notably less abundant compared to wood, with cellulose and hemicelluloses constituting only about 20% of cork's composition, as opposed to the 70 to 80% found in wood. Cellulose and hemicelluloses make up around 10% and 11%, respectively, of the structural components in cell walls. These low molecular weight elements provide cells their structural rigidity. These carbohydrates in cork are low molecular weight monosaccharides including glucose, arabinose, galactose, xylose, and mannose, connected by glycose linkages.[5,48]

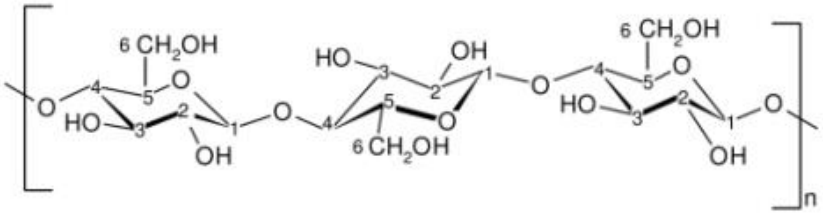

Figure 5. Cellulose structure. [5]

1.1.2.5. Waxes

Plant waxes typically serve as hydrophobic components forming an unstructured layer on the external surface of plants. These waxes are vital to plants, acting as a protective barrier against several environmental stressors.[49]

Waxes are cork´s lipophilic portion and make up about a third of all cork extracted components. Triterpenes constitute about half of the ingredients in wax; the remaining ingredients are n-alkanes, n-alkanols, and fatty acids.[19]

The utility of waxes spans across a spectrum, from consumer items like, cosmetics and candles, to industrial applications such as hot-melt adhesives for sealing cartons and additives for suspension polymerization processes. In the context of food, waxes find extensive use in coatings for food products to preserve quality and prolong shelf life.[49]

According to studies, waxes can be found in both black condensate and cork powder, but the first one has a higher concentration. Cork powder has a higher concentration of betulinic acid than black condensate, which has a large content of friedelin. This demonstrates that cork by-products can serve as sources of bioactive compounds.[50]

1.1.2.6. Phenolics

Around 6 to 9% of cork is made up of the phenolic portion, which contains tannins, flavonoids, coumarins, phenolic acids, and phenolic aldehydes. However, the amount of phenolic compounds can change based on the age of the oak tree and how far the sample was taken from the tree's trunk.[8,45]

Ellagitannins, including castalagin, grandinin, rouburins A and E, and vascalagin, make up the bulk of cork tannins. In the wine aging processes, members of the ellagitannin family are essential. Along with tannins, phenolic acids like gallic, ellagic, protocatechuic, vanillic, caffeic, and ferulic are also present, as well as coumarins like aesculetin and scopoletin. There are also protocatechuic aldehyde, vanillin, and coumaric acid.[5,19,20,51]

The focus on phenolic compounds centers on their anti-aging, anti-cancer, anti-inflammatory, anti-radical scavenger, anti-allergic, anti-cancer, anti-microbial, and anti-acne qualities. Because of their qualities, these natural ingredients have gained more attention in recent years for use in beauty products.[51]

1.1.3. Cork by-products applications

1.1.3.1. Antioxidant activity

Antioxidants play a crucial role in delaying the oxidation of molecules by inhibiting oxidation chain reactions, acting as free radical scavengers, and regulating the body's internal antioxidant system. Reactive oxygen species (ROS), which arise because of metabolic processes, contribute to these processes. While low concentrations of ROS are essential for intracellular functions, oxidative stress occurs when there is an imbalance between ROS and antioxidants. This imbalance can lead to oxidative damage, resulting in a range of skin conditions and the aging of the skin. [52,53]

The incidence of ultraviolet (UV) radiation at the skin's surface can compromise the immune system, heightening the risk of specific skin cancers and accelerating the aging mechanisms. These factors collectively contribute to lasting harm to the skin. A primary driver of such harm is the generation of ROS due to UV ray exposure. Consequently, antioxidants that shield the outermost skin layer from oxidative stress have the potential to thwart the processes of aging, cancer development, and genetic mutations. Although the epidermis employs its internal antioxidants for this protection, they are not infinite and can become depleted over time. These attributes underscore the suitability of antioxidants as valuable components for topical application.[52]

The bark of *Q. suber* is undoubtedly a candidate, since it is a rich source of phenolic acids, tannins and flavonoids, which are substances with a lot of antioxidant potential. Specifically, phenolic compounds

exhibit potent abilities to scavenge radicals and reduce oxidative stress within skin cells. This efficacy stems from their redox properties, as they serve as reducing agents, suppliers of hydrogen, quenchers of electrons, and neutralizers of singlet oxygen, thereby inhibiting the actions of oxidative enzymes. [5,54–56]

Cork studies confirmed that some of the phenolic acids present, like caffeic acid, gallic acid and ferulic acid are strong antioxidants.[19,56,57]

Studies have also shown that cork not only possesses a significant antioxidant capacity, like the well-known antioxidant compound ascorbic acid, but it also serves as a non-cytotoxic matrix for skin cells. Experiments using cork extracts and keratinocytes revealed that as the contact time between the cells and the extract increased, there was a corresponding increase in the inhibition of ROS. This demonstrates the antioxidant bioactivity and its impact on DNA protection by cork.[51,58]

Cork has in its constitution hydrolysable tannins, especially ellagitannins, that are also powerful antioxidants agents, due to their hydroxyl groups that amplify the extreme scavenging effort. There are also a flavanoellagitannin and a castalagin that have an antiradical and ion-reducing properties. Vanillin, friedelin and triterpenes (betulinic acid lupeol and b-sitosterol) also found in cork, showed antioxidant potential.[5,19,52–54]

Finally, two others naturally occurring bioactive cork constituents, vanillin and friedelin, have also shown antioxidant activity. Lupeol, betulinic acid, and β-sitosterol are three triterpenes present in cork that also have antioxidant properties.[5,52,53]

1.1.3.2. Sun protection properties

Excessive exposure of the skin to sunlight leads to the degradation of organic compounds, primarily due to UVA and UVB radiation, resulting in significant biological damage to the skin. This UV radiation can result in skin discoloration, sunburns, and skin dryness, among other issues. To address these skin inconveniences, UV absorbers play a vital role as a key material. [59–61]

Several sunscreens contain antioxidants as part of their fundamental ingredients, lignin, due to its phenolic structure and UV-absorbing elements such as phenolic units, ketones, and chromophores, and cellulose which is a readily available natural polymer known for its biodegradability, biocompatibility, and recyclability. These are components of cork that are very effective light blockers making them naturals UV protective ingredients.[59,62–64]

1.1.3.3. Anti-ageing activity

The issue of premature skin aging has become a significant concern today, prompting the need to develop products that can address this problem. Various environmental factors contribute to skin aging, including UV exposure and air pollution. These factors induce oxidative stress on cells, resulting in the accumulation of cellular damage and the eventual emergence of wrinkles on the skin.[28,52]

Skin loses both physiological and integrity functions due to photoaging, leading to epidermal thickness, unbalanced collagen, and histological changes. The human body naturally produces elastase and collagenase, which are enzymes that help break out elastin and collagen, respectively. A large ROS concentration causes a rise in the mentioned enzymes, hastening the aging of the skin.[8]

Cork extractives are crucial in the suppression of ROS formation, as was previously mentioned. Additional studies revealed that extracts containing terpenoids, flavonols, gallic, protocatechuic, and caffeic acids also have collagenase and elastase inhibitory activity, which provided further evidence for this. Additionally, cork extracts can inhibit metalloproteinases (MMP), an enzyme family that breaks down collagen, fibronectin, laminin, and proteoglycan, leading to wrinkles and a reduction in the suppleness of the skin. According to studies, the antiaging effects are dose-dependent and more significant in the inhibition of MMP-9; nonetheless, the gallic acid in cork extracts has been shown to have MMP-1 inhibitory activity.[8,28,29,52,58,65]

Suberin and friedelin, both found in cork, possess anti-aging properties. This is attributed to the hydroxycarboxylic acids, the primary component of suberin, which exert a smoothing and anti-wrinkle effect on the skin. When applied topically, suberin cork extract has demonstrated the ability to enhance skin texture, brightness, and firmness, showcasing its dual role as an anti-aging agent. An in vivo study further confirmed the benefits of cork extract by showing that it promotes viscoelastic recovery, reduces skin deformation, and increases skin stiffness.[8,58,66,67]

1.1.3.4. Skin depigmentation properties

Skin pigmentation is primarily attributed to melanin, a pigment produced by epidermal cells known as melanocytes. Melanin plays a crucial role in safeguarding the skin from the harmful effects of UV radiation. However, an excessive production of melanin can lead to conditions like melasma, freckles, and uneven skin tone. To prevent the excessive accumulation of melanin, it is essential to regulate melanogenesis.[58,68,69]

Tyrosinase serves as a pivotal enzyme in melanin formation, with its activity being stimulated by UV radiation. This enzyme directly regulates the quantity of melanin generated, as it plays a crucial role

in the intermediate production of L-Dopa and dopaquinone. Inhibiting tyrosinase activity curtails melanin synthesis and reduces melanin deposition.[28,58,68–71]

Phenolic compounds possess the capability to function as tyrosinase inhibitors or alternative substrates, enabling skin depigmentation. Specifically, flavonols and phenolic acids derived from cork demonstrate inhibitory activity against tyrosinase, as evidenced by research assessing their impact on melanocytes. Additionally, ellagic acid and ellagitannins effectively block tyrosinase activity. When topically applied, these substances prove effective in reducing UV-induced skin pigmentation. Notably, both phenolic acids limit melanocyte proliferation, subsequently blocking melanin production. Gallic acid, also present in cork, also exerts inhibitory effects on melanogenesis, highlighting its potency as an anti-tyrosinase agent.[28,68,69,72]

Cork extracts may contain compounds that are beneficial for skin melasma whitening because of their depigmenting activity and the cork's potential as an anti-tyrosinase agent.[58]

1.1.3.5. Anti-acne activity

Acne is a common skin disorder in which the sebaceous glands create more sebum than standard. Products that provide lipogenesis control action have become the preferred option for treating this condition.[58,73]

According to studies, cork extracts cause keratinocytes' lipidic buildup to be inhibited as well as the production of the SREBP-1 gene. This transcription factor has the ability to directly upregulate the lipogenesis-promoting enzyme and leads to the creation of fatty acids and cholesterol.[58,73]

Cork compounds, such as polyphenols have an inhibitory activity on the lipidic synthesis and SREBP-1 expression. Additionally, tannins, also cork compounds, have anti-acne properties. These characteristics make cork a useful source of components that are able to regulate sebum production and, consequently, can be use in the cosmetic industry as anti-acne agents. [73,74]

1.1.3.6. Anti-inflammatory properties

Inflammatory conditions, such as aging skin, acne, and diseases like cancer, cardiovascular problems, and neurodegenerative disorders, are characterized by an inflammatory state. In these conditions, cells experience upregulation of several enzymes, pro-inflammatory mediators, and signaling proteins. One by-product of inflammatory reactions is nitric oxide (NO), a highly reactive radical that generates a harmful oxidant detrimental to our body. Suppression of nitrite synthesis can help prevent cellular damage.[8]

A study shows that in the presence of a pro-inflammatory stimulus, cork extract demonstrated a strong capacity to inhibit NO. The iNOS enzyme, which is directly engaged in NO production during an inflammatory process, was found to be dropping in quantities. It was discovered that cork extract also prevented the stimulation of the transcription factor NF-kB. This serves a crucial role in the activation of genes that encode inflammatory response-related proteins. Additionally, cork extract lowers cytokine levels, which are inflammatory response factors like IL-6, TNF-, and CCL5. Therefore, it is safe to draw the conclusion that cork extract exhibited anti-inflammatory qualities.[58]

1.1.3.7. Antimicrobial properties

Bioactive components in the *Q. suber* species' constitution have antimicrobial action. According to studies, cork is efficient against bacteria like *Escherichia coli* and *Staphylococcus aureus*. The high phenolic content of cork, which is known to have antibacterial properties, may be the cause of this action.[51,54,75,76]

