Development of a cosmeceutical with natural bioactive compounds

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Muito obrigada

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Ab: Absorbance

AGS: Gastric adenocarcinoma

ANOVA: Analysis of variance

BACs: Bioactive components

BHA: Butylated hydroxyanisole

BHT: Butylated hydroxytoluene

CAA: Cellular antioxidant activity

CFU: Colony forming units

CMC: carboxymethyl cellulose

CIR: Cosmetic Ingredient Review

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

EC50: Extract concentration responsible for 50% of inhibition

EEO: Eucalyptus essential oil

EEP: Ethanolic extract of propolis

EMM: Estimated marginal means

FDA: Foods and pharmaceuticals

GI50: Sample concentration inhibiting 50% of cell growth

HPLC-DAD-MS: High-performance liquid chromatography coupled with *diode-array* detection and mass spectrometry

IARC: International agency for research on cancer

MBC: Minimum bactericidal concentration

MCF7: Breast carcinoma

MEB: Malt extract broth

MFC: Fungicidal concentration

MIC: Minimum inhibitory concentration

NCI-H460: Non-small-cell lung carcinoma

pH: Hydrogen potential

PLP2: Porcine liver primary culture

SRB: Sulforhodamine B assay

ST: Storage time

TBARS: Thiobarbituric acid reactive substances assay

TBHQ: Terbutyl hydroquinone

TSB: Tryptic soy broth

Tris-HCl : Tris hydrochloride

UVB rays : Ultraviolet B rays

Abstract

Preservatives are normally included in cosmetic formulations as they are important to fight microorganisms such as bacteria, fungi, and yeasts. Finding the right type of preservative or preservative system to add to each formulation that meets all standards for conservation and toxicological safety is a challenge when it comes to cosmetic microbiology. Thus, the main objective of this work was to develop cosmetic formulations by incorporating bioactive plant extracts and propolis extract, sources of natural antioxidants and antimicrobials. In a first phase, two extraction methods were applied according to the matrices requirements, namely maceration for the plants (e.g., rosemary, eucalyptus) and heated water bath for the propolis. Afterwards, different combinations of extracts in an attempt to search for possible synergistic effects were chemically characterized by HPLC-DAD-MS and subjected to bioactivity evaluation. The antioxidant activity was also evaluated by TBARs and CAA methods. Furthermore, the antimicrobial activity against pathogens was evaluated by the microdilution method, and the toxicity in normal cells was tested by the sulforhodamine B assay. Additionally, the anti-inflammatory and anti-tyrosinase activities of these extracts were also evaluated. Two of the combinations gave the greatest results, namely *eucalyptus/propolis* (50:50 *m/m*) and *eucalyptus/rosemary* (60:40 *m/m*), had the highest antioxidant and antimicrobial properties. These two combinations were incorporated into two different formulas (soap and cosmetic serum). The physical parameters of the developed formulas were analyzed, such as texture (tetrameter), color (colorimeter), and pH; as also the bioactivities, in order to assess their stability after incorporation into the final formulation.

The outcomes demonstrated that the new soaps have remarkable antibacterial and antioxidant activities. Throughout the period of storage, they managed to keep up their physical characteristics and bioactivities. The bioactivities of the artificially preserved serum formulas were higher than the other formulations. Nevertheless, the formulas only with natural extracts exhibited very strong capacities, being able to inhibit oxidation processes effectively.

Keywords: Preservatives, cosmetics, eucalyptus, propolis, rosemary, bioactivities, soap, serum.

Resumo

Agentes conservantes são normalmente incluídos em formulações cosméticas, pois são importantes para o controlo de microrganismos como bactérias, fungos e leveduras. Encontrar o conservante ou sistema conservante mais adequado que atenda a todos os padrões de conservação e segurança toxicológica, é uma dificuldade no que toca também à microbiologia cosmética.

Assim, o principal objetivo deste trabalho foi o desenvolvimento de formulações cosméticas pela incorporação de extratos vegetais bioativos e própolis, fontes de antioxidantes e antimicrobianos naturais. Dois métodos de extração foram utilizados de acordo com as exigências das matrizes, uma maceração no caso das plantas (ex.: alecrim, eucalipto) e extração aquecida em banho de água no caso do própolis. De seguida, diferentes misturas das três matrizes foram consideradas, numa tentativa de encontrar possíveis efeitos sinérgicos. Os extratos foram caracterizados quimicamente por técnicas de HPLC-DAD-MS e foram sujetos à avaliação da sua bioatividade. A atividade antioxidante foi avaliada pelo método de TBARs, a atividade antimicrobiana contra patógenos pelo método de microdiluição, e a toxicidade em células normais foi analisada pelo método da sulforrodamina B. Duas das misturas consideradas, revelaram os resultados mais promissores em termos de bioatividade, nomeadamente a mistura de eucalipto/própolis (50:50 *m/m*) e eucalipto/alecrim (60:40 *m/m*). Estas misturas foram posteriormente incorporadas em formulações cosméticas (sabonete e sérum) que foram posteriormente analisados quanto às suas propriedades físicas como textura (texturômetro), cor (colorímetro), pH, e quanto às propriedades bioativas, de forma a averiguar a sua estabilidade após a sua incorporação nos produtos. Os resultados demonstraram que os novos sabonetes têm notáveis atividades antibacterianas e antioxidantes. Durante todo o período de armazenamento, eles conseguiram manter suas características físicas e bioatividades. As bioatividades das fórmulas de sérum conservadas artificialmente foram maiores do que as dos soros sem conservantes artificiais. No entanto, as formulações incorporadas com extratos naturais exibiram uma atividade muito forte, capazes de inibir eficazmente a oxidação.

Palavras-chave: conservantes, cosméticos, eucalipto, própolis, alecrim, bioactividades, sabão, serum

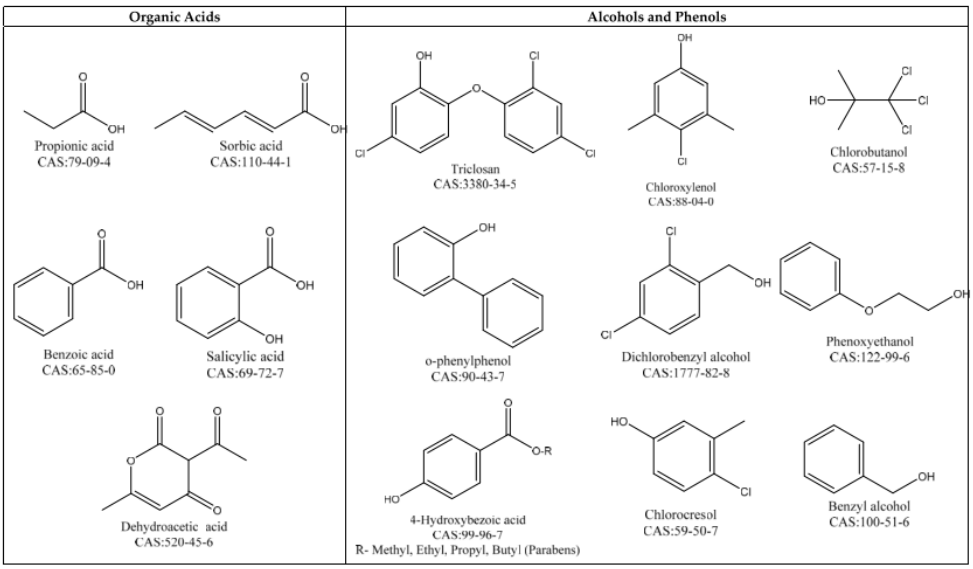
1. Introduction

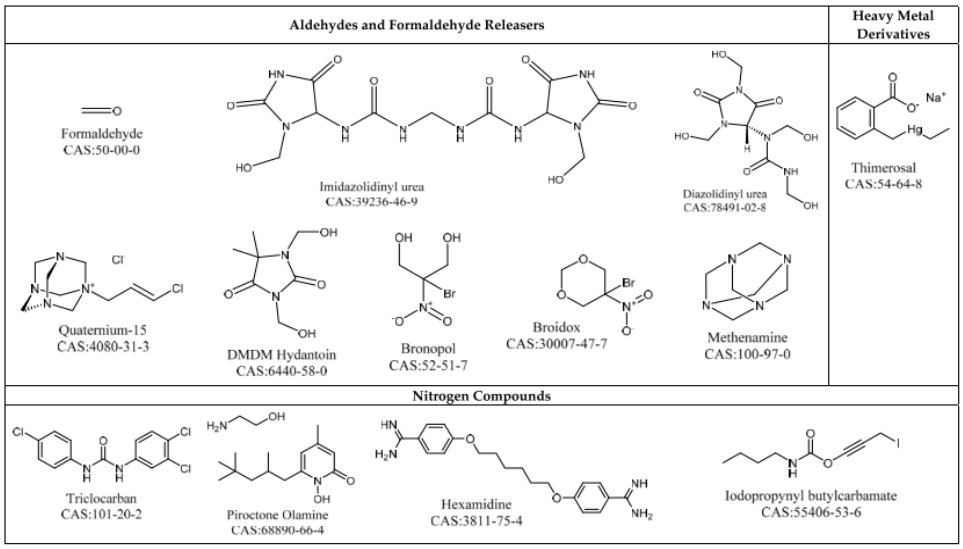
Preservatives have been identified and used as antibacterial and antioxidant compounds, which prevent microorganisms from degrading a product while also limiting oxidation and free radical formation. When preserving a product, it is critical to apply an antibacterial solution with high efficacy, and it should be harmless and compatible with the rest of the cosmetic ingredients (Halla et al., 2018; Kerdudo et al., 2016). Artificial preservatives, which are typically found in cosmetics are frequently associated with harmful effects. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and/or propyl gallate and terbutyl hydroquinone (TBHQ) are examples of chemicals that are toxic, carcinogenic, and damaging to human health (Gullon et al., 2020). In recent years, the "back to nature" trend has expanded, as the demand for the elimination of artificial preservatives in several industries has increased. This led cosmetic manufacturers to exploit novel compounds to replace synthetic stabilizers in existing cosmetic formulations (Dreger & Wielgus, 2013; Gullon et al., 2020; Sharmeen et al., 2021; Telichowska et al., 2020). These agents are usually found in Nature, being plants considered a source of bioactive molecules with bioactive effects because of their high concentration of phenolic compounds, which have strong antioxidant and antibacterial activities. Furthermore, propolis, a plant glue utilized by bees to defend the honeycomb from air currents, predators, and germs, has been demonstrated to be successful when used as a natural ingredient due to its potent capacity to prevent oxidation and microorganism damaging.

1.1. The use of preservatives in the cosmetic industry

Many pharmaceutical and cosmetic products are susceptible to microbial contamination, which can lead to degradation or alteration of their physical properties. Moreover, they may present a potential risk to consumer´s health. The incorporation of preservatives in cosmetic formulations is necessary to ensure longer shelf-life of the product. They are added to guarantee the microbial safety and stability of cosmetic products. The right type of preservative must be nontoxic, have good solubility in water and a broad spectrum of action on a pH range between 4 and 10 (Bernard et al., 2021; Carrascal et al., 2021; Park et al., 2020; Youenou et al., 2021).

acids, alcohols, phenols, aldehydes, formaldehyde releasers, isothiazolinones, biguanides, quaternary ammonium compounds (QAC), nitrogen compounds, heavy metal derivatives, and inorganic compounds can all be categorized based on their chemical composition. Figure 1. shows the most commonly used antimicrobial preservatives (Halla et al., 2018).





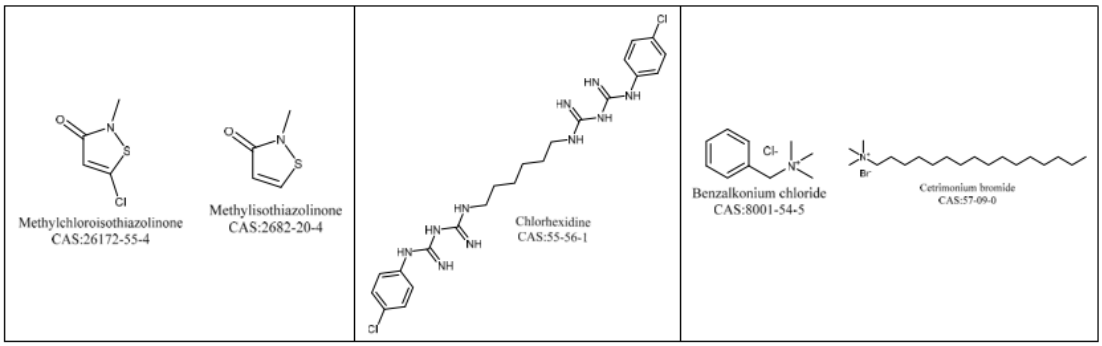


Figure 1: Chemical structures of some preservatives used in cosmetics (Halla et al., 2018).

Parabens are extensively used in the cosmetic industry, applied in about 35% of cosmetic products due to their powerful antibacterial and antifungal properties. These compounds could be naturally produced by plants or bacteria, however, all commercially used parabens are produced by synthetic methods. They are wildly applied in personal care and cosmetic products, as figure 2. shows, due to their lack of perceptible odor, or taste and possess high stability to pH and temperature variations, low price, marginal toxicity, and pronounced inhibitory activity against bacteria, molds, and yeasts. Propylparaben and methylparaben are commonly identified paraben compounds in surface waters due to their frequent use in cosmetics (Bilal et al., 2020; Carvalho et al., 2021).

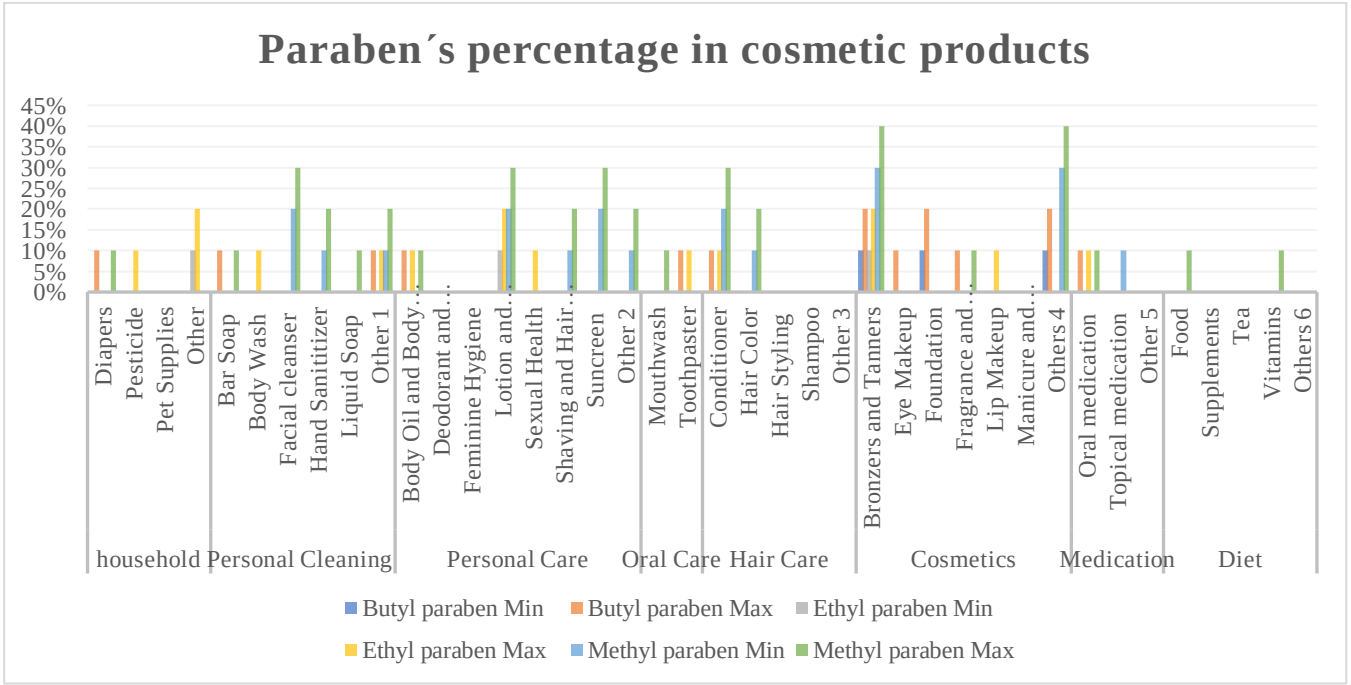


Figure 2: Parabens in cosmetic products (Fransway et al., 2019).