Antimicrobial properties of phenolic compounds, such as tannins and gallic acid, are well recognized. In comparison to ellagitannins, which also exhibit antimicrobial action against extracellular microbial enzymes, microbial metabolism, and the inhibition of oxidative phosphorylation, gallic acid is effective against *Salmonella thypi* and *Staphylococcus aureus*. Phenolic compounds exhibit resistance to bacteria and fungus as well.[19,51,77,78]

1.1.3.8. Anti-cancer potential

Cork offers a range of compounds that offer significant advantages in cancer treatment. Phenolic compounds derived from cork exhibit exciting potential in triggering apoptosis in cancerous cells and demonstrate a dose-dependent ability to inhibit the proliferation of these cells when assessed in human tumor cell lines. Notably, vescalagin exhibited inhibitory properties primarily in colon cancer cells, whereas castalagin demonstrated cytotoxic effects in leukemia cells as well.[19,79]

Skin cancer has become increasingly prevalent due to widespread human behavior involving excessive sun exposure and consequent UV radiation damage. Cork offers valuable components for skin cancer treatment, including ellagitannins and ellagic acid, which can inhibit cell proliferation by interrupting their cycle and inducing apoptosis in malignant cells. Additionally, cork contains apoptosis-inducing compounds such as caffeic acid and protocatechuic acid.[19,79,80]

1.2. The skin and the epidermis

The epidermis and dermis are the two distinct layers that make up the skin. Layers of differentiated keratinocytes make up the epidermis, which is the superficial layer of the skin. The stratum corneum is the top layer and it also contains keratinocytes, but these are terminally differentiated and enucleated. These keratinocytes are chemically crosslinked to bolster the skin barrier.[81]

Body sites also offer a variety of microenvironments with different UV exposure, temperature, pH, topography, and moisture levels. The areas can be divided into three distinct groups based on these characteristics: oily/sebaceous, which includes the face; chest and back; moist, which includes the elbow bend, back of the knee, and groin; and dry, which includes the volar forearm and palm. Sweat glands, hair follicles, appendages, and sebaceous glands all have an impact on the environment at these areas.[82,83]

As the biggest organ in the body, the skin main purpose is to safeguard the human body from external factors and to keep the body hydrated. The skin in the first defense against all physical, chemical, and biological stresses and it is home to the viruses, fungi and bacteria that make up the microbiome. Skin diseases, or even systemic diseases, may develop when the cutaneous barrier breaches or when the ration of commensal and pathogens is disrupted.[83,84]

The essential tissue that creates a strong, flexible, and self-healing barrier across the internal human body and the outside world is the epidermis. Its primary purpose is to shield the body from dehydration, nutrients loss and negative effects of any substances that contact the skin. Additionally, it helps in the defense of organisms such as animals, plants, fungus and microorganisms like bacteria and viruses.[85,86]

The skin can be divided into different defense lines: the epidermis makes up the physical, biochemical, and adaptive immunological barriers; the stratum corneum is part of the physical barrier and, finally, the biochemical barrier is also composed by macrophages, lipids, acids, antimicrobial peptides, and hydrolytic enzymes. The physical barrier is responsible for the protections from pathogens and mechanical harm, while the biochemical one has antimicrobial properties.[85,87]

The usual four-layer structure of the epidermis, consequent of cells ongoing maturation, shows that exists a differentiation process inside the keratinocytes meant to create a skin barrier. Since cornified keratinocytes constantly detach from the epidermis, the basal cell layer on going proliferation requires control, to maintain the homeostasis in the epidermis.[86]

1.2.1. Keratinocytes

As the most superficial layer of the skin, the epidermis is victim of several harms from environmental agents, so there is necessary to the skin to be able to repair itself efficiently. Keratinocytes are the dominant cell type in the epidermis, and they are responsible for various roles that are crucial in skin reparation.[88]

To have the barrier properties, the skin cells keratinocytes go through a differentiation process, starting with the proliferation of basal cells, which develop to epidermal stem cells, ending at the production of corneocytes. These cells are characterized as nucleus-free and highly differentiated and form the cornified envelope. This is an insoluble and rigid structure that is vital for the skin´s barrier properties. The process of cell differentiation highly depends on controlled communication mechanisms.[85,87]

They carry out the re-epitheliazation, in which the keratinocytes move, multiple and differentiate and, consequently, reestablish the barrier in the epidermis. Cytokines, MMPs and growth factors are some of the microenvironmental signals that can influence the cells transition in between states. Additionally, these cells collaborate with fibroblasts in wound healing.[88–90]

Undifferentiated keratinocytes undergo a differentiation process, as they move to the cornified envelope, becoming differentiated nondividing cells.[89]

Keratinocytes must properly and effectively regulate their gene expression in response to a variety of external stimuli to carry out the immunological activities outlined above. In this context, epigenetic mechanisms are receiving more and more attention. The epigenome is an essential link between the genome and the environment, which consists of molecular markers and regulatory molecules that modulate genomic activity without altering the DNA sequence.[91–93]

1.2.2. Skin Wounds

A wound is a sore on the mucous membrane or the skin that is marked by the collapse of the tissue. The epidermis, parts of the dermis and subcutaneous fat can disappear as consequence of a wound. This damage frequently appears as an area of inflammatory tissue and reddish skin. Lack of mobility can also result in wounds by putting sustained pressure on the tissues. Bedsore or decubitus ulcers are typical skin wounds caused by this type of stress on the blood circulation.[94,95]

The entirety of bacteria, their genomes, and interactions within a particular environment is known as the microbiome. It is essential to identify the specific types of wound microbiomes that promote the

healing process, considering that the epidermis serves as the primary defense against environmental factors.[89]

After the barrier is disrupted, the human skin microbiome also experiences some changes. According to a recent study, the microbiomes in the deeper stratum corneum is crucial to the microbial recolonization following tape-derived damage. It has also been proposed that chronic wounds microbiomes regardless of whether they exhibit clinical indications of an infection, are an important part in the healing process. [89,96–98]

1.2.2.1. Chronic wounds

Skin wounds that do not heal after three months are referred to as chronic wounds. This illness not only presents a severe physical and psychological toll on people, but also places a heavy financial strain on the community. Chronic wounds fail to exhibit the common pathways of wound healing, for example they frequently fail to move from the inflammatory to the proliferative phase. Majority of chronic wounds can be divided into three groups based on their fundamental conditions: venous/arterial ulcers, pressure ulcers, and diabetic foot ulcers.[88,94,99]

Consistent inflammatory, bacterial colonization, angiogenesis, delayed reepithelization, and increased reactive oxygen species are frequent characteristics of chronic wounds. Keratinocytes are hyper-proliferative, however non-migratory cells near the non-healing borders of chronic wounds.[91,100,101]

Systematic diseases, aging and recurrent trauma are some causes of chronic wounds in addition to poor circulation, neuropathy and moving difficulties. Additionally, comorbid conditions can aid in the development of these wounds. Immune suppression and emotional stress are comorbid conditions, where the first is consequence of illnesses or long-term use of medical drugs. The second one might affect the healing process of the chronic wound negatively, by increasing the blood pressure and cortisol levels, which are factors that affect the immune system by lowering it. Finally, another parameter that might aid in chronic wounds development is old age. [94,102–104]

It has been hypothesized that microbiome from chronic wounds has much less variety than the surrounding health skin. *Staphylococcus*, *Pseudomonas* and *Corynobacterium* were among the most common organisms in chronic wounds. *Bacteroides*, *Peptoniphilus*, *Fingoldia*, *Anaerococcus* and *Peptostreptococcus* spp. were also found in chronic wounds. [97,98]

Chronic wounds can be divided into four stages depending on some characteristics: warmth, swelling, hardness, redness and discoloration are prominent in stage one wounds; stage two is characterized by the penetration of the skin by the wounds; stage three refers to full-thickness damage

that does not pierce the layer that separates the skin and deeper tissues from the fat and skin, called fascia; finally, stage four wounds are described by muscle and adjacent tissue damages.[94]

1.2.3. Skin Healing

The skin´s ability to regenerate depends on the epidermis stem cells populations in the wounded area. These types of cells are found in certain microenvironments named stem cell niches, that control both their behavior and outcome. To date, the bulge hair follicle, basal layer of interfollicular epidermis and the sebaceous gland are all recognized as unique epidermis stem cells niches. Each niche acts unipotently and replenishes its own specific tissue compartment when under regular homeostatic circumstances. [89,105,106]

As previously mentioned, skin microbiome has a very important role in human health, including the skin healing process. This is a factor that presents impact in skin damages, as the microorganisms move from the skin surface to the wound in the dermis, triggering an inflammatory response.[91,105]

At neutral pH the recovery of the skin is postponed, which prevents the post-secretory conversion of glucosylceramides into ceramides. In contrast, the barrier repair after acute perturbations continues as usual when there is an acidic pH.[107,108]

Chronic wounds healing process can be divided into three stages: first is the inflammatory stage in the first few days of the wound. Inflammation, which includes redness, heat and swelling, is an obvious sign of the immune response. In an effort to stop the bleeding, the injured area contracts the blood vessels, attempting to go back to its usual state. In this stage the platelets and thromboplastin clot. The second stage is the proliferative stage, which its length depends on the wound severity, but usually it lasts up to three weeks. Here granulation takes place, indicating the collagen producing by the fibroblasts, in order to cover the damage skin. Blood vessels grow new ones, and the wound progressively closes, and a skin layer is applied to it. Lastly, the maturation and remodeling stage can last up to two years. In this time, there is new collagen forms, which reshape the wound and reinforce the area´s tissue. Nonetheless, the scar tissue is weaker than the original tissue, reaching only 80% of the strength.[94]

1.2.3.1. Chronic wounds dressings

Given the rising prevalence of chronic wounds and the associated morbidity, the crucial nature of wound care has increased significantly. The concept of debridement, which entails removing the non-viable tissue components, is crucial to wound care. The objective is to reveal live the vascularized tissue

that may repair and populate the wound region by epithelial cell migration. Preserving the necrotic debris, on the other hand, only promotes infections and slows the healing process of the damage skin.[109]

Numerous wounds dressings have been created with the dual purpose of safeguarding the healing wound against infections and facilitating the wound healing process. One such dressing is the moist occlusive one, which aids in the promotion of the inflammatory phase by establishing a low oxygen tension environment. Furthermore, maintaining a controlled level of exudate on the wound facilitates autolytic debridement, thereby contributing to the advancement of effective wound healing. Nevertheless, the use of conventional wound dressing, such as gauze, might impede this process and potentially inflict additional harm when they are removed.[109,110]

The most prevalent kinds of wound dressings are low adherent and semipermeable films, with the intention of preventing liquid and microbial entrance while permitting water vapor and air to pass through. Alginate dressings are an additional choice; these non-woven materials, which derived from seaweed are typically used for wounds that exude fluid due to their capacity to take in liquid. Consequently, unfavorable effects are visible in dry wounds that are covered in alginate. Lastly, chronic ulcers and persistent wounds have both been treated with collagen-based treatments. Although, this collagen can be derived from a variety of sources, such as bovine and porcine, it is not the intention to directly make this a replacement for collagen produced by injured tissue. Instead, it is believed to contribute to the environment needed to attract certain cell types that are essential in the healing process, while reducing harmful agents like proteases and free radicals.[109,111,112]

The incorporation of antimicrobial substances into the wound dressing itself has been the subject of recent breakthroughs in this topic. These materials blend antimicrobial ingredients like silver, chitin, or betaine with conventional dressings like foams and hydrogels. These materials might be useful for treating chronic ulcers in the lower leg, where there is a risk of infection, especially if there is biofilm formation.[109]

Many pathogens can form biofilms, which helps them avoid being eliminated by antibiotics. Addressing biofilms became significantly difficult in would healing as it not only provides a barrier to the healing process but also increases the risk that the inflammation phase delays.[109]