The preservative systems mostly applied in cosmetics include some organic acids like benzoic acid and its salt, sodium benzoate. Formaldehyde (FA) or its methylene glycol form is also frequently used in water-based cosmetic products. This additive is employed as a stabilizing or protective agent in cosmeceutical and personal care products. It is considered as an important class of preservatives utilized in a broad spectrum of cosmetic industries in the formulation of shampoo, liquid soap, body shower gel, skincare lotion and many other products. Triclosan (TCS) is also an antimicrobial agent that is extensively employed as a preservative in a variety of personal care products such as shampoos, toothpaste, detergents, hand soaps, deodorants, and sunscreens. Benzalkonium chloride (BAC), known as a quaternary ammonium compound (QAC) and commonly used as a potential preservative of various cosmetics, pharmaceutical and personal care products is also applied in this industry. Another synthetic compound, is the 1,4-Dioxane ($C_4H_8O_2$, dioxane), commonly used in personal cleaning and household products including baby lotion, shower gel, body lotion, toothpaste, mouthwash, and shampoo. It is used to enhance the foaming texture in personal care products and minimize the adverse effects of chemicals especially sodium lauryl sulfate. Imidazolidinyl urea and diazolidinyl urea are widespread antimicrobial fixative agents used in cosmeceutical industries and personal-care products, especially skincare products, childcare products, hair treatment products, skin and face makeup, and nails (Bilal et al., 2020; Carvalho et al., 2021; Mondejar-Lopez et al., 2021).

1.2. Artificial vs natural preservatives in the cosmetic industry

Chemically synthesized preservatives used for years, like *p*-hydroxybenzoic acid, phenoxyethanol, and imidazolidinyl urea, have several advantages, which include their low cost, broad spectrum of activity against bacteria and fungi, compatibility with other ingredients, and the fact that they generally do not interfere with fragrance, color, or other aspects of the formulations. However, while popular synthetic preservatives do help to extend the lifespan of items and keep them free of microbes, many of them have earned unfavorable reputations in the minds of consumers, and their usage has been increasingly controversial in recent years. Since they are frequently petroleum-based, they can be toxic and can significantly cause irritations or infections, especially on damaged skin or the delicate areas around the eyes. Some of these can even be harmful to one's health (Kerdudo et al., 2016).

Parabens, for example, are regarded as emerging contaminants. They rank seventh position in terms of allergens in shampoos. They have weak estrogen-like properties, antiandrogenic activity, estrogenicity, endocrine disruptors, cytotoxic and genotoxic effects on human lymphocytes, according to the study. Furthermore, there is a possible link between parabens and breast cancer. Furthermore, it may cause allergic reactions and reproductive problems. However, recent detailed toxicity studies have finally proven that they are noncarcinogenic (Atolani et al., 2016; Bilal et al., 2020; Dreger & Wielgus, 2013; Fransway et al., 2019; Palma et al., 2021; Parham et al., 2020).

Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tert-butyl-hydroquinone are other synthetic compounds that have traditionally been used as antioxidants and/or preservatives in cosmetics and pharmaceutical products (TBHQ). These preservatives are known to cause allergic reactions in human skin and have been classified as potential carcinogens by the International Agency for Cancer Research (Atolani, Olabiyi, et al., 2016). As previously stated, BHT can cause unfavorable effects in consumers, but some studies have discussed BHT toxicity and have shown the positive effects of this antioxidant, such as increasing intracellular levels of glutathione and related enzymes in rats and protecting against cancer due to its antioxidant activity (Atolani et al., 2016; Ousji & Sleno, 2020). In the case of BHA, several investigations have shown that it has anti-tumor and nephroprotective properties. Nonetheless, BHA has been found to be a carcinogen and an endocrine disruptor in mice (Ousji & Sleno, 2020).

Also, it has been discovered that Benzalkonium chloride concentration also increases the lap of cell division and DNA damage induction mediated by BAC (Bilal et al., 2020). In addition, imidazolidinyl urea and Diazolidinyl urea have been linked to a variety of negative side effects in humans, including DNA damage, antiandrogenic activity, estrogenicity, endocrine disruptors, cytotoxic and genotoxic effects on human lymphocytes, an increased risk of cancer, allergic reactions, reproductive disorders, and environmental and animal toxicity (Mondejar-Lopez et al., 2021).

The rising interest in natural ingredients has been a trend in the cosmetic sector. These natural components have the potential to replace synthetic ones since they are safer and more environmentally friendly. They are known for having minimal toxicity, being abundant in plants, and being less expensive (Cizauskaite & Bernatoniene, 2018; Dias et al., 2021). They perform a variety of functions, including antioxidant and antibacterial activity. These natural

alternatives have been found to contribute to the delay of microbiological and chemical reactions (Baptista et al., 2020; Halla et al., 2018; Silva et al., 2020). Natural substances, on the other hand, may have a variety of disadvantages to consider, such as dilution losses, pH-dependent action, volatility, lipophilic properties (essential oils), strong fragrances, coloring, and instability. Many natural ingredients may be incompatible with specific formulae because of these considerations (Bernard et al., 2021; Halla et al., 2018).

1.3. Legislation for preservatives in the cosmetic industry

Nowadays, more and more consumers have questioned the safety of chemical preservatives. It becomes a subject to stringent regulatory oversight in different regions. The European Union and Japan regulate the use of the preservatives by a positive list published by official guidelines. It constitutes the group of allowed substances for cosmetic preservation from microbial spoilage (Alvarez-Rivera et al., 2018). In the European Community framework, only preservatives listed in Annex V of Regulation (EC) No. 1223/2009 of the European Parliament and of the Council of 30 November 2009 can be applied in cosmetics and personal care products (Halla et al., 2018; Juliano & Magrini, 2018), While in Japan, Annex 3, of the "Standards for Cosmetics" of the Ministry of Health and Welfare (No. 331 of 2000) lists all preservatives authorized to be incorporated into cosmetics (Halla et al., 2018).

Regarding the United States, the use of preservatives is regulated by the same governmental agency as are foods and pharmaceuticals (FDA). It works to keep cosmetics safe with the cosmetics industry, consumers, and Cosmetic Ingredient Review (CIR) system. The CIR expert panel reviews and evaluates the safety of cosmetic ingredients (Alvarez-Rivera et al., 2018). In Canada, lists of banned and regulated ingredients is proposed, no positive lists is suggested (Alvarez-Rivera et al., 2018).

Nitrogen compounds, formaldehyde releasers, isothiazolinones, and the quaternary ammonium compounds are listed in different classes due to their specific properties. The nitrogen compounds used as preservatives according to Annex V of the EU Directive are zinc pyrithione, triclocarban, piroctone olamine, chloroacetamide, hexamidine, dibromohexamidine

isethionate, dimethyloxazolidine, climbazole, iodopropynyl butylcarbamate, 7-ethylbicyclooxazolidine, and ethyl lauroyl arginate hydrochloric acid (Halla et al., 2018).

The European authorities have continuously updated the use of preservatives. The guideline mention that benzalkonium chloride is allowed up to 3% concentration in rinse-off hair care products and 0.05% in other products and imidazolidinyl urea is authorized at a maximum concentration of 0.6% in cosmetics. these two ingredients were allowed to be used at a concentration between 0.1% to 0.3% in cosmetics products. The use of diazolidinyl urea is restricted up to 0.5% concentration in Europe (Ryu et al., 2018). The manufacture, import, export, and marketing of cosmetic products containing chloroacetamide ware banned by the French National Agency of Medicine and Health Products Safety (Halla et al., 2018).

The most widespread family of preservatives, the parabens, has been reviewed and re-reviewed in the EU Regulation, because it was mentioned in several reports that there is a hypothetical connection between these cosmetic preservatives, and breast cancer (Juliano & Magrini, 2018). The use of some of them (isopropyl-, isobutyl-, phenyl-, benzyl- and pentylparaben) has been forbidden by the European authorities but they are considered safe in the United States. in cosmetic products (Alvarez-Rivera et al., 2018).

BHT and BHA have been banned in Japan since 1958 and have restrictions in infant formulas in the UK. However, BHT, BHA, and TBHQ are currently allowed in Canada, United States, Korea, and certain countries within the European Union. They are categorized as "generally recognized as safe" (GRAS) by the U.S. Food and Drug Administration (FDA) and safe to use in cosmetics by the Cosmetic Ingredient Review (CIR) Expert Panel, while the International Agency for Research on Cancer (IARC) classifies BHT as non-carcinogenic (based on limited evidence), BHA as reasonably anticipated to be a human carcinogen and TBHQ as not carcinogenic (Ousji & Sleno, 2020).

Mercury based preservatives are prohibited in Canada, but they are permitted with restrictions in the EU. In the EU, chloroacetamide is allowed up to 0.3%. currently, is under public consultation because allergic reactions can be elicited at concentrations even lower than 0.3%. It is judged unsafe by the United States, and it is already prohibited in Canada (Alvarez-Rivera et al., 2018).

2. Plants as sources of natural preservatives

2.1. Main bioactive compounds in plants

The cosmetics business has recently seen an increase in interest in natural ingredients, primarily of plant origin. Bioactive phytochemicals derived from herbs, flowers, fruits, and seed oleates have the potential to improve therapeutic efficacy, reduce toxicity and side effects, and ensure environmental safety due to nontoxic compounds' biodegradability (Carvalho et al., 2021; Cizauskaite & Bernatoniene, 2018; Telichowska et al., 2020).

The advantages attributed to plant extracts aren't due to a single class of compounds, but rather to the numerous contributions of several bioactive components (BACs) such as Camphor, camphene, carnosol, carvacrol, thymol, -pinene, -cymene, 1,8-cineol, limonene, -terpinene, and terpinen-4-ol that represent some of the most common terpenes. The flavonols quercetin and kaempferol are the most common, but apigenin, isorhamnetin, luteolin, myricetin, and rutin all stand out. Finally, phenolic acids including caffeic, cinnamic, chlorogenic, ferulic, quinic, rosmarinic, and sinapic acids are some of the most well-known like figure 3 (Pateiro et al., 2021).

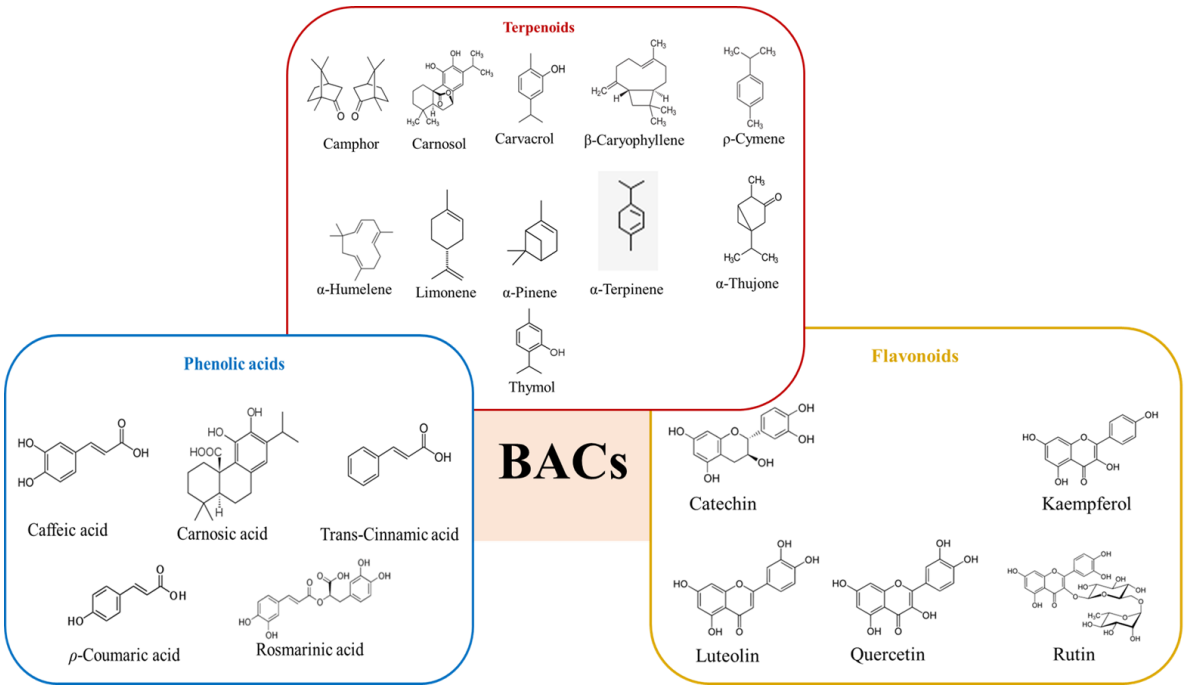


Figure 3: Main bioactive compounds (BACs) present in different plants extracts (Pateiro et al., 2021).

The efficiency of plant extracts and essential oils is mostly due to the presence of chemicals with high antioxidant activity, predominantly polyphenols and terpenoids, in their composition (Pateiro et al., 2021). Tannins are water-soluble phenolics that play an important antioxidant role due to their many phenolic hydroxyl groups. They may even have antibacterial properties. Phytic acid, which is found in a variety of plant byproducts such as cereals, legumes, vegetables, and nuts, is another bioactive component that can be used as a natural preservative (Tlais et al., 2020). Furthermore, Flavonoids, such as flavanols, flavones, flavanones, isoflavones, flavanonols, and anthocyanins, are secondary metabolites found in large quantities in plants, fruits, and seeds that because of their antibacterial and antioxidant properties, they can be utilized as food preservatives (Dias et al., 2021; Tlais et al., 2020).

Spice plants have gradually been employed as a source of bioactive compounds in developed countries. Their essential oils are described for their antioxidant and antibacterial effects as mentioned in Figure 4., which could differ according to the chemical composition of each plant (Diniz do Nascimento et al., 2020).

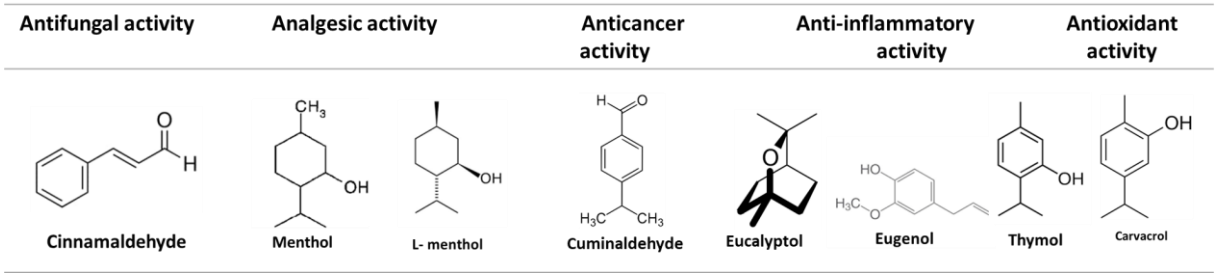


Figure 4: Natural compounds from essential oils and their reported biological activities with potential pharmaceutical applications (Diniz do Nascimento et al., 2020)

Mango kernel oil was also shown to have the ability to act as a natural antioxidant for the preservation of other fats and oils (Mwaurah et al., 2020). Hops have also been employed as a natural preservative in beer due to their high quantity of bitter acids and polyphenols, which hinder the growth of a wide range of microbes (Astray et al., 2020).