1.3. Cell Line

1.3.1. HaCaT cell line

The cells present in HaCaT cell line are immortalized human keratinocytes. Not only are these cells capable of thriving in conventional media, but they can also be sustained in culture over extended

durations. These cells offer numerous appealing qualities as *in vitro* model for keratinocyte differentiation, as they possess the complete functional differentiation characteristics of the regular keratinocytes. Moreover, when cultured, these cells can switch between the differentiated and basal states as, consequence of the alterations in calcium concentration within the medium. Therefore, these present relevant markers for differentiation.[113,114]

While various factors can prompt the differentiation of basal keratinocytes, calcium serves as the most physiologically relevant agent capable of inducing differentiation in both *in vitro* and *in vivo*. *In vivo*, basal keratinocytes encounter a low calcium concentration, while differentiated keratinocytes are sustained by an increasing concentration gradient. Upon sensing a heightened level of calcium, basal keratinocytes respond by ceasing their cell cycle and transitioning towards termina, differentiation. [113,115]

1.3.2. L929 cell line

L929 cells, originating from mice fibroblasts, have gained significant prominence in the realm of biomedical investigation, particularly in the domains of cell biology, immunology, virology, and cancer research. These cells display a unique fibroblast-esque visual aspect, characterized by their stretched, spindle-shaped form.[116,117]

The utility of L929 cells extends to serving as a valuable model for delving into studies pertaining to cell proliferation, intercellular interactions, cytokine generation, and cellular signaling pathways. These cells find frequent application in cytotoxicity assays, facilitating the assessment of the impact of drugs, chemicals, or biological agents on cellular viability and growth. Additionally, their contribution extends to research on inflammation, wound healing, and tissue regeneration studies.[116,117]

1.3.3. Human Embryonic Kidney cells

Human Embryonic Kidney cell lines, abbreviated as HEK cell lines, find widespread application in both biological and biomedical research endeavors. Originating from human embryonic kidney tissue, these cells have gained prominence due to their amenable culture and manipulation, establishing them as a cornerstone in research investigations. HEK cells are proven useful for tasks such as protein expression, virus generation, drug evaluation, and functional analyses.[118–120]

A notable attribute of HEK cells is their robust proliferation in cell cultures, rendering them suitable for large-scale experimental ventures. Notably non-tumorigenic, these cells maintain stable genetic characteristics, a crucial factor in ensuring consistent outcomes.[121,122]

The notable feature of high transfection efficiency sets HEK cells apart, serving as invaluable tools for producing recombinant proteins and investigating gene functions. Moreover, these cells hold significance in drug screening assays, facilitating assessments of compound impacts on cellular pathways and receptors.[122]

However, it is important to acknowledge that the use of HEK cells raises ethical considerations due to their origin from human embryonic tissue.

2. Aims

Our project´s primary objective is to repurpose the cork´s by-products originated from the cork wine stoppers making, which would be disposed in landfills otherwise. We aim to harness the potential of these by-products in the healthcare sector, with a particular focus on bio compounds, including phenolic compounds, known for their skin-beneficial properties, by determining not only the cork composition as a raw material, but also determining important characteristics of the cork´s extracts like the total phenolic content, flavonoid content, and antioxidant capacity. Our goal extends to the evaluation of the cork´s effectiveness in treating chronic skin wounds by determining the influence of the cork extracts in the inhibition of two important skin enzymes: tyrosinase and elastase; and also, in different cell lines viability.

Through this initiative, we seek to achieve two key outcomes. First, we aim to significantly reduce the waste generated by the cork industry, particularly in Portugal. Second, our efforts align with the principles of the circular economy and sustainability, both of which are of paramount importance in today's world.

3. Material and Methods

3.1. Raw material and Reagents

The cork used for this experiment was acquired from Tech4Med with a granulometry fraction of 1 mm to 1.6 mm diameter. Ethanol 96% purchased from AGA. Petroleum ether; Sulfuric acid (H_2SO_4) purchased from Chem-Lab, Belgium. Sodium carbonate (Na_2CO_3), Folin-Ciacalteau reagent, Aluminum chloride ($AlCl_3$), Sodium nitrate ($NaNO_3$), Catechin, TPTZ ($C_{18}H_{12}N_6$), DPPH ($C_{18}H_{12}N_5O_6$), Trolox, ABTS salt ($C_{18}H_{18}N_4O_6S_4$), Potassium persulfate ($K_2S_2O_8$), Iron(III) chloride ($FeCl_3$), enzymes tyrosinase and elastase, and all reagentes for the enzymatic activities, Resazurin ($C_{12}H_7NO_4$) purchased from Sigma-Aldrich, Germany. Sodium hydroxide (NaOH), Acetic acid (CH_3COOH) purchased from Merck Millipore, Ireland. Hydrochloric acid (HCl) purchased from Fisher Scientific, USA. Ultrapure water was obtained from a Milli-Q Water Purification System, Germany. Phosphate-buffered saline 10x (PBS), DMEM medium, FBS Supreme bovine serum, Trypsin 0.05% in PBS and Penicillin-Streptomycin were purchased from PAN Biotec, Germany.

3.2. Raw material characterization

3.2.1. Lipid quantification with SOXTEC

The classic Soxhlet method, first devised by Frank Von Soxhlet in 1879, lays the groundwork for the modern solvent extraction called Soxtec™. In Soxtec, the immersion technique, specifically the Randall modification, is utilized to accelerate the extraction of oil and fat. This approach cuts approximately 20% of the time needed for a traditional Soxhlet extraction.[123]

Briefly, aluminum extraction cups with glass beads and cellulose thimbles were previously oven-dried for 12 hours at 105°C. 2.5 g of cork, weighted in triplicates, were placed in the cellulose thimbles, which were re-weighted with the samples in them. The thimbles were covered with cotton.

The aluminum cups and the thimbles were placed into the Soxtec 8000 Extraction Unit by FOSS and in this process, each extraction cup was loaded with 50 mL of petroleum ether before the extraction process initiated. The process is composed by three steps: boiling at 70°C for 40 minutes; rinsing, for 11 hours, and recovery, for 1 hour, lasting around 14 hours. Following the completion of the extraction cycle, the cellulose thimbles and extraction cups were retrieved. The extraction cups were subjected to an oven-drying process at 105°C, for 12 hours. The samples from the thimbles were transferred to aluminum containers, which were also oven-dried with the same mentioned conditions. The aluminum containers

and cups were weighted three times, and the total lipid content was calculated as a percentage by mass of the dry biomass.

3.2.2. Cork humidity and ashes

The humidity content was determined by the mass difference before and after heating the cork at 105 °C overnight. The ash content was determined by placing the dry cork into a muffle at 600 °C overnight. The final weight represents the ash content.

3.2.3. Extractives quantification with Soxhlet

The Soxhlet system allows the determination of raw material extractives. The transfer equilibrium is promoted by the recurrent contact between a sample and new sections with the extractant. The heat supplied to the distillation flask, which partially warms the extraction cavity, also contributes to maintaining a relatively high temperature in the system. Since multiple extractions can be performed simultaneously, and filtration is not required after leaching, this method is considered cost-effective.[124]

So, cork extractives were analyzed by the Soxhlet. The cork powder was poured to the cellulose thimble until it reached a 1/3 of its height. Upon the system assemble, as demonstrated in **Figure 6,** the system was left to run overnight using water as solvent and for 6 hours using as a solvent ethanol.

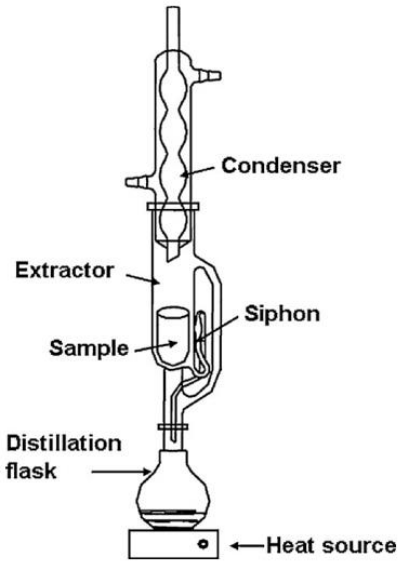

Figure 6. Conventional Soxhlet extractor. [124]

3.2.4. Sugars quantification by high-performance liquid chromatography

The distilled material obtained at the Soxhlet was used to determine the sugar content. For that 4 g of the extracts were transferred to a flask, to which 23.96 g of distilled water was added followed by sulfuric acid 97% until a weight of 25 g. The flask and its content were autoclaved for 20 min at 121°C. After it cooled down, the flask was weighted to ensure if that there was material loss. The cooled down material was filtered using a 0.22 μm membrane. The filtered material was then injected into a high-performance liquid chromatography equipment (HPLC) to quantify sugars and uronic acids. Glucuronic, galacturonic and mannuronic were used as standards for the uronic acids. Glucose, arabinose, xylose, mannose, arabinose, rhamnose, fucose and mannitol were the standards used for carbohydrates.

The HPLC analysis was performed using the following conditions: AminexHPX-87H column at 60°C and a mobile phase of 0.05 mol/L H2SO4 at a 0.6 mL/min flow rate and analyzed through a refractive index detector and UV detector at 210 nm.

A second sample was prepared for HPLC, in this case an extract-free cork was subjected to an acid hydrolysis. 0.5 g of cork was placed into a hydrolysis tube with 5 mL of 72% sulfuric acid, at a 30°C for 60 min in a water bath. The hydrolysis tube was carefully stirred every 10 min. The tube content was transferred to a flask and weighted prior being autoclaved at 121°C for 60 min. After it cooled down, the flask was weighed again to ensure that none of the content was lost. The sample was then filtered using porous crucibles and analyzed by HPLC with the same conditions mentioned previously, but in this case the sample absorbance was also read at 240 nm to determine the soluble lignin. The solid remaining in the porous crucibles was weighed to determine the insoluble lignin.

3.3. Phenolic compounds extraction from cork

The cork powder was sieved to obtain a granule size ranging from 1 to 1.6 mm of diameter.

The extraction of the phenolic compounds was performed using water, ethanol 40% and 80% as solvents at different temperatures, 40 °C and 80 °C and different agitation times, 30 min and 60 min.

The sieved cork powder was mixed with the solvent at a ratio of 1:10, with one part cork to ten parts solvent.

The container was weighed and then immersed in a water bath at 40 °C and 80°C with agitation at 150 rpm for 30 min and 60 min. Each condition was duplicated.

The samples were then weighted, the evaporated volume replaced by the respective solvent.

The samples were filtered using vacuum and paper filters with 20 to 25 μm pores.

The extracts were stored in the cold and dark pending analysis.

3.4. Dry weight determination

To determine the extract dry weight, 1 mL of the extract was placed in a previously oven-dried and weighted cup. The cup with the sample was then weighted and oven-dried at 105°C overnight. The dry weight was obtained by the difference of the weight of the cup with the sample before and after the oven-drying process.

3.5. Total phenolic content determination using the Folin-Ciocalteau method

The total phenolic content was determined using the Folin-Ciocalteau assay. This method is characterized by the chemical reduction of the mixture of molybdenum oxides and tungsten. The outcomes of the metal oxide reduction are visible as a blue color and show a wide range of light absorption, with a peak at 765 nm. The phenolic content present affects the extent of light absorption at the mentioned wavelength.[125]

Initially, standards of gallic acid were prepared with different concentrations (0-200 mg/L). Then, 20 µL of standard solutions and respective samples were added to each well, plus 100 µL of the Folin-Ciocalteau reagent (1:10 H_2O) and 80 µL of sodium carbonate (Na_2CO_3) (7.5%). Afterwards, the microplate was incubated at 40 °C, protected from light for 30 min. The absorbance was read at a wavelength of 750 nm in the microplate reader (Thermo Fisher Scientific, Lisbon, Portugal). All standards solutions and samples were analyzed in triplicate. The content in total phenols are expressed in milligrams of gallic acid per gram of sample (mg GA/g sample).

3.6. Determination of flavonoids content

Flavonoids are a group of polyphenolic compounds that result from plants' secondary metabolism. Due to the biological activities of these compounds, they are considered a subject of study. The content of flavonoids is commonly determined using colorimetric methods, such as the aluminum chloride complexation method. This procedure involves the formation of Aluminum (III) flavonoid chelates, with aluminum acting as a complexing agent.[126]

Primarily, standards of catechin were prepared with different concentrations (0-200 mg/g). Then, 24 µL of standard solutions and respective samples were added to each well, plus 28 µL of sodium nitrite ($NaNO_2$) (50 g/L). After a wait of 5 min at room temperature, 28 µL of aluminum chloride ($AlCl_3$) (100

g/L) was added. And 6 min later, 120 µL of sodium hydroxide (NaOH) (1.0 M) was added. Immediately after, the absorbance was read at a wavelength of 510 nm in the microplate reader (Thermo Fisher Scientific, Lisbon, Portugal), with a shaking of 30 seconds. All standards solutions and samples were analyzed in triplicate. The content in total phenols are expressed in milligrams of catechin per gram of sample (mg CAT/g sample).

3.7. Antioxidant activity

3.7.1. FRAP (Ferric Reducing Antioxidant Power)

Ferric Reducing Antioxidant Power, also known as FRAP, is a method used to determine iron reduction in biologic fluids and aqueous solutions of pure compounds. In this method, the reduction of Fe3+ to Fe2+ (ferric to ferrous ion reduction) takes place, at a low pH (**Figure 7**). This results in a blue colored ferrous-tripyridyltriazine complex, whose absorbance can be read at 593nm. The absorbance values are linear with the antioxidants´ concentration.[127,128]

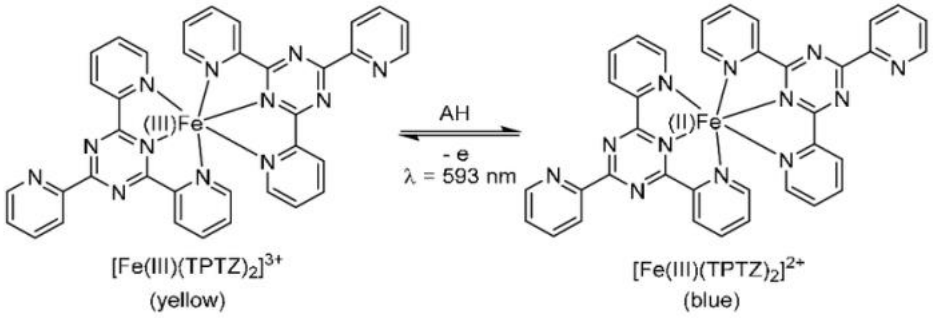

Figure 7. Reduction of Fe^{3+} to Fe^{2+}. [129]

Initially, the FRAP working solution was prepared by mixing 10 volumes of acetate buffer (330 mM, pH = 3.6), 1 volume of TPTZ (2,4,6-Tripyridyl-s-Triazine) (40 mM) previously dissolved in HCl (40 mM) and 1 volume of ferric chloride (20 mM). This solution is prepared daily and heated 10 minutes before use.

Standards of Trolox were prepared with different concentrations (0– 0.625 mmol/L). Then, 140 µL of the FRAP solution are added, plus 10 µL of each standard solution and sample. The reaction was incubated at 37 °C protected from light for 30 min and the absorbance was read at 593 nm, in the microplate reader (Thermo-Fisher Scientific, Oporto, Portugal). All standards solutions and samples were analyzed in triplicate and the results of the antioxidant power capacity were expressed as millimoles of Trolox per gram of sample (mmol TEAC/g sample).

3.7.2. DPPH method

DPPH assay is used to determine if a substance has antioxidant potential by taking advantage of the free radical α,α-diphenyl-β-picrylhydrazyl. Due to the spare electron´s delocalization over the entire molecule, which prevents it from dimerization, DPPH is classified as a stable free radical. The delocalization also contributes to the deep violet color, which, when in ethanol solution, absorbs at 520nm. In this assay, the samples with higher antioxidant activity will turn to a yellow tone, while the samples with a lower antioxidant content will keep the purple color. The higher antioxidant potential is correlated with the higher inhibition percentage and, consequently, with lower absorbance.[129,130] This is a method that can be applied to the general antioxidant capacity like cysteine, ascorbic acid, glutathione, among others.[131,132]

A mixture was prepared by diluting 0.5 mL of DPPH (8.87 mM) into 26 mL of MeOH/H_2O (70:30, v/v), adjusting its absorbance to 1.000 at a wavelength of 520 nm. Standards of Trolox were prepared with different concentrations (0.078 – 1.25 mmol/L). Then, 190 µL of the $DPPH^{\cdot}$ solution are added, plus 10 µL of each standard solution and sample. A blank was also prepared by adding 10 µL of MeOH/H_2O (70:30, v/v) to the $DPPH^{\cdot}$ solution.

The reaction was incubated at room temperature protected from light for 15 min, and the antioxidant activity was evaluated by measuring absorbance at 520 nm, using a microplate reader (Thermo-Fisher Scientific, Oporto, Portugal). To calculate the inhibition percentage of each standard solution and sample, the following formula was used:

$$\% \text{ Inhibition} = 100 \times (Abs520BLANK - Abs520SAMPLE / Abs520BLANK)$$

All standards solutions and samples were analyzed in triplicate and the results of the radical scavenging capacity were expressed as millimoles of Trolox per gram of sample (mmol TEAC/g sample).

3.7.3. ABTS method

Antioxidant activity can also be determined by the ABTS method, which is frequently used to evaluate the antioxidant activity of food products and plant extracts. The $ABTS^{\cdot}+$ cation, with an absorption maximum between 600 nm and 734 nm, is produced by oxidizing ABTS, also called 2-2´-Azino-bis (3 ethylbenzothiazoline-6-sulfonic acid).[133]

This procedure compares antioxidant power to eliminate the $ABTS^{\cdot}+$ cation produced in the reaction, to a Trolox standard solution. By suppressing the long wave absorption spectra, the blue green $ABTS^{\cdot}+$ cation is reduced by a hydrogen-donor antioxidant, and the radical cation transforms back into the colorless neutral state. In this assay, the samples with higher antioxidant content will show a lighter

color, as the ABTS assay let us know the inhibition percentage. Lower absorbance implies a higher inhibition percentage and, consequently, a higher antioxidant capacity.[134,135]

Initially, 88 µL of potassium persulfate (148 mM) are added to an ABTS solution (7 mM), and the mixture is left to rest for 12-16 hours protected from light and at room temperature, to reach its stable oxidative state. After this period, the working solution was prepared by diluting the $ABTS^{\cdot}$+solution in sodium acetate buffer (20 mM, pH = 4.5) until an absorbance of 0.700 ± 0.020 is obtained at a wavelength of 734 nm. Standards of Trolox were prepared with different concentrations (0 – 0.900 mmol/L). Then, 188 µL of the $ABTS^{\cdot}$+solution is added, plus 12 µL of each standard solution and sample. Blank was also included by adding 12 µL of distilled water to the $ABTS^{\cdot}$+solution. The reaction was incubated at room temperature protected from light for 30 min. Then, the antioxidant activity was evaluated by measuring absorbance at 734 nm, using a microplate reader (Thermo-Fisher Scientific, Oporto, Portugal). To calculate the inhibition percentage of each standard solution and sample, the following formula was used:

$$\% \text{ Inhibition} = 100 \times (Abs734BLANK - Abs734SAMPLE / Abs734BLANK)$$

All standards solutions and samples were analyzed in triplicate and the results of the radical scavenging capacity were expressed as millimoles of Trolox per gram of sample (mmol T/g sample).

3.8. Cells maintenance and assays

3.8.1. Cells thawing

Cell lines are stored in liquid nitrogen to maintain their viability, and it is necessary to thaw them for them to regain biological activity. For that, the cryovial containing the cells are thawed quickly in a water bath at 37 °C. Upon which the cells are transferred to a tissue culture flask containing cell culture medium with foetal bovine serum, antibiotic, and antifungal agents. The cells are then placed into an incubator at 37°C with 5% CO_2 atmosphere.

3.8.2. Cell culture

HaCaT, L929 and HEK cells were cultured in tissue culture flasks with essential medium, composed by 89% DMEM medium, 10% bovine serum and 1% antibiotic. The cells were maintained in a 5% CO_2 atmosphere, at 37°C and observed on a daily basis using an inverted microscope. The cell culture was subcultured when it reached 70 % confluence.

3.8.3. Sample preparation

The cork extract was lyophilized. A stock solution of 1 mg/mL was prepared by diluting the lyophilized extract in water. From this solution, successive dilutions were made to achieve concentrations of 0.5; 0.25; 0.125; 0.0625 mg/mL. These solutions were further diluted in essential culture resulting in the following concentrations: 0.3; 0.15; 0.075; 0.0375; 0.01875 mg/mL.

3.8.4. Cell viability assay

The resazurin assay is widely used colorimetric and fluorometric method to assess cell viability and metabolic activity. Its nomenclature finds its roots in the chemical entity resazurin also known as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), the pivotal constituent of the assay.[136,137]

Functionally, the resazurin assay hinges on the transformation of the blue, non-fluorescent resazurin dye into the intensely pink, highly fluorescent resorufin compound, catalyzed by viable cells. The transformation occurs through the transfer of electrons from the cellular reducing environment to resazurin, resulting in a shift from a blue to a pink hue and a simultaneous increase in fluorescence.[137,138]

The versatility of the resazurin assay makes it applicable in various domains, including areas such as cell biology, drug discovery, and toxicology.

For the resazurin assay, the different cell lines were seeded into 96-well plates at a concentration of $0.32x10^4$ cells/mL and incubated overnight at a 37 °C and 5% CO_2 atmosphere.

The cell culture medium was removed and replaced by cell culture medium containing various concentrations of extracts for analysis. Two negative controls were used, in the first control cells were incubate with medium and PBS to mimic the protein dilution that occurs when the extract was added to the culture medium; in the second control cells were incubated with essential medium. The cell culture was incubated at 37 °C in a 5% CO_2 atmosphere for a period of 24h.

Upon the 24h the medium was removed from each well and washed with Phosphate-buffered saline (PBS) twice.

Resazurin at 0.5mM in PBS was diluted in essential medium in a 1:10 ratio. 200 μL of resazurin solution was placed in contact with the cells and incubated in a 5% CO_2 atmosphere at 37°C for 2 hours – The fluorescence was measured using an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

3.9. Enzymes assays

3.9.1. Tyrosinase inhibition assay

The tyrosinase inhibition assay is a frequently employed technique in the fields of biochemistry and pharmacology for evaluating the capacity of compounds to hinder the activity of the enzyme known as tyrosinase. This enzyme plays a pivotal role in the melanin production pathway by facilitating two essential reactions: the conversion of tyrosine into L-DOPA (dihydroxyphenylalanine) through hydroxylation and the subsequent oxidation of L-DOPA into dopaquinone. During this assay, the level of tyrosinase activity is measured in the presence of a potential inhibitor and then compared to both a negative and a positive control. Inhibition efficacy is quantified by measuring the rate of dopaquinone generation or by monitoring the reduction in absorbance at a wavelength of 490 nm. A decline in tyrosinase activity serves as an indicator of the inhibitory effect of the tested compound.[139–141]

In this assay, two controls were used: 10 µl of DMSO (10% in water) served as the negative control, and 10 µL of kojic acid (1 mg/mL) was used as the positive control. Next, 10 µL of each sample was placed in a 96-well microplate. To this, 20 µl of tyrosinase (1000 U/mL) and 170 µl of a mixture containing L-tyrosinase solution (1mM), phosphate buffer (50 mM, pH 6.5) and distilled water in a 10:10:9 ratio were added. Finally, the microplate was incubated for 10 min at 37°C, and the absorbance was measured at 490 nm.

All the samples and controls were added in triplicate.