It is commonly recognized that the greatest class of phytochemicals present in plants, phenolic compounds, are primarily what give plant extracts their physiological effects. Due to their potential significance in the prevention of human diseases, these compounds' antioxidative

activity is receiving more and more attention. Due to an increase in the synthesis of the skin pigment (melanin), hyper-pigmentation is a frequent skin disorder where the skin is darker than usual. Numerous skin problems, such as Acanthosis nigricans, Cervical Poikiloderma, melasma, periorbital hyperpigmentation, lentigines, neurodegeneration associated with Parkinson's disease, and skin cancer risk, are characterized by an enhanced melanin production and accumulation of these pigments. The oxidation of tyrosine to dihydroxy-phenylalanine (DOPA) and from DOPA to DOPA-quinone is catalyzed by the enzyme tyrosinase, which is implicated in melanogenesis. The cosmetic and pharmaceutical sectors have utilized a wide range of melanogenic inhibitors. Vitamins, retinoids, kojic acid, hydroquinone, arbutin, and other well-known pharmaceutical melanogenesis inhibitors are also available. Vitamin C (ascorbic acid), one of these inhibitors, is recognized to have a variety of physiological and pharmacological roles in antioxidation. Additionally, it is utilized to reduce hyperpigmentation, and experts believe that this effect is correlated to its antioxidant properties. Contrary to other vitamins, ascorbic acid is known to have relatively poor stability and hence rapidly deteriorates in response to oxidants, metal ions, and UV radiation. (Lee et al., 2011; Zerbinati et al., 2021). Furthermore, due to their tyrosinase inhibiting action, various plant extracts, such as Morus alba L. (Moraceae) or Glycyrrhiza glabra Linneva (Leguminosae), have been utilized as whitening agents in cosmetic preparations. Several substances, including terpenoid, phenyl, pyridine, piperidine, pyridinone, hydroxypyridinone, thiosemicarbazone, thiosemicarbazide, azole, thiazolidine, kojic acid, benzaldehyde, and xanthate derivatives, were identified as strong tyrosinase inhibitors according to the findings. (Fawole et al., 2012 ; Özer et al., 2008 ; Priani et al., 2019 ; Zolghadri et al., 2019)

Additionally, propolis from several countries (Australia, Brazil, China, Hungary, Japan, Ukraine, and Uruguay) has been shown to inhibit tyrosinase activity. It has been shown that certain flavonoids can chelate active sites and inhibit mushroom tyrosinase. The chelation interactions between the vicinal 30,40-dihydroxyl group of catechin and the Cu^{2+} on tyrosinase, for example, or the structure of the 3hydroxy-4-keto moiety in certain flavonoids, such as quercetin, are two examples. According to other research, caffeic acid phenethyl ester does not directly block tyrosinase activity in order to reduce the synthesis of melanogenic enzymes. (El-Guendouz et al., 2016).

2.1.1. Rosemary

Rosmarinus officinalis L. (figure 5) is commonly known as rosemary, belongs to the Lamiaceae family, which comprises up to 200 general and about 3500 species. It is a wild, strewing evergreen perennial plant. Rosemary has been a significant aromatic herb commonly cultivated in Mediterranean countries such as Morocco, France, Spain, but it is distributed in all parts of the world region, mainly due to its medicinal, culinary, food preservative, and cosmetic applications. It was traditionally used as an antiseptic, astringent, and preservative in food. The European Union has approved rosemary extracts as a safe and effective natural antioxidant for food preservation due its low toxicity levels. Moreover, this species is highly known for the antioxidant, antibacterial, and antifungal, anti-inflammatory, antitumor, antithrombotic, antinociceptive, antidepressant and antiulcerogenic activities associated with its extract (de Macedo et al., 2020; Diniz do Nascimento et al., 2020; El Kharraf et al., 2021; Hosseini et al., 2021; Kaur et al., 2021; P N Dinh, 2020).

Figure 5: The rosemary (R. officinalis) (Ahmed & Babakir-Mina, 2020)

These characteristics of rosemary are due to its chemical constituents, namely rosmanol, carnosol, carnosic acid, ursolic acid, rosmariquinone, caffeic acid, betulinic acid, oleanolic acid, micromeric acid methyl ester, rosmaridiphenol, and rosmarinic acid (de Macedo et al., 2020; Kaur et al., 2021).

Several studies reported the distribution of rosemary flavonoids (eriocitrin, luteolin 3'-*O*-β-D-glucuronide, hesperidin, diosmin, isoscutellarein 7-*O*-glucoside, hispidulin 7-*O*-glucoside, and genkwanin) in flowers, roots, and stems during different stages of the plant's

growth. Other studies reported a high concentration of flavonoids, polyphenols, and terpenes in R. officinalis leaves (de Macedo et al., 2020).

Rosemary essential oil is mentioned as having antifungal, antibacterial, and hepatoprotective properties. Its chemical compositions diversify, but the main compounds to find are eucalyptol, β-pinene, α-pinene, borneol, γ-cadinene, α-terpineol, myrcene, and camphene 1, 8-cineole, borneol, camphor, limonene, and verbenone. Some studies reported 1,8-Cineole, camphor, α-pinene and borneol as the main components of Rosmarinus officinalis originated from Tunisian (de Macedo et al., 2020; Diniz do Nascimento et al., 2020; Elbanna et al., 2018; Seyed Mohammad Vahdat, 2019).

Many research studies reported that rosemary extract chemical composition can vary according to the growth stage and origin. It contains wide bioactive molecules, including triterpenes (ursolic and oleanolic acid.), tricyclic diterpenes (carnosic acid and carnosol), phenolic acids (caffeic and rosmarinic acids), and essential oils. Flavonoids are also found, such as genkwanin, hispidulin-*O*-glucoside, cirsimaritin, luteolin, isoscutellarein 7-*O*-glucoside and, the latter constituting, caffeic and rosmarinic acids, the main components in the hydrosoluble fraction. In figure 6 it is possible to observe the different chemical structure of some Rosmarinus officinalis secondary metabolites. Rosemary extracts are employed as a natural antioxidant to improve the shelf life of foods (Baptista et al., 2020; de Macedo et al., 2020; Diniz do Nascimento et al., 2020).

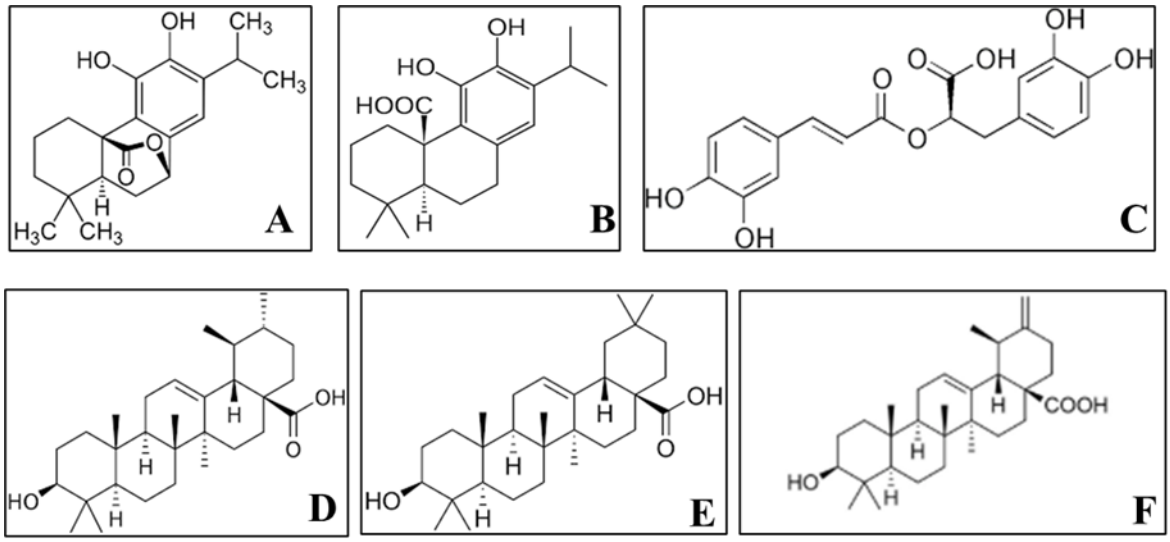


Figure 6: Chemical structure of some Rosmarinus officinalis secondary metabolites: carnosol (A), carnosic acid (B), rosmarinic acid (C), ursolic acid (D), oleanolic acid (E), and micromeric acid (F) (de Macedo et al., 2020).

Many studies have mentioned the potential benefits of using rosemary and its extracts due to its antioxidant and antimicrobial activity, being widely used for medicinal purposes as also for beauty care (Park et al., 2020).

These authors studied mixed *R. officinalis* extract (containing carnosic acid) with extracts of 2 other plants (Zanthoxylum clava-herculis (containing magnoflorine and laurifoline) to formulate a Ginoid Lipodystrophy (GLD, Cellulite) cream. The formulation led to an improvement in the appearance of the cellulite. Also, in another study the authors used hydroalcoholic extracts of rosemary to treat mice with testosterone-induced alopecia (loss of some or all hair) and it showed a significant increase in hair growth after 16^{th} day of treatment (de Macedo et al., 2020).

The *R officinalis* derived ingredients have multiple functions in cosmetics. Most of the ingredient's function are as a skin conditioning agent and fragrance ingredient. Most cosmetic formulations contain very low concentrations of the R officinalis extracts, often much less than 0.1%. However, the leaf extract is indicated to be used at up to 10% in body and hand products and 3% in eye shadow formulations and bath soaps and detergents. Rosemary flower extract is reported to be used in 32 cosmetic formulations, but no use concentration data were reported. Products based on rosemary may be applied to baby skin (0.012% rosemary leaf extract in baby lotion, oils, and creams), used in products that could be incidentally ingested (0.012% rosemary

leaf in lipstick formulations), or used near the eye area (up to 3% Rosmarinus officinalis rosemary leaf extract in eye shadow formulations) or mucous membranes (up to 3% rosemary leaf extract in bath soaps and detergents). Furthermore, rosemary derived ingredients are used in cosmetic sprays and powders (Fiume et al., 2018).

Table 1: Major natural compounds of *R. officinalis* (Diniz do Nascimento, Moraes et al. 2020).

Plant Organ	Major Compounds (%)	References
Leaves	eucalyptol (15.2%), c amphor (15.1%) and β-pinene (11.0%)	(Baj et al., 2018)
	α-pinene (14.69–20.81%), eucalyptol (5.63–26.89%) and camphor (4.02–24.82%)	(Bajalan et al., 2017)
	eucalyptol (42.86–46.76%), camphor (16.26–23.42%), α-pinene (6.37–9.19%), camphene (2.27–4.37%), and borneol (4.00–4.33%)	(Wang, 2018)
	γ-cadinene (29.93%), camphor (14.76–28.42%) and eucalyptol (10.60–15.36%)	(Conde-Hernández et al., 2017)
	eucalyptol (35.15–50.28%) camphor (12.71–13.08%), α-pinene (5.60–13.92%), borneol (7.42–9.81%) and α-terpineol (1.76–15.40%)	(Kowalski et al., 2018)
	eucalyptol (11.33–37.29%), camphor (8.81–40.35%) and α-pinene (2.60–28.68%)	(Mezza et al., 2018)
Aerial parts	camphor (14.80–42.50%), eucalyptol (8.00–26.40%), α-pinene (4.10–13.20%) and myrcene (1.00–30.30%)	(Pistelli, 2018)
	eucalyptol (35.32%), (E)-caryophyllene (14.47%), borneol (9.37%), camphor (8.97%), α-pinene (7.90%) and α-thujone (6.42%)	(Selmi et al., 2017)
	eucalyptol (23.67%), camphor (18.74%) and borneol (15.46%)	(Bouyahya et al., 2017)

2.1.2. Eucalyptus

The genus Eucalyptus is a member of the Myrtaceae family. It was native to the Mediterranean, Australia, and Tasmania area. this genus encompasses more than 900 species and subspecies, spreading worldwide occupying>20 million hectares (Aleksic Sabo & Knezevic, 2019; Gullon et al., 2020; Palma et al., 2021; Parham et al., 2020).

Eucalyptus represents a promising source of high-value phytochemicals. This medicinal herb is an excellent source of essential oils, sterols, alkaloids, glycosides, flavonoids, tannins, and phenols (Aleksic Sabo & Knezevic, 2019; Gullon et al., 2020). Its extracts and essential oils have been used in the pharmaceutical, health, agricultural, cosmetic and food industries because of their medicinal properties (Gullón et al., 2019).

Eucalyptus globulus L. leaves contain a diverse range of compounds, including monoterpenes, sesquiterpene-phloroglucinol derivatives, flavonoids, tannins and related polyphenols. About 11 terpene compounds were identified from this species. The compound 1, 8-cineole (Eucalyptol) was the major terpene in methanol extracts and essential oil followed by pinocarvone, pinocarveol and viridiflorol (Gullón et al., 2019; Palma et al., 2021). The major constituents in *Eucalyptus oleosa* leaf essential oil were spathulenol and γeudesmol followed by *p*-cymene, 1,8-cineole, *p*-cymen-8-ol, cis-sabinol, *p*-cymen-7-ol and verbenone, while in oleosa fruit essential oil 1,8-cineole, γ-eudesmol, α-selinene and pcymene were the main compounds. *Eucalyptus microtheca* leaves and flowers and *Eucalyptus viminalis* leaf (Southeast of Iran) present in their essential oil a high concentration of several compounds including α-phellandrene, aromadendrene, α-pinene, globulol, ledene, p-cymen, and β-pinene, while in the oil of *E. microtheca* flowers, α-pinene, o-cymen, β-pinene, aromadendrene, áphellandrene, globulol, and 9-octadecenamide were the major components. *E. viminalis* leaf were consisted of the following major compounds: 1,8-cineole, αpinene, limonene, and globulol (Stankov et al., 2020). For *Eucalyptus camaldulensis* (figure 7) chemical composition, it was reported that leaf contain 0.1–0.4% essential oil, of which 77% is 1,8-cineole and it was also mentioned that leaves contain 5–11% of tannins. The kino (a class of wood exudates), contains 45% kinotannic acid as well as kino red, glucoside, catechol, and pyrocatechol. Flavonoids and sterols were detected in leaves and fruits and the bark contains 2.5–16% tannin, the wood 2–14%, and the kino 46.2–76.7% (Aleksic Sabo & Knezevic, 2019).

Figure 7: Eucalyptus camaldulensis (river red gum), (Sabo & Knezevic, 2019).

The main compounds of Eucalyptus essential oils in Northern and Northwestern Tunisia are monoterpenoids, being the essential oil of the species *Eucalyptus grandis* richest in 1,8-cineole and *p*-cymene. In Argentina, *E. grandis's* major components were α-pinene and 1,8-cineole. However, Alloocimene and α-pinene were the major constituents of EEO found in Hainan eucalyptus (China) (Zhou et al., 2021).

The secondary metabolites of *E. camaldulensis* leaf extracts from Nigeria revealed the presence of tannins, saponins, and cardiac glycosides. Anthraquinones, flavonoids, saponins, and terpenoids were detected in crude methanolic extract from the leaves of *E. camaldulensis* from India (Aleksic Sabo & Knezevic, 2019).

Different species of Eucalyptus are used in folk medicine as antiseptics due to their antimicrobial properties, and to treat respiratory infections. As well, its essential oils are known to display several bioactivities such as antibacterial, antifungal, analgesic, and anti-inflammatory properties applied in cosmetic and pharmaceutical products also used in the field of medicine, food, and the chemical industry (Gullon et al., 2020; Zhou et al., 2021).

This medicinal species possesses a strong antibacterial ability against *Staphylococcus aureus*, *Listeria monocytogenes*, Bacillus sp., *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Salmonella Enteritidis*, *Propionibacterium acnes* and *Escherichia coli*. Furthermore, the antioxidant activity is due to the presence of polyphenol, oenothein B, gallic acid, ellagic acid, flavonoids, and hydrolysable tannin dimer in its extract. Eucalyptus has been noted that it has

an antiviral activity against herpes simplex virus (HSV) and influenza virus (Parham et al., 2020; Veerasophon et al., 2020).

The World Health Organization (WHO) has reported that Eucalyptus has different medicinal uses including as an expectorant or to treat asthma, influenza, diarrhea, and fungal infections among others (Gullon et al., 2020). Its leaves and also the essential oils have multiple application because owing to its properties. It can treat gastrointestinal symptoms, respiratory disease and arrest bleeding. In addition, in industry, the wood of *E. camaldulensis* has been used for heavy construction, railway sleepers, flooring, framing, fencing, plywood, and veneer manufacture, wood turning, firewood, and charcoal production (Aleksic Sabo & Knezevic, 2019).

3. Propolis

Propolis (figure 8*)* means the "city's guardian". It is a resinous mixture made by honeybees. Propolis as a final product comes from tree or other plant buds, exudates, or resins found in the stem, branches, or leaves of different plants collected by the bees and mixed with substances secreted by their metabolism, such as wax and salivary secretions and other materials (Ahangari et al., 2018; Dezmirean et al., 2020; Oses et al., 2020).

Its composition and color vary according to the botanical source and geographical origin. Poplar-type propolis color varies greatly from yellow orangish, to reddish and brown, or dark brown. It melts on 60 °C to70 °C while some of its kinds melt on 100 °C (Anjum et al., 2019; Dezmirean et al., 2020; Oses et al., 2020).

Figure 8: Propolis (Almuhayawi, 2020)

3.1. Main compounds present in propolis

The chemical composition of propolis is highly influenced by altitude, illumination, seasonal variations, and the botanical sources around the hive (Almuhayawi, 2020). So far more than 350 compounds have been recognized in propolis belonging to polyphenols, terpenoids, steroids, sugars and amino acids (Dezmirean et al., 2020). Generally, contains about 50% to 70% resin and vegetable balsam (phenolic compounds), 30% to 50% oil and wax and 5% of pollen and 5% of other substances (aminoacids, vitamins, minerals, salts) including organic debris, Figure 9 (Almuhayawi, 2020; Oses et al., 2020).

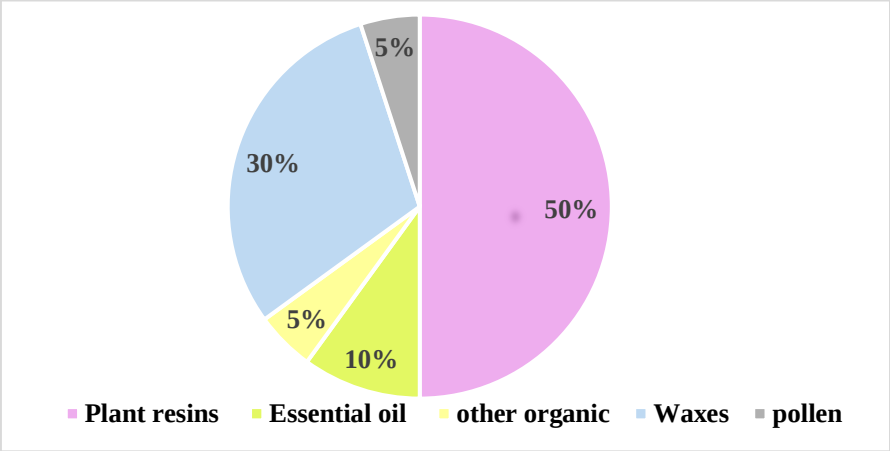


Figure 9: General chemical composition of propolis (Almuhayawi 2020).

Polyphenols and terpenoids are the most active components. Total phenolics of propolis from various geographical origins have a wide range of values in the literature, depending on the standard and solvent employed. Propolis's phenolic content ranged from 65.49 mg GA/g to 228.40 mg GA/g, while flavonoid group values ranged from 27.89 to 108.18 mg GA/g (Oses et al., 2020). The composition includes the flavonoids chrysin, pinocembrin, apigenin, galangin, kaempferol, quercetin, tectochrysin, pinostrobin and phenolic acids such as ferulic, cinnamic, caffeic, benzoic, salicylic and *p*-coumaric acids. Also, micro and macro-elements (Mn, Fe, Si, Mg, Zn, Se, Ca, K, Na, Cu) and vitamins B1, B2, B6, C and E can be found in propolis (Oses et al., 2020; Przybylek & Karpinski, 2019). In term of volatiles,hydrocarbons are the major components of propolis, such asalkanes, alkenes, alkadins, monosesters, diesters, aromatic esters, fatty acids and steroids have been identified in propolis.

Hydrocarbons are the major components of propolis. Alkanes, alkenes, alkadins, monosasters, diesters, aromatic esters, fatty acids and steroids have been identified in propolis.

Reports have shown that minerals are rare elements (such as calcium, magnesium, aluminum, carbon, iron, manganese, nickel, and zinc), as well as toxic elements (mercury, carbide, and lead). According to the studies, vitamins E, C, B1, B2, B6 have been identified in propolis (Ahangari et al., 2018).

It has been reported that propolis from temperate zones are rich in pinocembrin, pinobanksin, galangin, chrysin, caffeic, and ferulic acids. Phenolic acids and their esters and flavonoids were identified in propolis samples from France. Flavonoids also were revealed as predominant components in hydroalcoholic extracts of propolis from Spain. Portuguese propolis characterization revealed methylated and/or esterified or hydroxylated derivatives of poplar flavonoids, Table 2 (Dezmirean et al., 2020).

Based on the diverse profile of physicochemical properties revealed in recent studies of Portuguese propolis from various regions of Portugal, multiple types of propolis have been identified. Some Portuguese propolis samples have comparable phenolic content, with obvious differences in concentrations. It was reported that samples from the north, central coast, Azores Archipelago, and south had higher phenolic content, containing simple phenolics up to 261 mg/g and flavonoids up to 460 mg/g. The remaining samples, which comprised propolis from the north, central interior, south, and Madeira Island samples, showed a low phenolic compound composition overall (Falcão et al., 2013; Falcao et al., 2013).

Recent research on Portuguese propolis has identified two main types: common temperate propolis, which contained the typical poplar phenolic compounds including flavonoids and their methylated/esterified forms, phenylpropanoid acids and esters, and uncommon temperate propolis, which had the typical poplar flavonoids and a significant amount of unusual flavonoid glycosides, some of which had never been characterized in propolis (Falcao et al., 2013).

Over than 85% of the Portuguese propolis samples studied were defined as common temperate propolis with a common phenolic matrix. This group contained all the samples from the central coast and the Azores archipelago, as well as most samples from the north and a few from the central interior, south, and Madeira. All samples from the central coast, the south, and a sample from Madeira had an extra flavonol designated kaempferide. Pinobanskin derivatives were scarce in samples from the center interior and south, although they did include a kaempferol-dimethyl ether, which is uncommon in temperate propolis. In overall, 62 compounds were identified in common temperate propolis type samples (Falcao et al., 2013).

Different samples of Portuguese propolis were investigated in the Falcao studies, and it was discovered that six of them had four additional phenolic compounds that were not detected in the common propolis samples: ellagic acid, luteolin, a dimethoxylated flavonol, and a dihydroxy-dimethoxyflavone. Furthermore, flavonoid glycosides such as Quercetin-3-O-rutinoside, quercetin-3-*O*-glucuronide, quercetin-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, isorhamnetin-3-*O*-rutinosode, isorhamnetin-*O*-pentoside, quercetin-3-*O*-rhamnoside, isorhamnetin-*O*-glucuronide, kaempferol-methylether-*O*-glucoside isorhamnetin-*O*-acetylrutinoside, rhamnetin-*O*-glucuronide, Quercetin-dimethylether-*O*-rutinoside, quercetin-dimethylether-*O*-glucuronide, kaempferol-*O*-*p*-coumaroyl rhamnoside were discovered in the uncommon propolis type (Falcao et al., 2013).

Moreover, the phenolic profile of both poplar tree male and poplar tree female ethanolic extracts was identical to that of the Portuguese common temperate propolis type previously reported. Caffeic acid, 3,4-dimethyl-caffeic acid, caffeic acid isoprenyl ester and its isomer, caffeic acid benzyl ester, and caffeic acid phenylethyl ester are among the phenolic acids and derivatives found in the composition. Pinocembrin, chrysin, pinobanksin-3-*O*-acetate, and galangin were identified in the poplar tree extracts, as well as flavonoids and their derivatives. Once the phenolic profiles of the two genders were compared, it was observed that the female poplar had the chemicals pinobanksin-5-methyl-ether-3-*O*-pentanoate and 3-hydroxy-5-methoxyflavanone, , pinobanksin-3-*O*-butyrate, pinobanksin-3-*O*-pentenoate, pinobanksin-3-*O*-pentanoate, pinobanksin-3-*O*-hexanoate, which had already been reported in Portuguese propolis, whereas the male poplar had none (Falcão et al., 2013).

Table 2: Chemical composition of poplar-type propolis of the major producing poplar-type propolis of the world (Dezmirean, Pasca et al. 2020).

Origin	Separated Compounds
Algeria	Pinostrobin chalcone, galangin, naringenin, tectochrysin, methoxychrisin, prenilated coumarin, pectolinaringenin, pilosin, ladanein, chicoric acid, caftaric acid, 2-hexanal, myristic acid, linoleic acid, spathulenol, isooctgane, hexadecane, *p*-cymene, palmitic acid, 4-terpineol, carvacol, α-cedrol
Bulgaria	Dihydrocaffeic acid, dihydroferulic acid, pinostrobin, dimethyl kaempferil, benzyl alcohol pinobanksin, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, quercetin, myricetin, kaempferol, rutin, catechin, quercetin-3-β-glucoside, alcohols, aromatic acids, organic acids, terpenoids

Canada	Chrysin, pinocembrin, ellagic acid, pinostrobin, benzyl caffeate, palmitic acid, naringenin, pinobanksin, isopentenyl caffeate, acacetin, caffeic acid, acacetin, caffeic acid phenethyl ester, other aromatic acids, fatty acids, esters, dihydrochalcones
France	Benzyl caffeate, pinocembrin, trans-*p*-coumaric acid, caffeic acid, *p*-coumaric acid, chrysin, pinobanksin, pinobanksin-3-acetate, galangin, kaempferol, tectochrysin
Germany	Chrysin, pinocembrin, naringenin, pinobanksin, kaempferol, luteolin, pinobanksin-5-methylether, coumaric acid, galangin, apigenin, pinostrobin, benzyl caffeate
Italy	Communic acid, isocupressic acid, acetylisocupressic acid, caffeic acid, *p*-coumaric acid, ferulic acid, quercetin, apigenin, kaempferol, chrysin, caffeic acid phenethyl ester, pinocembrin, galangin, benzyl salycilate, benzyl cinnamate, caffedic acid cinnamyl ester, pinobanksin-3-*O*-acetate
Morocco	Wogonoside, quercetin-arabinoseglucoside apigenin dihexoside, rhamnetin hexoside, baicalin, rhamnetin, isorhamnetin, saphnin, daphnitin, afzelechin-catechin dimmer
Portugal	Caffeic acid, ellagic acid, p-coumaric acid, ferulic and isoferulic acid, quercetin, luteolin, apigenin, kaempferol, rhamnetin, chrysin, galangin, acacetin, kaempferide, kaempferol dimethyl ether, other flavonoid glycosides, sesquiterpene, monoterpene, aliphatic and aromatic alcohols, fatty acids, carbonyl compounds, hydroxarbons
Poland	Aromatic acids, fatty acids, esters, flavonoids, and chalcones (85 constituents) 37 Phenolic compounds
Romania	Gallic acid, protocatechuic acid, syringic acid, caffeic acid, vanillin, *p*-coumaric acid, ferulic acid, cinnamic acid, rosmarinic acid, pinocembrin, chrysin, galangin, pinostrobin, caffeic acid phenethyl ester, rutin, quercetin, apigenin, resveratrol
Spain	Naringenin, genistein, kaempferol, apigenin, pinocembrin, galangin, acacetin, chrysin, benzoic acid, guaiol, pinostrobin, pinobanksin, galangin-7-methyl ether, pinobanksin-3-acetate, glyceryl trans-caffeate, henicosane, tricosane, pentacosane, hexacosane, heptacosane, nonacosane, tetracosanoic acid
Turkey	Apigenin, pinocembrin, pinobanksin, chrysin, galangin, quercetin, rutin, kaempferol, *p*-coumaric acid, ferulic acid, caffeic acid and their esters, *p*-hidroxybenzoic acid, vanillic acid, protocatechuic acid, cinnamyl cinamate, abietic acid, isopimaric acid, dihydroabietic acid, hydroxy fatty acids, phenolic glicerides
Tunisia	Chrysin, galangin, tectochrysin, pinocembrin, pinobanksin, dimethylallil caffeate, phenylethyl caffeate, myricetin 3, 7, 4', 5'-tetramethyl ether, quercetin 3, 7, 3'-trimethyl ether

3.2. Main applications of propolis

Propolis is one of the promising natural products, exhibiting a wide range of biomedical applications. It has been used as an antibacterial, antifungal, anti-inflammatory, antiviral, anesthetic, antioxidant, antitumoral, antiprotozoal, anticancer, antihypertensive, anticarcinogenic and anti-hepatotoxic in addition to possessing cytotoxic activity. The potential bioactive properties have been owed to its chemical compounds. Instead of individual components, there may be combined action, which leads propolis to have diverse biological performance (Almuhayawi, 2020; Anjum et al., 2019).

The bee glue has been used since ancient times in folk medicine. It was known by its antiputrefactive properties, the Egyptians used for embalming their cadavers. The romans and Greek doctors applied as an antiseptic for wound treatment and healing also as a mouthwash. Moreover, it was used in the battlefield like ointment or cream for the treatment of wounds of soldiers in battlefield. It has been impregnated in ointments containing butter, Vaseline olive, oil, or lanolin (Almeida et al., 2020; Almuhayawi, 2020). Nowadays, propolis is used in diverse fields as food industry biomedical applications, oral hygiene, and cosmetic products. Is reported to increase the shelf life of the products (Oses et al., 2020).

Several studies have reported the antibacterial activity of propolis against many types of Gram-positive and Gram-negative bacteria. Middle Eastern propolis has showed the best anti-bacterial activity. It was highly effective against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria. This activity was mostly tested on *E. coli*, *S. aureus, Salmonella* spp and aeruginosa. According to literature studies, (Almuhayawi, 2020; Dezmirean et al., 2020) ethanolic extracts of propolis were more effective against Gram-positive bacteria compared to Gram-negative ones. Brazilian propolis revealed antibacterial activity against some aerobic bacteria such as, *Bacillus cereus*, *B. subtilis*, *Enterococcus faecalis*, *Micrococcus luteus*, *Nocardia asteroids*, *Rhodococcus equi*, *Staphylococcus auricularis* (Anjum et al., 2019). The Turkish propolis has potential against various types of mycobacteria (Almuhayawi, 2020).

As mentioned before, propolis has antifungal activity. showing an impressive result against Mycobacteria, Candida, Trichophyton, Fusarium, and other skin infecting fungi. Also, propolis was tested on *Leishmania donovani*, *Trypanosoma cruzi*, *Giardia lamblia, Trichomonas vaginalis, Toxoplasma gondii* and *G. duodenali* presenting antiprotozoal activity.

Additionally, ethanolic extract of Brazilian and Turkish propolis had a significant role in dental pulp repair. Phenolic compounds present in propolis such as galangin and pinocembrin protect cells from oxidation reactions and food storage from oxidation and poisoning. Portuguese propolis has the antioxidant property and prevents lipid peroxidation in red blood cells of humans (Anjum et al., 2019; Zabaiou et al., 2017).

Propolis components has therapeutic capability on tissue repairing and regeneration of injury due to the presence of bioflavonoids, arginine, vitamin C, provitamin A, B complex as well as some minerals. (Anjum et al., 2019) Propolis is a very powerful antioxidant, and it is one of the most promising candidates for use as an antitumoral agent due to its antiproliferative activity (Dezmirean et al., 2020; Elisa et al., 2017). Several studies had reported that propolis such as Turkish propolis and Chinese propolis act as an anti-tumor by enhancing program cell death (Anjum et al., 2019; Hiroshi Izuta, 2008; Watanabe et al., 2011). Flavonoids can be active against breast cancer, lung cancer, oral cancer as well as esophagus, stomach, colorectal, prostate, and skin cancer. Besides, the water extract of propolis from Thailand has shown anticancer activity against colon carcinoma cell line SW620. (Anjum et al., 2019) Propolis can be used as an alternative canal irrigant for the elimination of *Enterococcus faecalis* and *Candida albicans*. Also, proved very effective on *Enterococcus faecalis* after seven to ten days and can be used as an intra-canal medication. The use of propolis as an intracanal medication can change the clinical color of tooth crown (Ahangari et al., 2018). During dental trauma, the tooth is completely out of alveolar socket, so a storage medium is needed to carry the tooth to the dental clinic and to maintain the vitality of the PDL cells.