3.9.2. Elastin inhibition assay

The elastase inhibition assay is a technique used to measure the ability of a substance to inhibit the activity of elastase enzymes. Elastase is a type of enzyme that breaks down elastin, which is a protein found in connective tissues like skin and blood vessels. Inhibition of elastase activity is interesting in various fields, including drug discovery, as it can be related to conditions such as inflammation, tissue degradation and certain diseases. The enzyme elastase is produced by neutrophils and is responsible for maintaining the elasticity of the skin and blood vessels. In this assay, a synthetic substrate that mimics elastin, the target of elastase, is used. When elastase is active, it cleaves the substrate, resulting in a measurable change is some property of the substrate. To assess the inhibitory effect of a substance, it is added to the reaction mixture along with elastase and the substrate. The role of the inhibitor is to interfere with or block elastase activity.[36,142]

To determine the elastin inhibition, 50 μl of each sample was placed into a 96-well plate, followed by 160 μL of Tris-HCl buffer (0.2 mM, pH 8) and 20 μL of Ala substrate (0.8 mM) and incubated at room temperature protected from light for 10 min. 20 μL of elastase enzyme (1 U/mL) was added to each well and incubate for 20 min. Tris-HCl buffer was used negative control. The absorbance was read at 410 nm.

All the samples and controls were added in triplicate.

3.10. Data analysis

The results were presented as the mean values of three independent experiments ± standard derivation. For statistical analysis of data, GraphPad Prism 8.0.1 (GraphPad Software. Inc.) was used. The 3way ANOVA tests, 2way ANOVA tests were performed to consider statistical significance when necessary. When *p*-value was < 0.05 significant differences were contemplated. For TPC and antioxidant activity, Pearson correlation was done using Microsoft Excel.

4. Results and Discussion

4.1. Cork composition

To evaluate the composition of cork powder, several assays were conducted, including assessments of lipid, ash, sugar, and protein content.

Table *1* describes the e composition of the raw material. As it can be seen the raw material has approximately 3% of ashes. Depending on the tree where the cork was extracted, the chemical content might vary; the ash content found on the literature, varies from 1% to 5%.[4,143–145]

As for crude protein, cork has less than 5% of protein content. Additionally, it did not show a high lipidic content as the crude lipid is only 1.48%, since it is a tree bark. These results followed the ones reported for *Pinus pinaster* bark, as the protein and fat contents are also low with 1.64 ± 0.03% and 2.54 ± 0.26%, respectively.[146]

The sample showed a 5% of water extractives content, which was similar to the ethanol extractives that summed to about 4%. As already mentioned, cork composition varies with the extraction source and in the literature, water extractives values are mentioned to vary between 3% and 10% and ethanol extractives values varying from 2% to 6%. The components that are extracted with ethanol and water are polar compounds, such as phenolics and polyphenolics.[4,143,145]

Cork has in its composition a high content of carbohydrates such as glucose, xylose, rhamnose and arabinose, which it is the opposite of what happens when uronic acids are analyzed. Cork has xylose,

being the one with the higher percentage, followed by glucose. Rhamnose and arabinose are also present in the cork composition, however with lower content. Literature shows that glucose is usually the most prominent sugar in cork with approximately 50% of total monosaccharides, followed by xylose going between 28% to 37%, arabinose with 6% to 12% and, finally, rhamnose with 1% to 2%.[4,145] The samples have a lower sugar content and the most prominent sugar is xylose. While the typical uronic acid content in cork is approximately 12%, the samples exhibited a significantly lower value of 0.92%, which contrast to the figures reported in the literature.[48]

The lignin in the samples is shown to be 70% of the cork composition, which consists of the combination of insoluble lignin and soluble lignin. The insoluble lignin is more prominent making up 60% of the composition, and the soluble lignin is approximately 10% of the total cork composition. Literature shows that insoluble lignin can range from 13% to 24% and soluble lignin can vary from 1% to 3%.[4,143,145] The samples have a lignin content higher than the one present in the literature.

The difference in the content between the samples and the literature might lie in some problem that could have happen in the hydrolysis process or it can be due to the raw material not being directly extracted from the tree, but being a by-product from the cork stoppers process, which could have changed the samples composition.

Table 1. Chemical characterization of Cork, expressed as percentage of dry raw material weight (composition by 100 g).

Composition (%)	
Ash	2.98 ± 0.41
Crude Protein	4.89 ± 0.02
Crude Lipid	1.48 ± 0.05
Water extractives	5.23 ± 0.85
Ethanol Extractives	4.22 ± 1.60
Carbohydrates	24.06 ± 0.90
Uronic acids	0.92 ± 0.04
Glucose units	7.19 ± 0.37
Xilose units	13.54 ± 0.45
Rhamnose units	0.32 ± 0.01
Arabinose units	2.09 ± 0.06

Acid Insoluble Lignin	60.17 ± 1.24
Acid Soluble Lignin	10.93 ± 0.63
Lignin	70.80 ± 1.86

4.2. Extraction yield

The extraction procedure aimed at extracting phenolic compounds, therefore several conditions were chosen. These conditions encompassed the use of different solvents (H_2O, ethanol 40%, and 80%), varying temperatures (40°C and 80°C), and different extraction times (30 min and 60 min). The inclusion of multiple extraction conditions allowed us to assess the influence of different variables on the extraction of phenolic compounds.

The extraction condition that showed the highest yield was ethanol 80%, at 60 min and 80°C (**Figure 8**) with 19.13 ± 0,124%. When the temperature subsided to 40°C there was a decrease in the extraction yield. When the extraction time decreased to 30 min there was also a decrease of the yield percentage.

Within all the different extraction conditions, water shows a significant decrease in the extraction yield compared to the ethanolic extractions. Whereas the ethanolic extractions only show a significant difference in the conditions 30 min 40°C and 60 min 80°C.

The samples showed an extraction yield between 5% to 20%, which goes in agreement with what is stated in the literature, in which yields range from 7% to 11%. In the literature, water extractions show a 8% yield, which is similar to the water samples; ethanol 40% and ethanol 80% are stated to have 9% and 10% yield, respectively, which are close to the 40°C extractions yield, and lower than the 80°C extractions, in the respective solvents.[147]

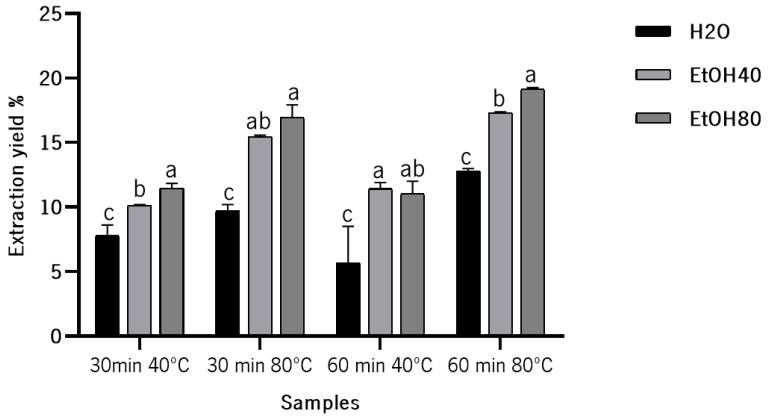

Figure 8. Cork extraction yield (%). The extractions were done with different solvents (H2O, Ethanol 40% and 80%), different temperatures (40°C and 80°C) and different extraction times (30 min and 60 min). Values are expressed as mean ± SD. Different letters show different significances (*p*-value < 0.05) between groups for the same condition.

4.3. Total phenolic content

Cork, as a tree bark, is known to have phenolics for its content, therefore the total phenolic content salvage was assessed for the cork extracts achieved with different solvents, temperatures, and extraction times. The results regarding the cork extracts total phenolic content (TPC) can be found in **Figure 9**.

The total phenolic content of the extracts was determined by the Folin-Ciocalteu method. Gallic acid was used as standard, for the calibration curve (**Appendix I** – Gallic acid standard curve (Folin-Ciocalteau method)).

The sample with the highest phenolic content was ethanol 40% at 60 min and 80°C (**Figure 9**), with 29.80 ± 2.694 mg GAE/g. With the decrease of temperature and extraction time, the phenolic content suffers decrease to values of 21.24 ± 3.11 mg GAE/g and 25.69 ± 2.758 mg GAE/g, respectively. These results are not within the limits reported in literature in analysis to pine bark extracts.[146] The phenolic content reported was more than ten times higher than the TPC obtained from the cork extracts. However, the trend is shown to be similar, where water and high percentage ethanolic extracts have less phenolic than lower ethanolic extracts.[146]

Within the same condition, water extracts show significantly less phenolic content than ethanolic extracts. However, the only conditions where ethanol 40% and 80% extracts are significantly different are 30 min 80°C and 60 min 80°C.

The presence of phenolic compounds in the 80°C extracts renders cork a compelling material, considering that these compounds typically undergo degradation at temperatures exceeding 50°C. This

occurrence may be attributed to the existence of one or more cork components capable of shielding the phenolic compounds from degradation caused by elevated temperatures.[148]

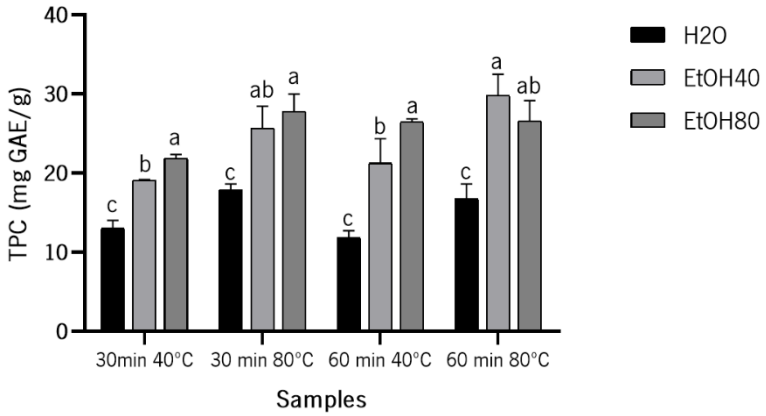

Figure 9. Total phenolic content (TPC). TPC (mg GAE/g sample) determined by Folin-Ciocalteu method, for cork extracts obtained with different solvents, temperatures, and extraction times. Values are expressed as mean ± SD. Different letters show different significances (p-value < 0.05) between groups for the same condition.

4.4. Flavonoids content

Flavonoids are extractable components of the cork; therefore, the flavonoid content was assessed for the cork extracts achieved with different solvents, temperatures, and extraction times. The results regarding the cork extracts flavonoid content (FC) can be found in **Figure 10**.

The flavonoid content of the extracts was determined by the Aluminum chloride assay. Catechin was used as standard for the calibration curve (**Appendix II** – Catechin standard curve for flavonoids content determination).

The sample with the highest FC was ethanol 80% at 60 min and 80°C (**Figure 10**) with 36.96 ± 1.787 mg CAT/g. When the temperature and extraction time declined there was decrease in the FC to 32.04 ± 1.669 mg CAT/g and to 31.71 ± 2.303 mg CAT/g, respectively.

In all extraction conditions, the ethanolic extracts show a significantly higher flavonoid content than water extracts. Whereas, between ethanol 40% and 80%, the only conditions where there is a significant difference are 30 min 40°C and 60 min 80°C.

These results are not within what is assessed in the literature in analysis to pine bark extracts.[146]

The pine bark aqueous extract flavonoid content reported in literature was more than ten times higher than the aqueous cork extract, whereas the pine bark´s ethanolic extracts FC were about five times superior to the ethanolic cork extract FC. Additionally, the cork extracts do not follow the same trend as the pine bark extracts, where the highest FC is shown to appear at ethanol 50% and not at high ethanolic extracts.[146]

The existence of flavonoids in the high-temperature extracts makes cork an intriguing material, given that these compounds are typically prone to degradation as the temperature rises. This phenomenon can be ascribed to the presence of one or more cork components that possess the ability to protect the phenolic compounds from deteriorating under elevated temperatures.[149]

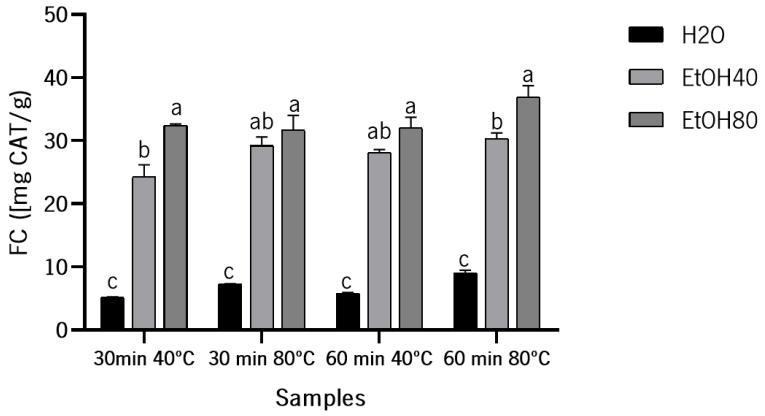

Figure 10. Flavonoid content (FC). FC (mg CAT/g sample) determined by Aluminum chloride method, for cork extracts obtained with different solvents, temperatures, and extraction times. Values are expressed as mean ± SD. Different letters show different significances (p-value < 0.05) between groups for the same condition.