This bee's product has been shown suitable preservative solution for a period of 6h and more propolis is more efficient than Hank's Balanced Salt Solution (HBSS), milk and serum, as more PDL cells survive (Ahangari et al., 2018). Green propolis extracts was used in sunscreen preparations of Gel Permulen TR-1 since it has shown an important SPF and antioxidant activity, which could guarantee a better skin protection to the final phytopharmaceutical product (Almeida et al., 2020). Propolis could be applied in the food industry due to its antioxidant and antimicrobial properties. It has the potential to delay lipid oxidation and as a result increase the shelf life of food products. Currently, propolis is used worldwide in several fields and its concentration varies depending on the application and should be enough to achieve bacteriostatic or bactericidal effects (Almuhayawi, 2020).

4. Application of natural preservatives in the cosmetic industry

Because of their complex active ingredients, powerful fragrance qualities, and natural marketing image, essential oils are commonly used in modern skincare products**.** It has been demonstrated that their antibacterial qualities make them useful as natural preservation agents in cosmetic preparations, either alone or in combination with other preservatives. They provide protection against germs and fungus in this way. Essential oils can increase the dermato-cosmetic qualities of the finished product, not only by fighting against microbial infections but also by helping to preserve the cosmetic formulation's preservation (Sharmeen et al., 2021).

An early study on the efficacy of *Thymus vulgaris* essential oil in two cream formulations revealed an unsatisfactory preservation effect in a challenge test. Despite the large amount of thyme oil used, the essential conditions were reached against bacteria strains and yeast, but not against *Aspergillus niger* (3 % Thymol levels in oil samples were high (38.6 % and 43 %, respectively), whereas carvacrol levels were low (9.8 % and 2.2 %). According to the authors, the appropriate ratio of both oil phenolic components would be more effective in antifungal activity. Another study found that essential oils of *Artemisia afra, Pteronia incana, lavandula officinalis,* and *Rosmarinus officinalis* were effective at reducing microbial contamination in aqueous cream formulation. The population of examined microorganisms was strongly controlled and regulated in the challenge test up to the seventh day. The most effective at all concentrations was *A. afra* oil reach in thujone (53 %) (0.5 %, 1.0 % and 1.5 %). In a comparison study of the efficacy of lavender, tea tree, and lemon essential oils alone and in combination with synthetic preservatives (1,3-dimethylol-5,5-dimethyllhydantoin, and 3-iodo-2-propynyl butyl carbamate) in body milks, the synergistic effect of the composed preservative system was clearly demonstrated, allowing for an 8.5-fold reduction in synthetic preservative contents. Synthetic preservatives tested at a lower dose (0.1 percent) without essential oils were ineffective against *Pseudomonas aeruginosa* and Candida sp. A further investigation using the same essential oils and preservatives in washing liquid and body balm enhanced with solubilizer verified the improved preservation efficacy only for *S. aureus*, Candida sp (Dreger & Wielgus, 2013).

The majority of studies suggest that when EOs are mixed with synthetic preservatives, chelators, and solubilizers, they become stronger, highlighting the synergistic effects of these natural ingredients with the synthetic applied agents. Synthetic preservative concentrations can

be decreased by using EOs at low concentrations or EOs in combination with solubilizers. When used alone, oil's antibacterial activity is usually insufficient, demanding higher doses to achieve a stronger effect. Phase separation, poor formulation viscosity, and a strong, unpleasant odor are all problems caused by a high concentration of EOs. When EOs are used in combination with synthetic preservatives, the total amount of both preservation agents is reduced, which appears to be a reasonable compromise. The primary benefit of such a solution is that it reduces the concentrations of potentially allergenic components, resulting in improved product safety. Furthermore, when there are less EOs in cosmetic perfumes, people accept them better (Dreger & Wielgus, 2013).

Furthermore, certain phytochemicals identified in sugarcane byproducts (most notably phenolic acids) have the potential to be used as skin-care ingredients. They could also be used in cosmetics as anti-aging agents and preservatives. (Carvalho et al., 2021) Additional study showed that wild cherry bioactive components had excellent antioxidant properties and can be employed in pharmaceutical creams, gels, and lotions (Telichowska et al., 2020).

Propolis is a phenolic compound-rich bee product with antibacterial, UV-protective, analgesic, antioxidative, and regenerative properties. It found that propolis allergy is uncommon 0.64–1.3% (Kurek-Gorecka et al., 2020). Due to these properties, the pharmaceutical and cosmeceutical industries are becoming increasingly interested in producing propolis-based products (Barros et al., 2019; Vassya Bankova, 2014). Sprays, colors, gels, dentifrices, soaps, and dermatological creams are among the commercial items that contain propolis in their formulations as mentioned in figure 10 (Barros et al., 2019). Shampoos containing bee glue can be a natural option for treating dandruff and avoiding recurrence due to its antifungal and anti-seborrheic properties. Propolis is also used in the manufacture of toothpaste. Aqueous or ethanol preparations of propolis are widely available. According to INCI nomenclature, it can be found in cosmetics under the INCI names propolis and propolis extract. The most often used propolis extracts are those in ethanol. Lipsticks are made with propolis mixed with lipids, while antifungal cosmetics are made with an aqueous extract. Skin cure based on propolis is effective against fungal skin disorders due to the presence of flavonoids (pinocembrin and pinobanksin), phenolic acids (caffeic acid), and terpenes (Kurek-Gorecka et al., 2020).

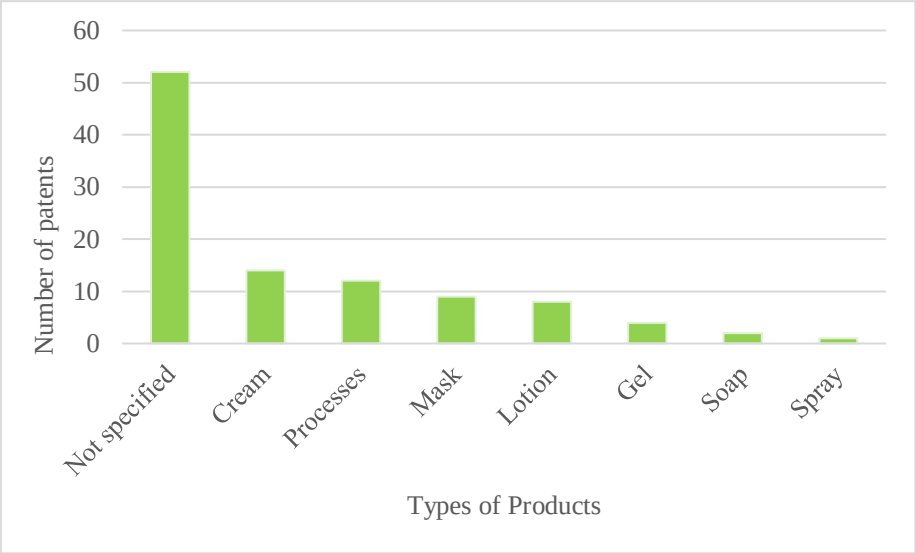


Figure 10: Type of propolis products for dermatological use deposited in intellectual property banks (Barros, Neto et al. 2019).

At different levels, the propolis dye was active, with the lowest inhibitory concentration being 0.625 percent. Because of its anti-inflammatory, antibacterial, healing, and immunomodulatory properties, the cosmetic industry has invested in the use of propolis in the treatment of acne, particularly in cosmeceuticals, where dermo-cosmetics containing propolis at a concentration of up to 4% can play a significant role in the prevention and treatment of acne vulgaris (Barros et al., 2019). Propolis ethanol extract inhibits Staphylococcus epidermidis in a similar way. Propolis is employed in the production of acne-fighting cosmetics as well as antibacterial and antifungal medications. Propolis contains regeneration and repair characteristics, as well as the capacity to defend against external causes, at a concentration of 5–20 %. It can be used in the production of anti-bedsores medications (Kurek-Gorecka et al., 2020).

Referring to Gregoris study, All the findings show that ethanolic extract of propolis (EEP) and its components provide adequate UVR protection, greatly beyond the UVR filters often used in skin care products as a standard. EEP, as well as some of its components, has been proven to have strong antioxidant capabilities. The combination of these two qualities qualifies EEP as a cosmeceutical, which means it might be used as an active ingredient in commercial sunscreen formulations for cosmetics with anti-aging and anti-aging effects (Gregoris et al., 2011).

This beeswax is a valuable component to sun defenders (creams, lotions, sticks, and lipsticks) because of its qualities as a natural filter, as well as its antioxidative, anti-inflammatory, and regenerative properties. Other researchers discovered that applying Romanian propolis to the skin of 30 Swiss mice produced photoprotective benefits against UVB rays. Propolis is also used to make protective lipsticks. It is both regenerative and antiviral in cold sores caused by the herpes simplex virus (Kurek-Gorecka et al., 2020).

Spanidi and his colleagues had recently developed a method that consists of liposomes and cyclodextrins containing propolis polyphenols for skin applications. The novel formulation has the advantage of encapsulating what occurs during extraction, allowing for a one-pot operation while also preserving the polyphenols from degradation within the system. Furthermore, if the raw propolis meets standards for initial polyphenol content, the encapsulation efficiency is sufficient, making the method cost reasonable for premium applications. Furthermore, the polyphenol content is released at a controlled rate once the item is placed in a suitable environment. The findings show that the encapsulation and delivery system can retain the anti-mutagenic, anti-oxidative, and anti-ageing effects of propolis polyphenols at levels comparable to those of propolis methanolic extracts from similar geographic origins, making it ideal for applications requiring non-toxic solvents and controlled polyphenol release (Spanidi et al., 2021). Polymeric nanoparticles containing propolis extract have recently been popular in the cosmetics industry due to their potential to increase solubility, stability, and skin permeability, vary the release patterns of cosmetic effectiveness components, and transport cosmetic efficacy components to target areas. The antiseptic, anti-inflammatory, antimycotic, antifungal, antibacterial, antiulcer, anticancer, and immunomodulatory properties of the polymeric nanoparticles developed in Joo Young, and his team study can be used in pharmaceutical applications that use propolis as an antiseptic, anti-inflammatory, antimycotic, antifungal, antibacterial, antiulcer, anticancer, and immunomodulatory agent (An et al., 2022).

Numerous studies have demonstrated the beneficial effects of bee products on the skin, especially the use of propolis, in wound healing shows their therapeutic usefulness (Kurek-Gorecka et al., 2020). When compared to the studied formulations, propolis has been shown to have a strong antibacterial effect, considerably improves the healing process of burn wounds, reduces convalescence time, has a positive impact on scars, and does not cause irritation or allergic reactions. Propolis ointment is one of the most effective treatments for healing wounds of different symptoms, as identified in the literature (Staniczek et al., 2021).

5. Objectives

The major purpose of this dissertation was the development of cosmetic formulations incorporated with bioactive extracts from plants and propolis in order to functionalize this product with natural antioxidants/antimicrobials.

The specific objectives can be defined as:

1) Extraction of bioactive compounds.
2) Bioactive evaluation, namely antioxidant and antimicrobial potential.
3) Chemical characterization in terms of phenolic compounds.
4) Consider mixtures of the three different matrices under study.
5) Incorporation of the most promising extract/mixture in cosmetic formulations.
6) Evaluation of the formulation´s stability.

6. Material and Methods

6.1. Samples origin

The propolis sample was collected in 2021, in the apiaries of the Agrarian Development Service of the Regional Secretariat for Agriculture and Rural Development in São Miguel, Açores, Portugal. After reception at the laboratory, propolis was stored at –20°C. Eucalyptus and rosemary were gently ceded by the enterprise Deifil – green biotechnology, Braga, Portugal. After reception, the samples were frozen and lyophilized, reduced to a fine powder and stored protected from light and humidity.

6.2. Extraction of bioactive compounds

6.2.1. Maceration

A mass of 1 g of each sample was extracted for 1 hour with 30 mL of ethanol: water 80:20 (*v/v*) at room temperature, under stirring at 150 rpm, and Whatman n° 4 filter paper was used to filter the solution. The residue was then extracted again using the same conditions. To eliminate ethanol, the mixed extracts were evaporated at 40 ° C (rotary evaporator Buchi R-210). The samples were then lyophilized after being frozen.

6.2.2. Heated water bath

A mass of 1 g of each sample was extracted for 1 hour with 10 mL of ethanol: water 80:20 (*v/v*) using a water bath at 70 °C, as previously developed by Falcao (Falcao et al., 2010). The mixtures were filtered, and the residues were extracted again under the same conditions. Following the second extraction, the filtered solutions were mixed, concentrated, and evaporated to remove ethanol at 40°C. The extracts were then frozen at -20°C and lyophilized.

The obtained extracts in each extraction methodology were mixed in different proportions to search for possible synergic effects. The different combinations used are presented in table 3.

Table 3: Different extract combinations

Extracts combinations	Proportions (%)
Eucalyptus	100
Propolis	100
Rosemary	100
Eucalyptus/propolis	(50:50); (60:40); (40:60)
Eucalyptus/rosemary	(50:50); (60:40); (40:60)
Rosemary/ propolis	(50:50); (60:40); (40:60)

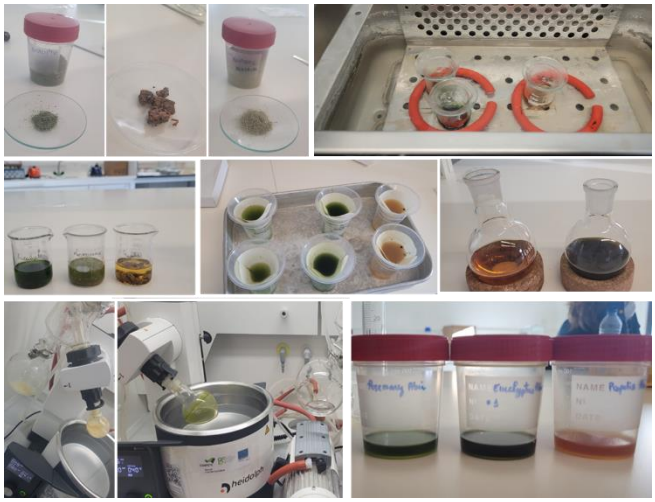


Figure 11: Extraction process.

6.3. Chemical characterization

6.3.1. Organic acids

For the organic acids, 1 g of each sample was combined with 25 ml of metaphosphoric acid (4.5%) in a goblet that was lined with aluminum foil. The mixture was then subjected to magnetic stirring for maceration for 20 minutes at room temperature. Following this procedure, a 0.2 µm nylon filter was used to filter the suspension.

The analysis was performed on an ultrafast liquid chromatography system (Shimadzu 20A series, Shimadzu Corporation Kyoto, Japan) coupled to a photodiode array detector (UPLC-PDA). Elution was done with sulfuric acid (3.6 mM) on a C18 reverse phase column (5 µm particle size, 250 × 4.6 mm; Phenomenex, Torrance, CA, USA). The identification of organic acids in the samples was made by comparing the retention time and UV- Vis spectrum

with those of the standards. A calibration curve obtained by plotting the peak area recorded at 215 nm against the concentration was used for the quantification. The results were expressed as g per 100 g of dry mass.

6.3.2. Phenolic compounds

6.3.2.1. Eucalyptus and rosemary

The phenolic compounds were extracted in accordance with the procedure detailed in 6.1.1. Thereafter, the extracts were re-dissolved in ethanol/water (20:80, *v/v*; 1 mL), and filtered through 0.22-μm disposable LC filter disks. The chromatographic analysis was achieved by using a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA), coupled to a diode array detector (280, 330, and 370 nm) and an electrospray ionization mass detector (Linear Ion Trap LTQ XL, Thermo Finnigan, San Jose, CA, USA), working in the negative mode. The chromatographic separation was performed using a Waters Spherisorb S3 ODS-2 C18 (3 μm, 4.6 mm × 150 mm, Waters, Milford, MA, USA) column at 35 °C. The compounds were identified considering their retention time, UV-Vis and mass spectra in comparison with available standards and literature data. Calibration curves of the available phenolic standards were plotted based on the UV-Vis signal in order to obtain quantitative analysis. In the case of unavailable commercial standards, the compounds were quantified via calibration curve of the most similar standard available. The results were expressed as mg/g of extract (Pinela et al., 2019).