4.5. Antioxidant activity

The presence of phenolic and flavonoids may exhibit antioxidant activity therefore, the antioxidant activity was assessed for the cork extracts achieved with different solvents, temperatures, and extraction times using three methods: FRAP, DPPH and ABTS. The results regarding the cork extracts antioxidant activity can be found in **Figure 11**. Trolox was used as standard for the calibration curves for all methods (**Appendix III** – Trolox standard curve (FRAP method), **Appendix IV** – Trolox standard curve (DPPH method) and **Appendix V** – Trolox standard curve (ABTS method)).

The sample that shows the highest antioxidant activity was ethanol 40% at 60 min and 80°C (**Figure 11**) with 0.309 ± 0.023 mmol TEAC/g when used FRAP assay, 0.299 ± 0.029 mmol TEAC/g when assessed by the DPPH method and 0.307 ± 0.024 mmol TEAC/g, when determined by the ABTS assay.

The sample that showed the lowest antioxidant activity was water at 60 min and 40°C (**Figure 11**) with 0.151 ± 0.016 mmol TEAC/g when determined by the FRAP assay, 0.128 ± 0.013 mmol TEAC/g when assessed by DPPH method and 0.144 ± 0.011 mmol TEAC/g when used the ABTS assay.

In the same extraction conditions, and in all three assays performed, water extracts show a significantly lower antioxidant activity when compared to the ethanolic extracts. It is also a trend in FRAP, DPPH and ABTS that there is a significant higher antioxidant activity between ethanol 40% and 80% extracts in the 60 min 80°C condition, as well as there is no significant difference between the ethanolic extracts in the 40°C extractions.

When comparing the three assays performed, FRAP, DPPH and ABTS, there seems occur a concordance between the results, where water extracts present the least antioxidant activity, and the ethanol 40% extracts are the ones with the highest antioxidant activity.

The results mentioned in the literature for the DPPH assay are 0.164 ± 0.001 mmol TEAC/g sample for the aqueous extract and 0.175 ± 0.041 mmol TEAC/g sample for the ethanolic extract.[150] The results from the cork extracts, on the DPPH assay, range from 0.128 ± 0.013 mmol TEAC/g to 0.194 ± 0.005 mmol TEAC/g for the water extracts, 0.204 ± 0.009 mmol TEAC/g to 0.299 ± 0.029 mmol TEAC/g for ethanol 40% extracts and 0.268 ± 0.005 mmol TEAC/g to 0.207 ± 0.014 mmol TEAC/g for ethanol 80%. The aqueous cork extract follows what is stated in the literature, whereas the ethanolic extracts show a higher antioxidant activity.

For the ABTS assay, the results reported in the literature are 0.118 ± 0.002 mmol TEAC/g sample for the water extracts and 0.425 ± 0.046 mmol TEAC/g sample for the ethanolic extract.[150] The cork extracts antioxidant activity determined by the ABTS assay ranges from 0.144 ± 0.011 mmol TEAC/g to 0.192 ± 0.010 mmol TEAC/g for the aqueous extracts, 0.192 ± 0.009 mmol TEAC/g to 0.307 ± 0.024 mmol TEAC/g for the 40% ethanolic extracts and 0.198 ± 0.006 mmol TEAC/g to 0.233 ± 0.015 mmol TEAC/g for the highest ethanolic extracts. The aqueous cork extract show a higher antioxidant activity than what is stated in the literature, however the ethanolic extracts show a decrease antioxidant compared to what Is shown in the literature.

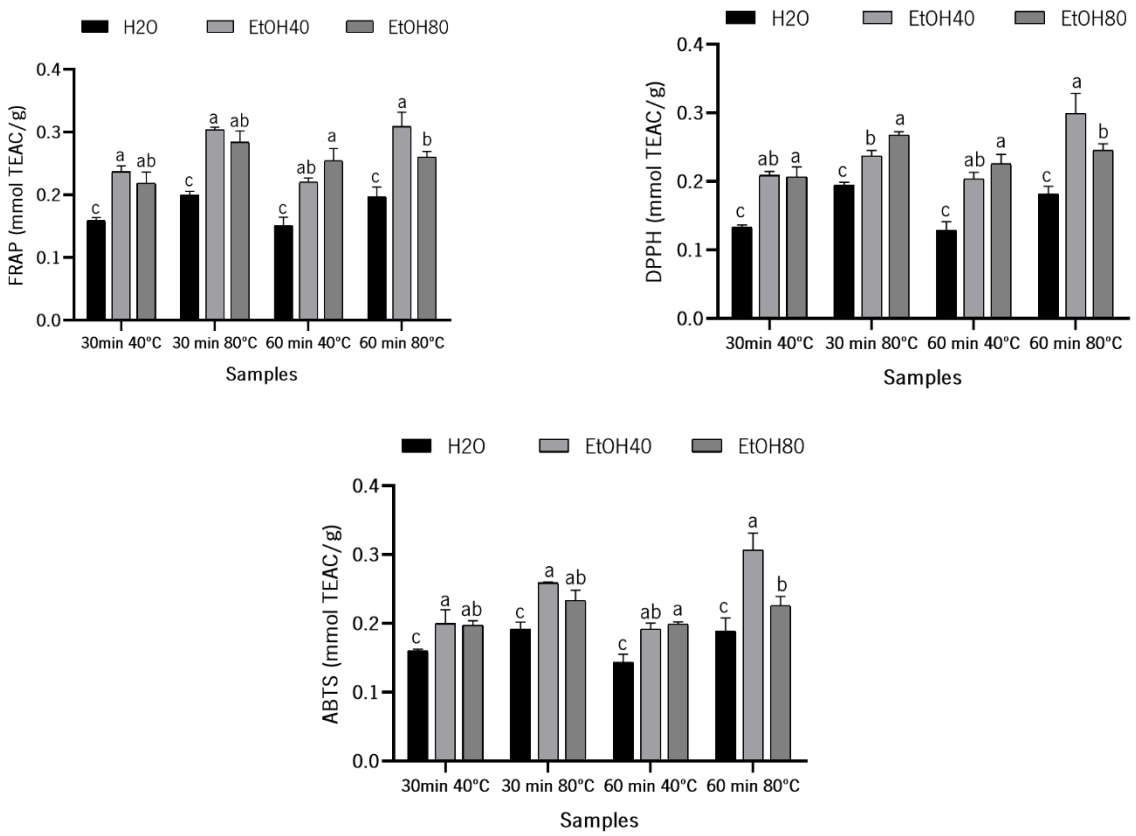

Figure 11. Antioxidant activity. Antioxidant activity obtained by FRAP, DPPH and ABTS (mmol TEAC/g sample) assays for the cork extracts obtained with different solvents, temperatures, and extraction times. Values are expressed as mean ± SD. Different letters show different significances (*p*-value < 0.05) between groups for the same condition.

4.6. Total phenolic content and antioxidant activity Pearson correlation

To analyze the how the variables antioxidant capacity (FRAP, DPPH and ABTS) and total phenols content correlated we used the Pearson correlation method. The Pearson´s correlation is the most popular technique for analyzing numerical variables, assigning values between -1 and 1, where -1 is total negative correlation, 0 means no correlation and 1 is total positive correlation.[151,152]

Table *2* shows that FRAP and ABTS assays have a correlation of 0.9485 and 0.9633, respectively, which is very close 1, and the DPPH method has a lower correlation to the TPC of 0.8608. In all assays there is a positive correlation with the total phenolic content of the samples, meaning that when the TPC increases, the antioxidant activity will also increase, demonstrating that phenolic compounds have a contribution to the antioxidant activity.

Table 2. Pearson correlation of Total phenolic content (TPC) and Antioxidant activity

Variables	TPC *vs* FRAP	TPC *vs* DPPH	TPC *vs* ABTS
Correlation value	0.9485	0.8608	0.9633

4.7. Enzymes inhibition assay

The samples chosen for the enzyme inhibition assay were the extracts at 40°C and extracted with water and ethanol 40% and 30 min and 60 min extraction times. This choice was based on the extract solubility in water.

As the main target for this project was the skin, the extract enzyme inhibition activity was assessed for two important skin enzymes: tyrosinase and elastase.

As the initial concentrations used for the tyrosinase inhibition assay was not known, the comparison of the inhibition percentages was not possible. However, as stated in **Table *3***, the cork extracts can inhibit this enzyme.

Table 3. Cork extracts tyrosinase inhibition percentage

Samples	Tyrosinase inhibition %
H_2O 30 min	8.64
H_2O 60 min	3.39
Ethanol 40% 30 min	22.23
Ethanol 40% 60 min	31.19

For the elastase inhibition activity, the cork extracts did not show the capability to inhibit this enzyme.

To our knowledge, this is a characteristic that is yet to be studied in cork extracts.

4.8. Cells viability

The samples chosen for the resazurin assay were the extracts at 40°C and extracted with water and ethanol 40% and 30 min and 60 min extraction times. This choice was based on the extract solubility in water.

4.8.1. HaCaT

HaCat cells, a renowned and extensively employed line of immortalized human keratinocytes, are derived from adult human skin. They serve as a valuable *in vitro* model for researching into the realms of skin biology, wound healing, and a wide spectrum of dermatological and skin-related research. Their capacity for continual division while retaining many characteristics of typical human keratinocytes renders them an invaluable asset for scientific exploration within the fields of dermatology and skin biology.[113,114]

The extraction condition that was able to increase cell metabolic activity the most was Water at 30 min extraction time and the concentration that was able to increase cell viability the most was 0.0375 mg/mL, with 155.5 ± 7.049% metabolic activity (**Figure 12**). With the samples concentration decrease there was an increase in cell metabolic activity. The 0.3 mg/mL samples decrease cell viability and the 0.15 mg/mL samples do not change the cell normal function.

When performed the 3way ANOVA, the difference concentration x solvent is significative with a *p*-value of 0.0131 (*), whereas the concentration x time is non significative (ns). This might happen due to different solvents being able to extract diverse compounds, that can affect cells in various ways. Also, with the time not being a significant variable, we are able to minimize the energetic costs and perform the extraction in less time.

The results for cell viability stated in the literature show that spruce extracts are able to increase cell viability. It Is shown that spruce extracts increased the cell viability to 130% compared to the control (100%) with the lower concentration extracts. Nonetheless, with high concentration samples, the cell viability remains the same as the control.[153] The cork extracts were able to induce cell metabolic activity up to 155 ± 7.049% compared to the 100% control. Additionally, the cork extracts follow the same trend as the spruce extracts, where increasing the samples concentration, decreases cell viability.

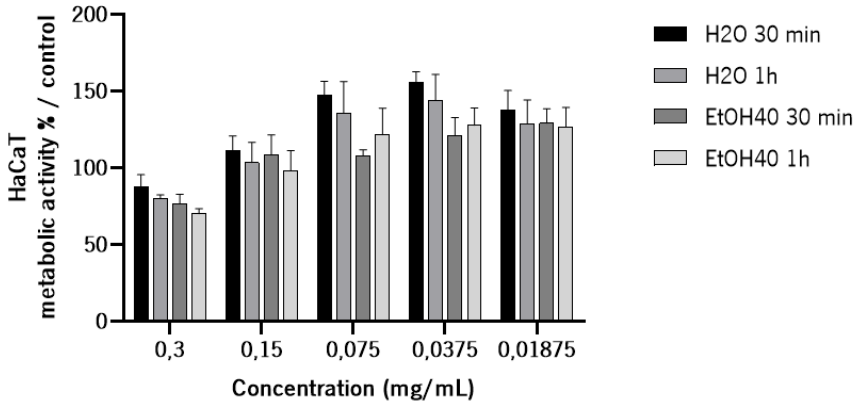

Figure 12. HaCaT metabolic activity. Cellular viability (%) of cork extracts, obtained with different solvents and extraction times, against skin fibroblast (HaCaT). Values are expressed as mean ± SD.

4.8.2. L929

L929 cells belong to an immortalized murine (mouse) fibroblast cell line. They have gained extensive utilization in diverse research investigations, with a notable presence in the realms of immunology, virology, and cell biology. Originating from the subcutaneous connective tissue of a C3H/An mouse, L929 cells were immortalized, granting them the ability to continuously divide and thrive in culture. A prominent feature of L929 cells is their susceptibility to interferon, rendering them a common choice in research pertaining to antiviral and immune responses. These cells serve as a model system for exploring cell growth, cell signaling, and the cells reactions to different stimuli. These cells have been harnessed to explore diverse subjects including viral infections, inflammation, cell proliferation, and apoptosis.[116,154]

The condition that was able to increase the cell viability the most was Water at 60 min in 0.075 mg/mL concentration with 197.6 ± 21.23% metabolic activity (**Figure 13**).

The solvent Ethanol 80% does not change the cell viability in the concentrations tested, whereas all the other solvents, in all concentrations are able to increase the cells metabolic activity.

When performed the 3way ANOVA, the difference concentration x solvent is significative with a *p*-value of 0.0002 (***), and the concentration x time is also significative with a *p*-value of 0.0012 (**). This might happen due to distinct solvents being able to extract various compounds, that lead to different cell responses.

The results for L929 cell viability asserted in the literature show that pine bark extracts can increase cell viability. It Is shown that pine bark extracts increased the cell viability to almost 150% with lower concentrations samples. With the increase of sample concentration, there was a decrease of cell viability.[146] The cork extracts were able to increase the L929 cells viability up to 197.6 ± 21.23% metabolic activity/control, which is close to what is mentioned in the literature. Furthermore, the L929 cells also follow the same trend stated in the literature, where the increase of samples concentration leads to a decrease in cell viability.

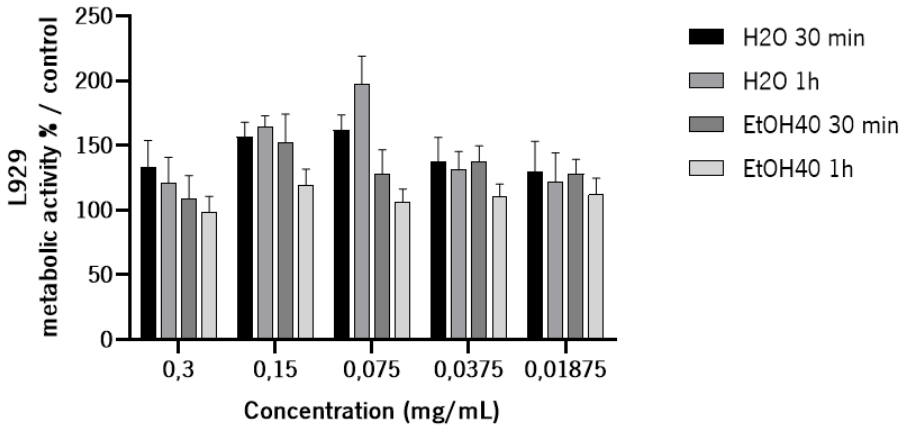

Figure 13. L929 metabolic activity. Cellular viability (%) of cork extracts, obtained with different solvents and extraction times, against mouse fibroblast (L929). Values are expressed as mean ± SD.

4.8.3. HEK

HEK cells, an abbreviation for Human Embryonic Kidney cells, represent a widely utilized human cell culture in the realm of biological and medical research. Despite their nomenclature, they are derived from the kidney tissue of an aborted fetus, specifically sourced from the human embryonic kidney. HEK293 cells, originated in 1973, are widely recognized in molecular biology and biotechnology. These are frequently utilized in protein expression and research across various domains, including vaccine development and gene function analysis. HEK cells are indispensable in a spectrum of scientific inquires and play a significant role in biotechnology, research focused on human genetic diseases and pharmaceutical advancements.[120,155,156]

The condition that was able to increase cell viability the most was Water at 30 min extraction time; however, the only exception is at 0.075 mg/mL, ethanol 40% at 30 min that is also the sample with the highest metabolic activity percentage of 159.8 ± 17.29% metabolic activity (**Figure 14**).

Water samples do not show variation of the metabolic activity in the different concentrations' tests; however, all the samples show an increase in cell viability. Whereas ethanolic samples show an increase of viability with the concentration decrease, followed by a slight decrease of activity in the two lower concentrations; additionally, the 0.3 mg/mL concentration does not increase cell metabolic activity.

When performed the 3way ANOVA, the difference concentration x solvent is significative with a *p*-value < 0.0001 (****), whereas the concentration x time is non significative (ns). This phenomenon can be attributed to the diverse solvents' capacity to extract various compounds, which can impact cells in numerous ways. Additionally, by not having time as a significant variable, we can reduce energy costs and expedite the extraction process.

The results for HEK cell viability affirmed in the literature show that pine bark extracts can induce cell viability. It Is exhibited that these extracts improved the cell viability to 150% with lower concentrations samples. With the increase of extract concentration, there was a decrease of cell viability.[146] The cork extracts were able to increase the HEK cells viability up to 159.8 ± 17.29% metabolic activity, which is close to what is mentioned in the literature. Nevertheless, the HEK cells in contact with cork extracts do not show the same behavior as the one mentioned in the literature and the variation of samples concentration does not show a constant trend in the different solvents used.

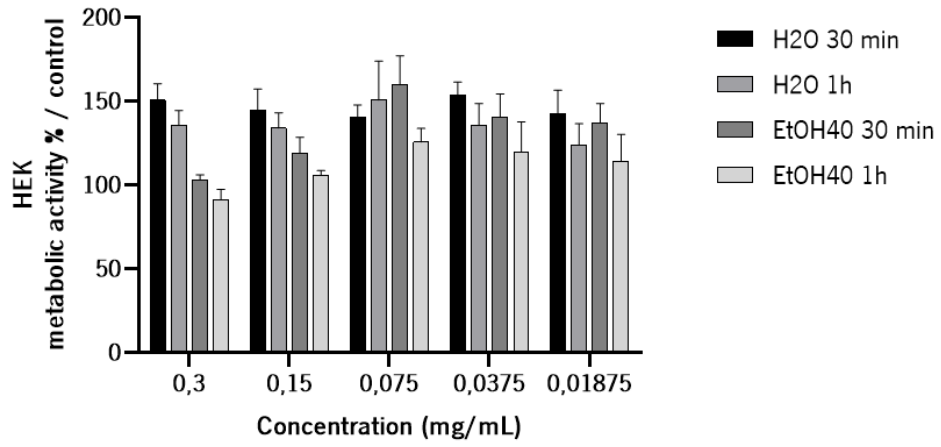

Figure 14. HEK metabolic activity. Cellular viability (%) of cork extracts, obtained with different solvents and extraction times, against human embryonic kidney cells (HEK). Values are expressed as mean ± SD.

5. Conclusion

With the biotechnology available today, we can effectively mitigate the environmental problems arising from industrial waste. By repurposing what would traditionally be considered by-products and introducing these materials to other industries, we can establish a circular economy and foster symbiotic industrial concepts.

In this work cork was used as the matrix and a promising source of phenolic compounds, aimed to be incorporated into the pharmaceutical industry.

Cork, as a raw material, was chemically characterized. It showed a low protein and lipid content of 4.89 ± 0.02% and 1.48 ± 0.05%, respectively. It also showed 5.23 ± 0.85% of water extractives and 4.22 ± 1.60% of ethanol extractives. Additionally, cork has 24.06 ± 0.90% of carbohydrates, that are divided into glucose, xilose, rhamnose and arabinose. Lastly, the lignin content reached 70.80 ± 1.86%, which is composed by soluble and insoluble lignin.

The bioactive compounds´ extraction was done using different solvents (Water, Ethanol 40% and 80%), temperatures (40°C and 80°C) and extraction times (30 min and 60 min). The extraction yield ranged from 5.674 ± 2.829% to 19.132 ± 0.124%.

To determine the total phenolic content of the extracts, the Folin-Ciocalteu method was performed and is showed that the extract with the highest TPC was ethanol 40% at 60 min and 80°C with 29.80 ± 2.694 mg GAE/g, while the one with the least TPC was Water at 60 min and 40°C with 11.81 ± 0.905 mg GAE/g. The extracts´ flavonoid content was also determined with the Aluminum chloride assay, which showed that the ethanol 80% at 60 min and 80°C has the highest flavonoid content with 36.96 ± 1.787 mg CAT/g, whereas the Water at 30 min and 40°C shows the least flavonoid content with 5.209 ± 0.049%.

To determine the antioxidant activity of the cork extracts, three assays were performed: FRAP, DPPH and ABTS. All assays showed that the extract with the highest antioxidant activity was ethanol 40% at 60 min and 80°C with 0.309 ± 0.023 mmol TEAC/g when doing the FRAP assay, 0.246 ± 0.009 mmol TEAC/g from the DPPH assay and 0.307 ± 0.024 mmol TEAC/g when performing the ABTS assay.

Since the target of this project was the skin, two important skin enzymes inhibition was studied: tyrosinase and elastase. The cork extracts were able to inhibit the tyrosinase enzyme, however, they were not able to inhibit elastase.

Furthermore, the extracts were tested in three skin cell lines: HaCaT (human immortalized keratinocytes), L929 (mouse fibroblasts), and HEK (human embryonic kidney cells), to assess how the extracts could modify the cell viability. Low concentration cork extracts were able to increase the cells

metabolic activity %/control; in HaCaT cells, the extracts improved cell viability up to 155.5 ± 7.079% metabolic activity/control, in L929 cell viability also rose to 197.6 ± 21.23% metabolic activity/control and, lastly, in HEK cells, the viability increased to 159.8 ± 17.29% metabolic activity/control.

This shows that cork can be a promising source for bioactive compounds that are benefic to the pharmaceutical industry, specifically for skin treatments. This strategy encourages the utilization of natural compounds in the cosmetics industry, which is in line with the growing societal demand for the use of natural resources in such products.

6. Future work

With this work we were able to finish the main tasks that were set at the beginning of the project, however as a continuation of the work we would like to set new tasks with the objective to enrich our research. As future work perspectives we would repeat the enzymes inhibition assays with known concentration extracts, and more repetitions. We would characterize the different extracts used in the cells and determine the different compounds present to understand which compounds are able to affect cell viability. We would also use more cell lines, for example MC3T3 and Mg63, and do more repetitions of the resazurin assay, and, additionally, we would do the migration test with the HaCaT cells. Finally, we would assess the antioxidant activity on the cell lines of choice.

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8. Appendix

8.1. Appendix I – Gallic acid standard curve (Folin-Ciocalteau method)

To create the standard curve (**Figure 15**), we used different known concentration of gallic acid, which is the common standard use for determining the phenolic content of samples. The concentrations used were (200; 150; 100; 75; 50; 25; 10; 5; 0) mg/L.