6.3.2.2. Propolis

The propolis phenolic compounds were extracted following the method described in 6.1.2. According to earlier work by (Falcao et al., 2010), reversed-phase high-performance liquid chromatography (HPLC) with UV detection was used to examine the propolis and plant source extracts. The Knauer Smartline separation module, a Knauer Smartline autosampler 3800, a cooling system set to 4 C, and a Knauer UV detector 2500 composed the chromatographic system's basic components. ClarityChrom® software (Knauer, Berlin, Germany) was used for data collecting and remote control of the HPLC machine. The column was a 250 mm x 4 mm id, 5 m particle diameter, end-capped Nucleosil C18 (Macherey-Nagel) with a temperature of 30 ° c The mobile phase was composed of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile, which had previously been degassed and filtrated. The solvent gradient started with 80% A and 20% B, reaching 30% B at 10 min, 40% B at 40 min,

60% B at 60 min, 90% B at 80 min, followed by the return to the initial conditions. For the analysis, the ethanolic extract (10 mg) was dissolved in 1 mL of 80% of ethanol. Salicylic acid, as the internal standard (IS), was added to all extracts. Each sample was filtered through a 0.2 µm Nylon membrane (Whatman) and then 10 µl of the solution was injected. Chromatographic data were acquired at 280 nm Caffeic acid, ferulic acid, quercetin, pinocembrin, chrysin, and caffeic acid phenylethyl ester calibration curves, obtained with seven concentration levels, were used to quantify the compounds. When a standard wasn't available, the chemical's quantification was stated as the equivalent of the phenolic compound that was structurally closest (Falcão et al., 2013). Quantification was achieved using calibration curves for caffeic acid (0.0187-0.4 mg/mL; $y = 6 x10^7x$-26360; $R^2 = 0.996$), *p*-coumaric acid (0.0187-0.4 mg/mL; $y = 9x10^6x$ - 35105; $R^2 = 0.999$), genistein (0.0375-0.8 mg / mL; $y = 1x10^6x + 48333$; $R^2 = 0.999$), kaempferol (0.075-1.6 mg/mL; $y = 1x10^6x – 5867$; $R^2 = 0.997$), pinocembrin (0.0375-0.8 mg / mL; $y = 2x10^6x + 5250$; $R^2 = 0.997$) and chrysin (0.0375-0.8 mg / mL; $y=4x10^6x$ - 18959; $R^2 = 0.999$). When the standard was not available, the compound quantification was expressed in equivalent terms of the structurally closest compound. The assays were performed in duplicate, and the results expressed as mg/g of sample.

6.4. Evaluation of the antioxidant activity

6.4.1. Thiobarbituric acid reactive substances (TBARS) assay

Thriobarbituric reactive substances (TBARS) is a colorimetric method for screening and monitoring lipid peroxidation. Malonaldehyde (MA) is an aldehyde formed during the breakdown of unsaturated fatty acids. When heated in an acidic solution, one molecule of MA interacts with two molecules of thiobarbituric acid to generate a unique Schiff base complex (MDA-TBA), a pink pigment. The presence of antioxidants inhibits lipid peroxidation in porcine brain homogenates as measured by the absorbance of the MDA-TBA complex at 532-535 nm or fluorescence (excitation 515 nm, emission 553 nm) spectrophotometer (Abeyrathne, 2021). In a ratio of 1:2 *w/v*, brain tissue homogenate was dissolved in Tris-HCl buffer (20 mmol/L, pH 7.4) and centrifuged at 3000 g for 10 minutes. Each dilution of the sample solutions (200 µL) was pipetted into test tubes, together with 100 µL of ascorbic acid (0.1 mmol/L), 100 L of $FeSO_4$ (10 mmol/L), and 100 mL of brain tissue homogenate supernatant. The tubes were incubated for 1 hour at 37 °C. Trichloroacetic acid (500 µL, 28% w/v) and 380 µL thiobarbituric

acid (TBA, 2% w/v) were added to stop the reaction, and the mixture was incubated at 80 °C for 20 minutes.

To remove the precipitated protein, the solutions were centrifuged at 3500 rpm for 5 minutes. At 532 nm, the absorbances of the supernatant samples were measured. The following equation was used to determine the inhibition percentage:

$$Inhibition\ ratio\ (\%) = \left[\frac{A-B}{A}\right] X100$$

(A: absorbance of the control solution and B: absorbance of the sample solution) were normalized to EC_{50} values, (extract concentration responsible for 50% of lipid peroxidation inhibition) expressed in mg/mL of extract.

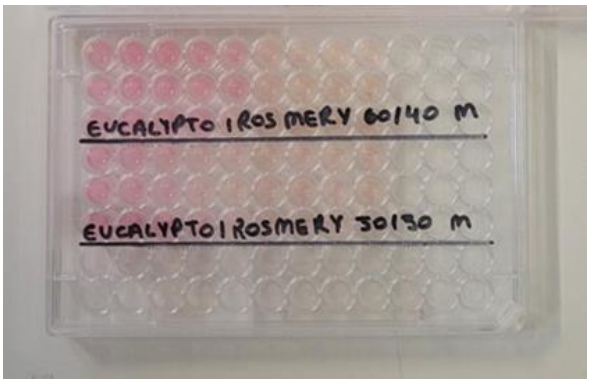


Figure 12: TBARS assay in microplate.

6.4.2. Cellular antioxidant activity

In order to measure the cellular antioxidant activity (CAA), the extracts were dissolved in water to a concentration of 8 mg/mL, from which 2',7'-dichlorohydrofluorescein (DCFH) was made with ethanol and diluted with HBSS (50 µM), giving the concentrations to be tested (500 - 2000 M), acording to KELLY L WOLFE (2007), this method was carried out.

The cell line employed was RAW 246.7 (murine macrophage cells), which were kept in an incubator at 37°C, 5% CO_2, a humidified environment, and DMEM culture media supplemented with L-glutamine, penicillin (100 U/ml), streptomycin (100 g/ml), fetal serum bovine (10%), and non-essential amino acids (2 mM). The contents of murine macrophages were transferred to a falcon using a cell scraper, followed by centrifugation for 5 minutes at

1200 rpm. The medium was discarded, and the amount of new medium used was determined by the size of the pellet produced. The solution was then made at a cell density of 70,000 cells/mL. An aliquot (300 L) of the obtained solution was transferred to black microplates with transparent bottoms (SPL Lifesciences) and incubated for 48 hours. The cells were then treated with several extract concentrations (32.5 - 2000 M) and incubated for an additional hour after the incubation time. The media was then discarded, following HBSS washing, a solution of 2.2 2'-azobis(2-methylpropionamide) dihydrochloride (AAPH) was added to the cells. For one hour, the fluorescence was measured using a Biotek FLx800 microplate reader at 485 nm excitation and 538 nm emission. Quercetin was the used positive control, whereas a negative control included dichlorohydroflurescein and DMEM culture media.

6.5. Cytotoxicity for normal cells

The sulforhodamine B (SRB) assay, was performed in accordance with the previous procedure described by (Vichai & Kirtikara, 2006). This approach is based on the ability of SRB to bind stoichiometrically to proteins under mildly acidic conditions before being removed under basic conditions. As a result, the amount of bound dye may be used as a standard for cell mass, which can then be generalized to measure cell growth.

This test was conducted in this research to assess the effects of the bioactive samples on non-tumor cells, which are porcine liver primary culture (PLP2) that was established at the Mountain Research Center and used as the standard cell line. Briefly, the cells were frequently maintained as adherent cell cultures in RPMI-1640 containing glutamine (2 mM), penicillin (100U/mL), streptomycin (100g/mL), heat-inactivated FBS (10%). They were then incubated at 37°C with humidified air and 5% CO_2 for an additional period. The 96-well microplates containing the cell line and the various sample dilutions to be examined (6.25-500 ug/mL) were incubated at 37°C with 5% CO_2 for 48 hours to determine the cytotoxicity. After 60 minutes of incubation at 4°C, the adhering cells were fixed by adding 10% of previously refrigerated trichloroacetic acid (100 µl). After that, distilled water was used to wash and dry the microplates. Eventually, 10mM Tris (200 µl) was used to solubilize the attached SRB, and the absorbance was measured using an ELX800 microplate reader at a wavelength of 540 nm. Following that, the results were represented as GI50 values (sample concentration inhibiting 50% of cell growth).

6.6. Anti-inflammatory activities

According to the method described by (Taofiq et al., 2019), the anti-inflammatory activity was evaluated. DMEM media supplemented with 10% heat-inactivated fetal bovine serum, glutamine, and antibiotics was used to cultivate the mouse macrophage-like cell line RAW264.7 at 37 °C in 5% CO2 and humidified air. A cell scraper was used to separate the cells for each experiment. The Trypan blue dye exclusion test revealed a cell density of 5 X105 cells/mL and a percentage of 5% dead cells. 150,000 cells/well of cells were placed in 96-well plates, and the cells were left to adhere to the plate overnight. Cells were then exposed to various doses of each extract for 1 hour before being further stimulated with LPS (1 g/mL) for an additional 18 hours. A positive control was applied, which was dexamethasone (50 µM). Nitric oxide (NO) basal levels may have been altered by the effects of all investigated substances when LPS was not present. In DMEM with supplements, both substances and LPS were dissolved. Compounds were dissolved in 50% DMSO for the suppression of NO and then subjected to successive dilutions (400 to 50 ug/mL) to determine the effective concentration required to exhibit anti-inflammatory action. Sulphanilamide, N-(1-napthyl) ethylenediamine hydrochloride (NED), and nitrite solutions were employed from the Griess Reagent System kit. The cell culture supernatant (100 L) was transferred to the plate and mixed with the sulfanilamide and NED solutions, each for 5–10 minutes at room temperature, according to the reference curve of nitrite (sodium nitrite 100 M to 1.6 M; y=0.0066x+0.1349; R2=0.9986). By measuring the absorbance at 540 nm (microplate reader ELX800 Biotek) and comparing the results to the reference calibration curve, the NO production was determined.

6.7. Anti-tyrosinase activity

The tyrosinase enzyme inhibition activity of the extracts was evaluated using L-DOPA (5mM) as substrate. All the tested samples including butylresorcinol used as positive control were submitted to a series of dilutions. Shortly, 137 µL of 0.1M phosphate buffer (pH 7.1), 60 µL of L-DOPA, 87 µL of MBTH (20.7mM), and 10 µL of test samples at different concentrations were added to each well. The microplate was incubated for 10 minutes at 25°C. Afterwards, 6µL of mushroom tyrosinase enzyme (142 units/mL) was added to each well, and the plates were incubated for 30 mins at 25°C. The formation of the dopaquinone-MBTH complex was evaluated at 505 nm using a microplate spectrophotometer (SPECTROstar Nano

Multi-Detection Microplate Reader; BMG Labtech, Ortenberg, Germany). The percentage of tyrosinase enzyme inhibition was calculated using the following equation:

$$\%\ Inhibition = \frac{Ab\ of\ Buffer\ Control - Ab\ of\ test\ sample}{Ab\ of\ Buffer\ Control} \times 100$$

Ab-Absorbance.

The best combinations, eucalyptus/propolis (50:50) and eucalyptus/rosemary (60:40), chosen for incorporation into the cosmetic formulations after being tested for the other bioactivities, were assessed for their anti-tyrosinase activity. A preliminary screening is required because this test is costly.

6.8. Antimicrobial properties

6.8.1. Antibacterial activity

For all the studied bacteria, the minimum inhibitory concentration (MIC) was obtained using the microdilution technique and the rapid *p*-iodonitrote-trazolium chloride (INT) colorimetric assay (Pires et al., 2018).The following Gram-negative bacteria were used: *Escherichia coli, Klebsiella pneumoniae, Morganella morganii, Proteus mirabilis, propionium acnes*, *Pseudomonas aeruginosa* and Gram-positive bacteria: *Enterococcus faecalis, Listeria monocytogenes, methicillin resistant Staphylococcus aureus (MRSA).* The bacterial strains were clinical isolates obtained from patients hospitalized in various departments at the Hospital Center of Trás-os-Montes and Alto Douro (Vila Real, Portugal).

The samples were first dissolved in 5% (v/v) dimethyl sulfoxide (DMSO) and 95% autoclaved distilled water, resulting in a final concentration of 20 mg/mL for the stock solution. In the first well of a 96-well microplate, 90 µl of this concentration and 100 µl of tryptic soy broth (TSB) were added in triplicate. 90 µl of TSB medium were added to the remaining wells. The concentration ranges were then obtained by serial dilution of the samples (10 to 0.03125 mg/mL). In order to complete the process,10µl of inoculum (that was standardized at 1.5 x106 Colony Forming Units (CFU) / ml) was added at each well, ensuring the presence of 1.5 x 105 CFU. A TSB control and an extract control were both prepared as negative controls. Three

positive controls were used. For all of the studied bacteria, ampicillin, imipenem and vancomycin were utilized. The microplates were incubated for 24 hours at 37°C. After adding (40 µl) of p-iodonitrotetrazolium chloride (INT) at a concentration of 0.2 mg/ml and incubating for 30 minutes at 37°C, the MIC of the samples was found. The minimum inhibitory concentration (MIC) was identified as the lowest concentration that inhibits visible bacterial growth, as evaluated by a change in color from yellow to pink if the microorganisms are viable. MBC was determined by plating 10 µl of liquid from each well that revealed no change in color on solid medium, Blood agar (7% sheep blood), and incubating it at 37°C for 24 hours. The MBC is determined by the lowest concentration that produced no growth. The MBC concentration was defined as the lowest concentration required to kill bacteria.

6.8.2. Antifungal activity

As indicated by (Heleno et al., 2013), the antifungal activity was carried out. The fungi *Aspergillus fumigatus* (ATCC 204305) and *Aspergillus brasiliensis* (ATCC 16404), Frilabo in Porto, Portugal, were used. The micromycetes were cultured on malt agar and the cultures were kept at 4 °C before being transferred to fresh media and incubated at 25 ° c for 72 hours.

To test the antifungal activity, sterile 0.85% saline with 0.1% Tween 80 (v/v) was used to wash the fungal spores off the surface of agar plates. In a final volume of 100 µl per well, the spore suspension was adjusted with sterile saline to a concentration of nearly 1.0×10^5. The samples were first dissolved in 5% (v/v) DMSO and 95% autoclaved distilled water to yield a stock solution with a final concentration of 20 mg/ml. Following that, 90 µl of this concentration was added in duplicate to the first well (96-well microplate) with 100 µl of Malt Extract Broth (MEB). 90 µL of medium MEB were put into the remaining wells. The samples were then serially diluted to achieve concentration ranges ranging from (10 to 0.03125 mg/ml).

The minimum inhibitory concentration (MIC) was determined using a serial dilution approach in a 96-well microplate. MICs were established as the lowest concentrations at which no observable growth was seen (using a binocular microscope). The fungicidal concentration (MFC) was evaluated by repeated sub-cultivation of 2µl of tested compounds dissolved in medium and inoculated for 72 h into microplates containing 100 µl of MEB per well, followed by another 72-h incubation at 26 °C. MFC was defined as the lowest concentration with no observable growth, signifying 99.5% death of the initial inoculum. As a positive control, the commercial fungicide ketoconazole (Frilabo, Porto, Portugal) was utilized.