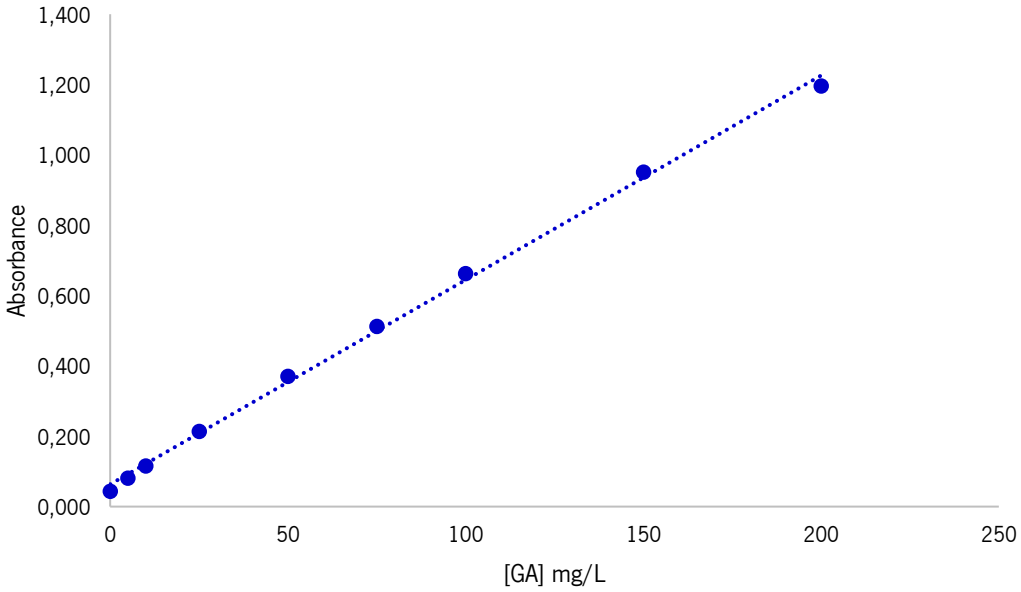

Figure 15. Gallic acid standard curve. The curve was obtained by determining the absorbance at 750 nm of known Gallic acid concentrations. The standard curve equation is y=0.0058x + 0.0634, where y is the absorbance and x is the gallic acid concentration (mg/L). R^2= 0.9981.

8.2. Appendix II – Catechin standard curve for flavonoids content determination

To create the standard curve (**Figure 16**), we used different known concentration of catechin, which is the common standard use for determining the flavonoids content of samples. The concentrations used were (200; 150; 100; 75; 50; 25; 10; 5; 0) mg/g.

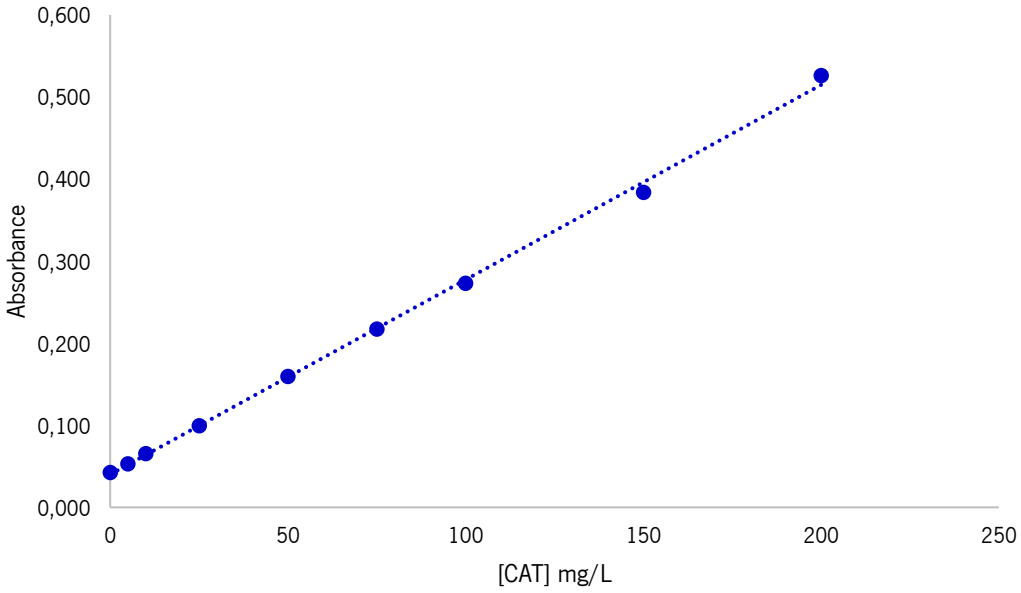

Figure 16. Catechin standard curve. The curve was obtained by determining the absorbance at 510 nm of known Catechin concentrations. The standard curve equation is y=0.0024x + 0.0401, where y is the absorbance and x is the catechin concentration (mg/g). R^2= 0.9988.

8.3. Appendix III – Trolox standard curve (FRAP method)

To create the standard curve (**Figure 17**), we used different known concentration of Trolox, which is the common standard use for determining the antioxidant activity of samples. The concentrations used were (0.625; 0.45; 0.313; 0.156; 0.078; 0.039; 0.0195; 0) mmol/L.

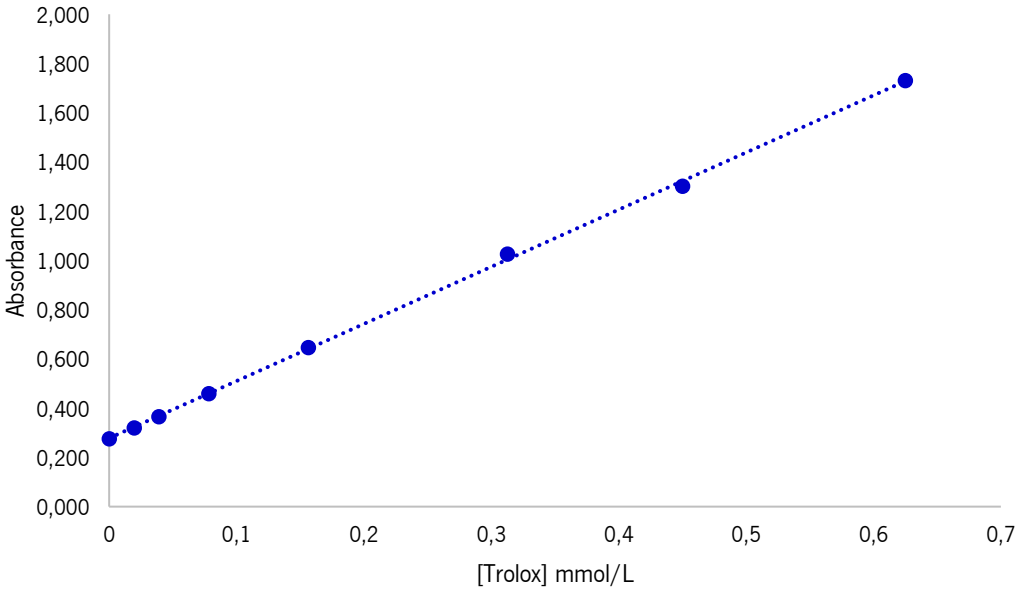

Figure 17. FRAP Trolox standard curve. The curve was obtained by determining the absorbance at 593 nm of known Trolox concentrations. The standard curve equation is y=2.3204x + 0.2782, where y is the absorbance and x is the Trolox concentration (mmol/L). R^2= 0.9995.

8.4. Appendix IV – Trolox standard curve (DPPH method)

To create the standard curve (**Figure 18**), we used different known concentration of Trolox, which is the common standard use for determining the antioxidant activity of samples. The concentrations used were (1.25; 0.9; 0.63; 0.31; 0.157; 0.078) mmol/L.

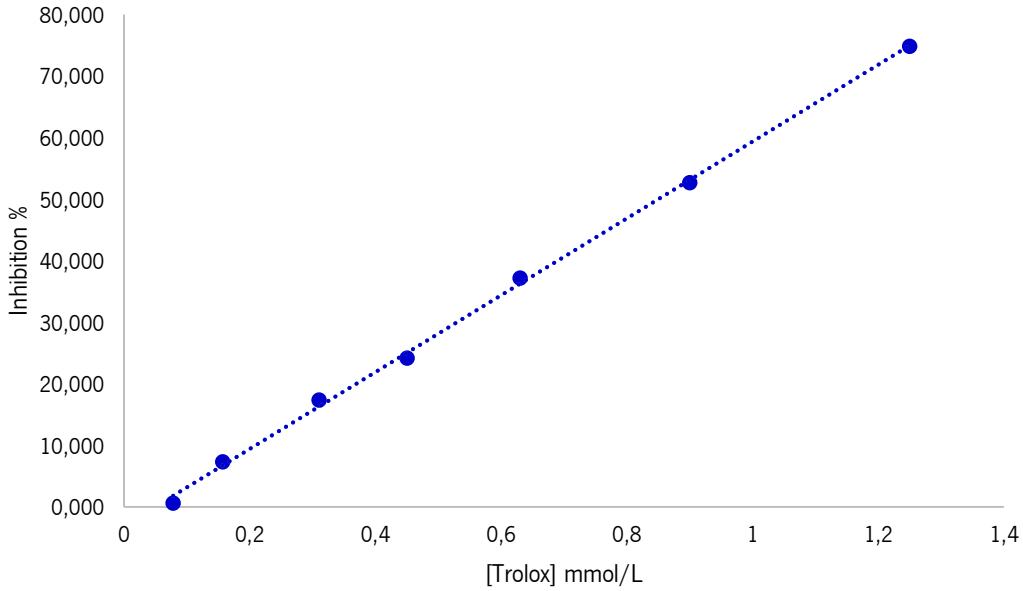

Figure 18. DPPH Trolox standard curve. The curve was obtained by determining the absorbance at 520 nm of known Trolox concentrations. The standard curve equation is y=62.425x - 3.0738, where y is the inhibition percentage and x is the Trolox concentration (mmol/L). R^2= 0.9989.

8.5. Appendix V – Trolox standard curve (ABTS method)

To create the standard curve (**Figure 19**), we used different known concentration of Trolox, which is the common standard use for determining the antioxidant activity of samples. The concentrations used were (0.9; 0.625; 0.3125; 0.15625; 0.078; 0.039; 0.019; 0) mmol/L.

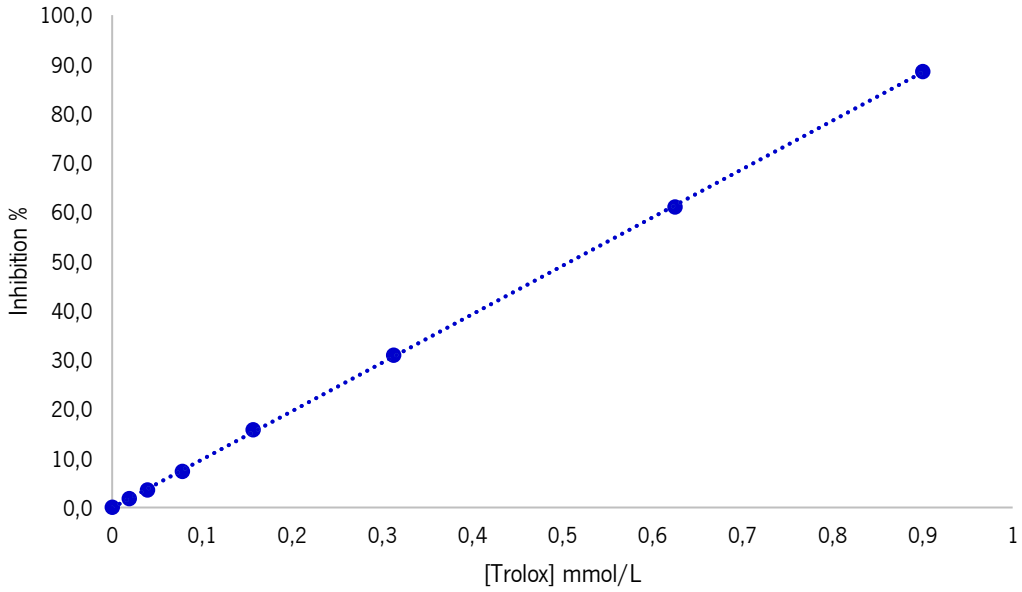

Figure 19. ABTS Trolox standard curve. The curve was obtained by determining the absorbance at 734 nm of known Trolox concentrations. The standard curve equation is y=98.284x – 0.0646, where y is the inhibition percentage and x is the trolox concentration (mmol/g). R^2= 0.9999.