6.9. Incorporation of extracts as natural ingredients into cosmetic formulations

6.9.1. Preparation of the soap samples

The reaction between a neutral fatty acid and an alkali to make soap and glycerol is known as the basic saponification reaction (G. Sucharita, 2020). Commercial base soap was utilized in this investigation. Two soaps were created using various extracts (Eucalyptus: Propolis 50/50 (*m/m*), Eucalyptus: Rosemary 60/40 (*m/m*)). Afterwards, 30 mL of solidified basic soap was placed in a 100 mL beaker and melted in a water bath. The water bath temperature was kept constant at 65°C. After converting the soap base to liquid form, the extract mixture was added to the preceding composition. The resulting mixture was boiled once more at the same temperature. The mixture was then transferred to the soap molds and placed in the freezer for up to 2-3 hours. The formulated soaps were analyzed for their bioactivities and their physical parameters at two different times after preparation ($\mathbf{T_0}$), after 2 months of preparation ($\mathbf{T_1}$). They were kept at a constant temperature of 4 ºC.

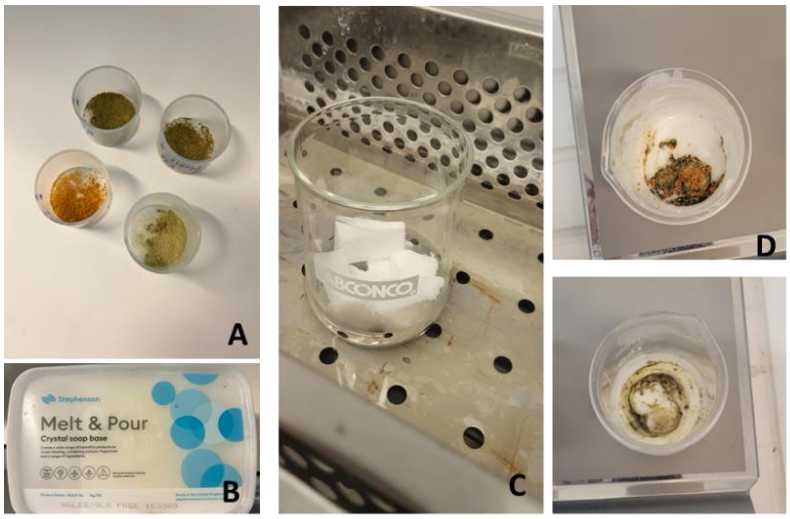


Figure 13: Soap formulation steps, **A**: extracts; **B**: Soap base: glycerin; **C**: Melting the soap base; **D**: Adding the extracts.

Serum definition:

In cosmetology, serum is a concentrated preparation that is frequently used. In professional cosmetology, the name is self-explanatory. In water or oil, the cosmetic serum has the same level of concentration as any other cream. A concentrated product like serum or another option has ten times as much organic material as cream (Miss. Purva et al., 2022).

6.4.1. Preparation of the serum samples

To manufacture the serum, 0.2 g/mL of citric acid solution was mixed with 0.2 g/mL caustic soda until the pH reached 5.5 (skin pH). After that, 0.15g of carboxymethyl cellulose was dissolved in 14.75 g of distilled water in a beaker. In another beaker, 14.75 g of distillate water was mixed with 0.3 g of glycerin. A volume of 3 mL of citrus buffer was added to the second mixture. Six serum formulations were prepared as mentioned in table 4. In the first serum, 0.3g of *eucalyptus/propolis* extract (50:50, *m:m*) was mixed into the glycerin beaker. Furthermore, 15g of potassium sorbate and 0.15g sodium benzoate, two artificial preservatives, were added to the same mixture. In the second serum, 0.3g of *eucalyptus/rosemary* extract (60:40, *m/m*) was added to the mixture. In the third serum, instead of the extract, 0.3g of ascorbic acid was added, resulting in the serum that served as a control. The fourth and fifth serums are natural serums. They include the same components as the first and second serums, but without the two artificial preservatives. The reagents in formula six are the same as those in formula three, except there are no conservatives.

Table 4: Formula of cosmetic serums.

REAGENTS		**F1**	**F2**	**F3**	**F4**	**F5**	**F6**
Phase 1	CARBOXYMETHYL CELLULOSE (CMC)	0.5%					
	Distilated water	48.2%			48.7%		
	Distilated water	48..2%			48.%		
	Glycerin	1%					
	Citric acid buffer	1%					
Phase 2	Extracts/ Ascorbic acid	0.1%					
	Sodium benzoate	0.5%			0%		
	Potassium Sorbate	0.5%			0%		

F- formulation. F1: eucalyptus/propolis (50 :50) + conservatives; F2: eucalyptus/rosemary (60/40) +conservatives; F3: ascorbic acid+ conservatives; F4: eucalyptus/propolis (50 :50); F5: eucalyptus/rosemary; F6: ascorbic acid.

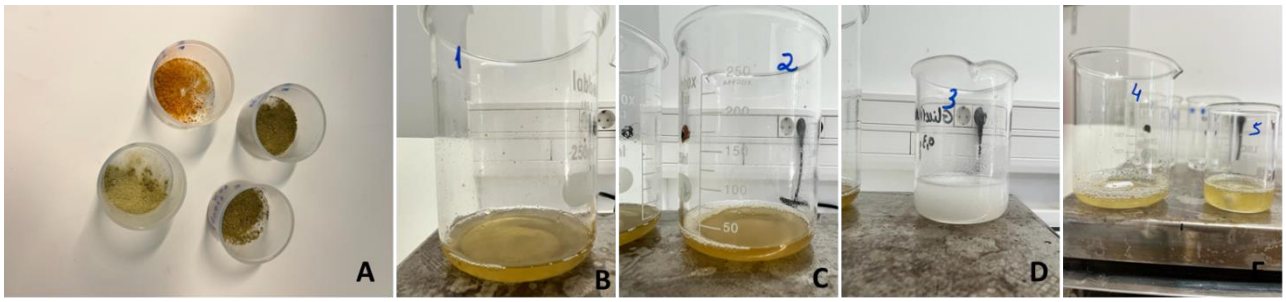


Figure 14: Serum *formulations; **A**: extracts; **B**:* serum (*Eucalyptus/Propolis*) *+conservatives;* **C:** serum (*Eucalyptus/Rosemary*) +conservatives; **D:** ascorbic acid+ conservatives' serum; **E:** serum (*Eucalyptus/Propolis*) and (*Eucalyptus/Rosemary*)

6.5. Evaluation of the physical parameters

6.5.1. Evaluation of the color of the soap and the serum during storage time

Using a colorimeter, the exterior color of each sample of soap and serum was measured at three distinct positions on the top. This study applied a portable Konica Minolta CR400 colorimeter with the D65 illuminant, a standard illuminant created by the International Commission on Illumination (CIE) (Chiyoda, Tokyo, Japan). This luminant reflects the European midday light (daylight illuminant). With a 10o observer angle and an 8 mm aperture that was previously calibrated against a standard white tile, the CIE L* a* b* color space from 1976 was utilized. L* stands for lightness, a* for redness (red- green), and b* for yellowness (yellow- blue) (Ângela Fernandes, 2012).

6.5.2. Evaluation of the pH of the soap and the serum during storage

Using an HI 99161 pH-meter (Hanna Instruments, Woonsocket, Rhode Island, USA), the samples' pH was also determined at three distinct places inside the samples.

6.5.3. Evaluation of the texture of the soap during storage time

According to (Carocho et al., 2019), texture analysis was performed using the P/45 45mm aluminum cylinder probe on a Stable Micro Systems (Vienna Court, Godalming 191 UK) TA. XT Plus Texture Analyzer with a 30 Kg load cell.

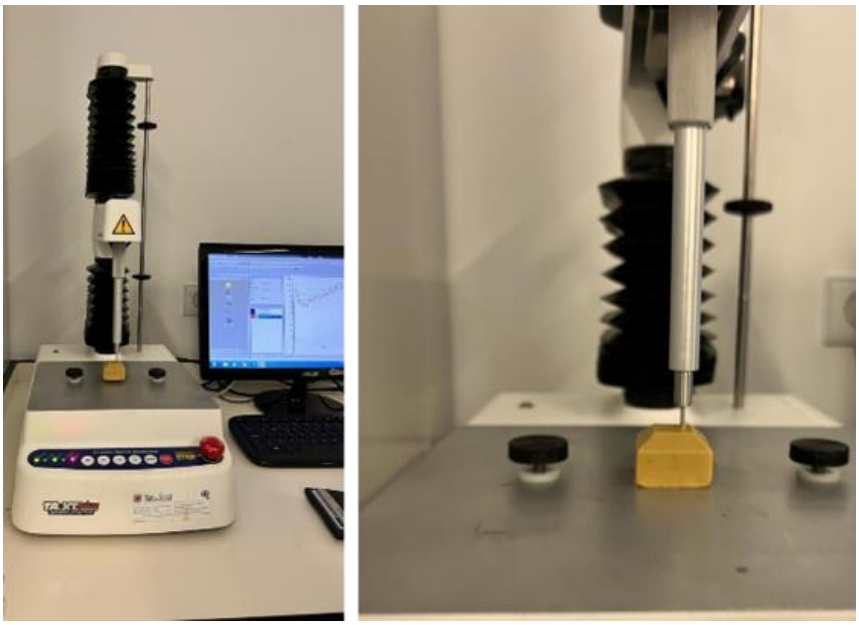


Figure 15: Soap texture evaluation.

6.5.4. Evaluation of the viscosity of the serum over storage time

The viscosity of the formulation is determined by the B-ONE PLUS Viscometer at 10 rpm using spindle type 2. For the measurement, 40ml of the serum was placed in a falcon with the spindle dipped in it for about 5 minutes before the measurement. The fluid was subjected to shear rate (rotational speed) and shear stress (motor torque) measurements. The values of shear rate and shear stress then make it possible to calculate the viscosity using the Newton equation and the constants associated with the mobile used.

Equation of Newton is: $\mu = \frac{\tau}{\dot{\gamma}}$

With μ for viscosity in Pa/s, τ for shear stress in Pa and γ¨for shear rate in S^{-1}.

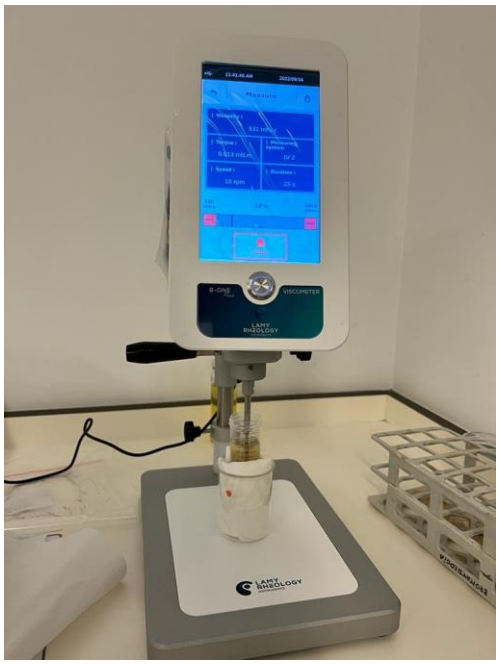


Figure 16: Serum viscosity evaluation.

6.6. Bioactive evaluation of the developed formulations

The soaps and the serums were evaluated for their antioxidant and antimicrobial properties to analyze the stability of the extracts/propolis. The antibacterial activity of the various serum formulations, the ingredients used to make the formulations, and the soaps were evaluated against four bacteria commonly associated with skin disorders: *Escherichia coli, Pseudomonas aeruginosa*, *MRSA*, and *Propionibacterium acnes*. With minor adjustments, the assessment procedure described for this activity is in 6.5.1. Instead of Tryptic Soy Broth (TSB), the medium employed is Muller-Hinton Broth (MHB). The microplates were also incubated for 24 hours at 37°C, except for *P. acnes*, which was incubated for 72 hours.

6.7. Statistical analysis

Throughout the whole document, all data was expressed as mean ± standard deviation. An analysis of variance (ANOVA) was used to analyse the samples, relying on a Tukey's test or a Tahmane T2 for post-hoc classification, depending on the homoscedasticity of the samples. For the soap samples, considering the two factors included, storage time (ST) and incorporation (I), a two-way ANOVA with type III sums of squares using the SPSS Software, version 25 was used. This multivariate general linear model treats the two factors, ST and I as independent, thus allowing the effect of each one to be analyzed independently, providing more insight on

their contribution towards the changes. If a significant interaction (<0.05) was recorded among the two factors (ST×I), these were evaluated simultaneously, and some general conclusions and tendencies were extracted from the estimated marginal means (EMM). If there was no significant interaction (>0.05), each factor was evaluated independently using a Tukey's or Tamhane's T2 for non-homoscedastic samples. Homoscedasticity was evaluated using a Levene's test. All analyses were carried out using a significance level of 0.05.

7. Results and discussion

The main objective of this research was to develop cosmetic formulations using bioactive plant extracts (rosemary and eucalyptus) and propolis, which are natural antioxidants and antimicrobials. Two extraction procedures were used, one by maceration on a magnetic stir plate and the other by using a heated water bath, both using a hydroethanolic solution (80:20 *v/v*, ethanol: water). The collected extracts were used in varying amounts to evaluate their bioactivity (antibacterial, anti-tyrosinase, anti-inflammatory, toxicity, in addition to being characterized in terms of phenolic compounds and organic acids.

7.1. Chemical characterization

7.1.1. Organic acids

Table 5 displays the organic acids present in the extracts, expressed in g/100g dw. Four molecules were identified, namely oxalic, malic, succinic and fumaric acids. Among these, in both extracts, succinic acid was the most abundant. Overall, rosemary showed statistically different amounts of oxalic, malic, and succinic acid, showing higher amounts for succinic acid, while eucalyptus showed higher abundances for succinic and oxalic acids. Fumaric acid, which was the least abundant in both samples, did not show any statistical differences.

There were no organic acids found in propolis samples, which contradicts previous research (Hroboňová et al., 2008). The lack of findings might be explained by the fact that the concentration of propolis in the tested sample was below the detection limit of the technique used.

Table 5: Organic acids present in the extracts, expressed in g/100g dw

	Oxalic Acid	Malic Acid	Succinic Acid	Fumaric Acid
Rosemary	1.92±0.09*	19.7±0.3*	19.9±0.5*	0.4±0.5
Eucalyptus	5.4±0.5	2.7±0.2	68.9±0.9	0.4±0.5

An asterisk (*) in each column means a statistically significant difference with a p-value of 0.05.

7.1.2. Phenolic compounds

Table 5 lists the phenolic compounds (mg/g) that were measured in propolis using LC-DAD-ESI-MS^n at 280 nm. By comparing phenolic compounds chromatographic behavior, UV spectra, and MS data with those of reference compounds, this approach enabled the clarification of phenolic compounds. When standards were not available, the structural information was verified using UV data along with previously published MS fragmentation patterns. In our work, The HPLC analysis permitted the identification of 40 compounds, as shown in figure 16 and table 5, indicating that the propolis sample included a wide range of phenolic chemicals. 16 phenolic acids and 24 flavonoids were among them (9 dihydroflavonols; 8 flavonols; 4 flavones; 3 flavanones). These phenolic compounds were previously reported (Falcao et al., 2013; Falcao et al., 2010). The major flavonoids were pinocembrin (35.91 ± 0.07 mg/g), chrysin (25.82 ±0.01 mg/g), galangin (22.73 ±0.05 mg/g), and pinobanksin-3-*O*-acetate (22.67 ±0.02 mg/g), which were all detected in greater amounts than phenolic acids. However, low amounts of ferulic acid, *p*-coumaric acid isoprenyl ester, caffeic acid derivative, *p*-coumaric isoprenyl ester (isomer), and quercetin-tetramethyl-ether (less than 1 mg/g) were found.

Table 6: Characterization of the phenolic compounds from Azorean propolis phenolic extract obtained by LC/DAD/ESI-MS^n.

Nr	t_R (min)	λ_{max} (nm)	$[M-H]^-$ m/z	MS^2 (% base peak)	Proposed compound	mg/g
1	6.8	293, 324	179	135	Caffeic acid[a,b]	2,59 ± 0,04
2	9.7	310	163	119	*p*-Coumaric acid[a,b]	1,95 ± 0,02
3	10.6	298, 323	193	133 (100), 149 (49), 177 (15)	Ferulic acid[a,b]	0,21 ± 0,02
4	11.2	298, 323	193	133 (100), 149 (49), 177 (15)	Isoferulic acid[a,b]	2,17 ± 0,02
5	16.0	298sh, 322	207	192 (100), 163 (62)	3,4-Dimethyl-caffeic acid[a,b]	4,44 ± 0,04
6	19.3	288	285	267 (100), 239 (25), 252 (16)	Pinobanksin-5-methyl ether[b,c]	5,50 ± 0,01
7	19.7	256, 370	301	179 (100), 151 (60)	Quercetin[a,b]	4,52 ± 0,04
8	21.1	309	177	163 (100), 119 (16)	*p*-Coumaric acid methyl ester[a,b]	2,73 ± 0,01
9	21.5	256, 355	315	300	Quercetin-3-methyl ether[b,c]	1,87 ± 0,01
10	23.9	292	271	253 (100), 225 (22), 151 (8)	Pinobanksin[b,c]	8,56 ± 0,04
11	26.1	290	269	255 (48), 227 (100), 165 (30)	Pinocembrin-5-methyl-ether[b,c]	1,28 ± 0,02
12	27.3	267, 335	269	225 (100), 151 (20)	Apigenin[a,b]	1,55 ± 0,01
13	27.9	265, 364	285	285 (100), 257 (13), 151 (20)	Kaempferol[a,b]	2,28 ± 0,06
14	29.3	253, 370	315	300	Isorhamnetin[a,b]	0,94 ± 0,01
15	30.0	267, 352	299	284	Kaempferol-methyl ether[b,c]	3,92 ± 0,02
16	32.0	256, 349	359	344	Quercetin-tetramethyl-ether[b,c]	0,71 ± 0,01
17	32.7	311	173	129	Cinnamylidenacetic acid[b,c]	2,07 ± 0,07
18	36.0	256, 367	315	165	Rhamnetin[b,c]	1,13 ± 0,01
19	39.3	298, 326	247	179 (100), 135 (16)	Caffeic acid isoprenyl ester[a,b]	4,61 ± 0,06
20	40.9	298, 326	247	179 (100), 135 (16)	Caffeic acid isoprenyl ester (isomer)[a,b]	6,63 ± 0,04
21	41.5	298, 326	269	178 (100), 135 (96)	Caffeic acid benzyl ester[b,c]	3,81 ± 0,09
22	43.3	290	255	213 (100), 211 (55), 151 (36)	Pinocembrin[a,b]	35,91 ± 0,07
23	45.8	268, 311	253	209	Chrysin[a,b]	25,82 ± 0,01
24	46.3	295	313	253 (100), 271 (20)	Pinobanksin-3-*O*-acetate[b,c]	22,67 ± 0,02
25	46.5	298, 325	283	179 (100), 135 (28)	Caffeic acid phenylethyl ester[a,b]	2,73 ± 0,03
26	47.1	266, 300sh, 359	269	269 (100), 241 (61)	Galangin[a,b]	22,73 ± 0,05
27	49.1	268, 331	283	269	Acacetin[a,b]	1,24 ± 0,01
28	49.6	265, 300sh, 350sh	283	269	6-Methoxychrysin[b,c]	2,52 ± 0,05
29	51.0	294, 311	231	163 (100), 119(12)	*p*-Coumaric acid isoprenyl ester[b,c]	0,37 ± 0,04
30	51.9	311	231	163 (100), 119 (12)	*p*-Coumaric isoprenyl ester[b,c] (isomer)	0,74 ± 0,03
31	52.5	295, 324	295	178 (100), 135 (60)	Caffeic acid cinnamyl ester[b,c]	2,20 ± 0,02
32	53.5	295, 323	295	178 (100), 134 (24)	Caffeic acid cinnamyl ester[b,c] (isomer)	3,51 ± 0,04
33	56.4	289	269	254 (100), 251 (54), 165 (22)	3-Hydroxy-5-methoxyflavanone[b,c]	2,48 ± 0,03
34	58.3	292	417	297 (100), 402 (85), 267 (67)	Pinobanksin-methyl-ether-3-*O*-phenylpropionate[b,d]	1,18 ± 0,05

35	59.3	293	475	415	Pinobansin-3-*O*-acetate-5-*O*-hydroxyphenylpropionate[b,d]	2,12 ± 0,04
36	60.1	290	475	415	Pinobansin-3-*O*-acetate-7-*O*-hydroxyphenylpropionate (isomer)[b,d]	23,29 ± 0,01
37	63.4	292	355	253	Pinobanksin-3-*O*-pentanoate or 2-methylbutyrate[b,c]	4,49 ± 0,01
38	65.1	292, 322	315	179 (100), 135 (31)	Caffeic acid derivative[b,c]	0,57 ± 0,03
39	65.4	293	403	253 (100), 271 (21)	Pinobanksin-3-*O*-phenylpropionate[b,c]	1,66 ± 0,01
40	67.0	292	369	253 (100), 271 (14)	Pinobanksin-3-*O*-hexanoate[b,c]	1,09 ± 0,03

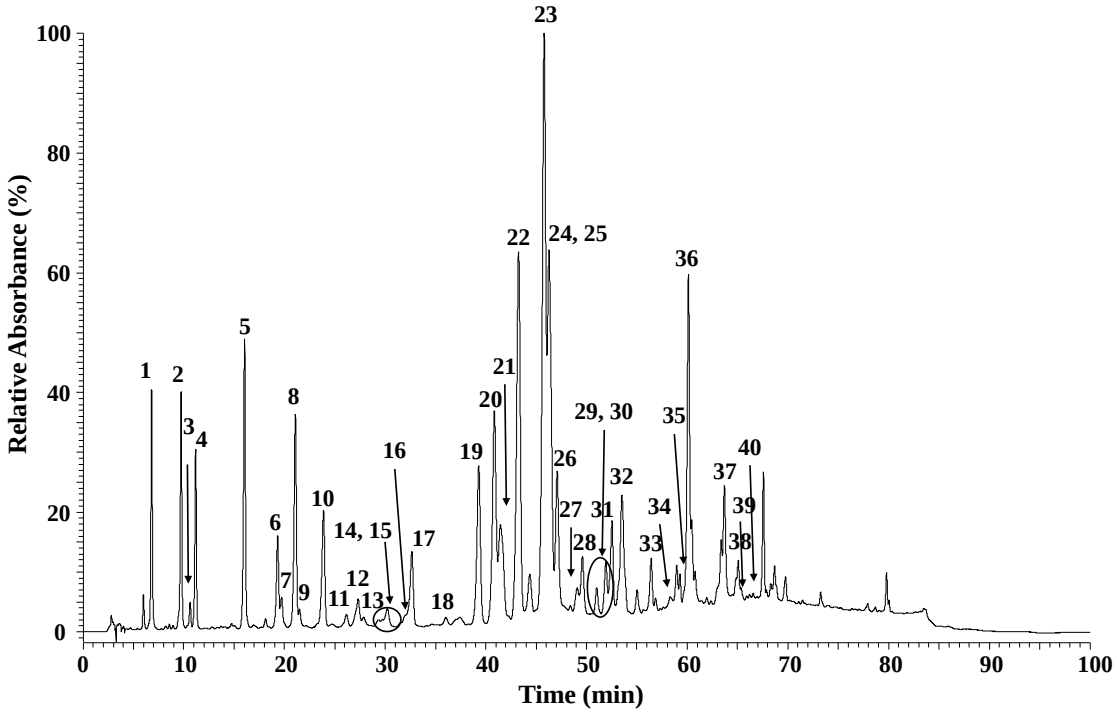


Figure 17: Chromatographic profile of Azorean propolis phenolic extract at 280 nm.

Table 7: Characterization of the phenolic compounds from Rosemary phenolic extract obtained by LC/DAD/ESI-MS^n.

Nr	t_R (min)	$[M-H]^-$ m/z	MS^2	MS^3	Proposed compound	mg/g
1	4,45	783	301, 481,	(301)271, 257, 229, 185	quercetin derivative	0,91 ± 0,019
2	4,74	353	191, 179, 173, 135	(191)172, 173, 127, 109	3-caffeoylquinic acid	0,14 ±0,00013
3	6,7	353	173, 179, 191, 161, 135	(173) 155, 137, 115, 111, 93, 71	4-caffeoylquinic acid	0,19 ±0,01
4	7,21	353	191, 179, 173, 135	(191) 173, 171, 153, 127	5-caffeoylquinic acid	0,32 ± 0,002
5	16,21	597	491, 509, 329, 311, 267 250, 197, 179	(491) 311	Yunnaneic acid isomer	0,49 ± 0,02
6	17,51	477	315	(315) 300	isorhamnetin-galactoside	0,85 ± 0,04
7	17,51	609	301, 271, 197	(301) 273, 271, 257,229, 179, 152, 151	Quercetin-3-rutinoside	0,45 ± 0,11
8	17,83	497	331	169, 125	Globulusin B or Eucaglobulin or Cypellocapin A	0,67 ± 0,04
9	17,83	571	527. 509, 329	(509) 311, 285, 241, 197, 179	Yunnaneic acid isomer	0,91 ± 0,10
10	18	477	301	(301) 273, 257, 239, 229, 179, 151	Quercetin-glucoronide	1,24 ± 0,06
11	18,58	461	285	(285) 241, 242, 201, 199, 175	Luteolin-glucoronide	5,80 ± 2,16
12	18,81	717	519, 475, 365	(519) 475, 365, 339, 321,	salvianolic acid E	0,63 ± 0,01
13	20,29	477	315, 357, 367, 301	(315)300	Isorhamnetin-galactoside	1,76 ± 0,31
14	21,76	751	509, 707	(509) 311, 329, 285, 267, 239, 197, 179	Yunnaneic acid derivative	0,87± 0,02
15	22,62	359	161, 223, 197, 179		Rosmarinic acid	9,37 ± 0,24
16	24,73	461	285	(285) 241, 225, 217, 151	Luteolin-glucoronide	26,87 ± 0,41
17	28,82	503	399, 443	(399) 355, 285, 283	Luteolin -acetyl-*O*-glucuronide isomer 1	8,71 ± 3,59
18	29,36	503	399, 443	(399) 355, 285 283	Luteolin-acetyl-*O*-glucuronide isomer 2	17,82 ± 4,29
19	31	503	399, 443	(399) 355, 285 283	Luteolin-acetyl-*O*-glucuronide isomer 3	9,15 ± 0,88

Using the same procedure, the phenolic profile of the ethanolic extract of rosemary was assessed. This process allowed for a thorough analysis of the phenolic component of the rosemary extract and the potential identification of up to 19 phenolic compounds (Table 7).

In this extract, flavonoids were the phenolic component class with the highest representation. Additionally, certain phenolic acids were identified. The most prevalent substances were luteolin-glucoronide (26,87± 0,41 mg/g), luteolin-acetyl-*O*-glucuronide isomer 1 (8,71 ±3,59 mg/g), luteolin-acetyl-*O*-glucuronide isomer 2 (17,82 ±4,29 mg/g), luteolin-acetyl-*O*-glucuronide isomer 3 (9,15± 0,88 mg/ g) and rosmarinic acid (9,37 ± 0,24 mg/g).

The least abundant molecules, ranging (0.14-0.32 mg/g), were caffeineylquinic acids. The amount of phenolic compounds found in various rosemary extracts was previously reported to be relatively varied, with some extracts displaying a content similar to that is shown here. It should be noted that the method and solvent applied for extraction have been found to affect the quantity and relative contribution of each type of polyphenolic compounds to rosemary extracts. The final phenolic content of rosemary extracts may also be influenced by irrigation conditions, harvest timing, storage conditions, and drying procedures (Mena et al., 2016).

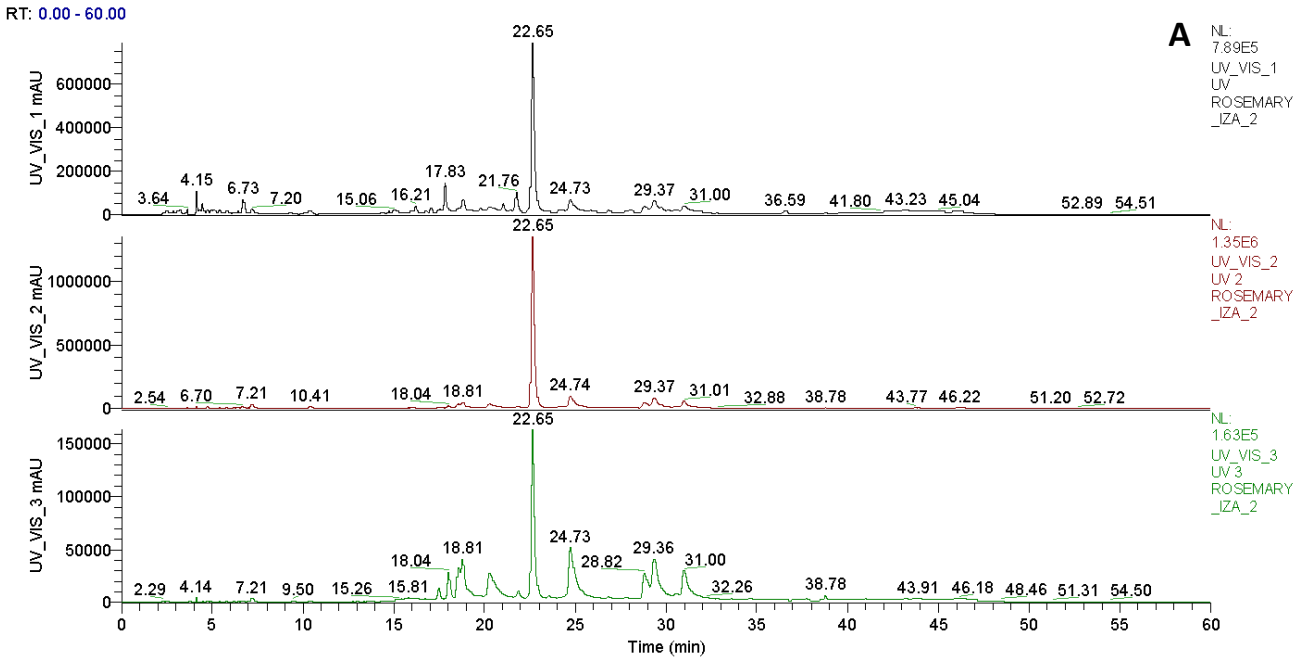


Figure 18: Chromatographic profile of Rosemary phenolic extract at **A**: 280 nm, **B**: 330 nm, **C**: 370 nm

B

The eucalyptus phenolic extract was also examined in the current study. The HPLC analysis enabled the identification of 15 phenolic compounds, as shown in table 8. The two main components were quercetin-*O*-glucoronide (4,40 ± 0,26 mg/g) and luteolin-*O*-glucoronide (8.17 ± 1.32 mg/g). However, Cypellogin A or B was shown to be the less prevalent substance in our extract. According to some research, eucalyptus extract contains polyphenols, oenothein B, gallic acid, ellagic acid, flavonoids, and hydrolyzable tannin dimer. Additionally, quercetin and kaempferol-3-*O*-β-D-glucuronides are present in eucalyptus extract (Parham et al., 2020; Park et al., 2004; Santos et al., 2012). Some of these compounds as gallic acid, quercetin-*O*-glucoronide, kaempferol-*O*-rutinoside were found in the present study.

Table 8: Characterization of the phenolic compounds from Eucalyptus phenolic extract obtained by LC/DAD/ESI-MS^n

Nr	t_R (min)	$[M-H]^-$ m/z	MS2	MS3	Proposed compound	mg/g
1	7,27	353	191, 179, 173, 135	(191) 173, 171, 153, 127	5-caffeoylquinic acid	1,23 ± 0,04
2	17,46	497	331, 313 169	169	Cypellocarpin A	0,84 ± 0,31
3	17,74	497	331, 313, 169	169	Globulusin B or Eucaglobulin or Cypellocapin A	0,75 ± 0,01
4	17,98	477	301	(301) 273, 257, 239, 229, 179, 151	Quercetin-*O*-glucoronide	4,40 ± 0,26
5	17,98	497	331, 437, 438, 211, 313 169	169	Eucaglobulin	0,53 ± 0,05
6	18,24	483	169, 439, 313	(169) 125, 111	Globulusin B or Eucaglobulin or Cypellocapin A	0,48 ± 0,07
7	18,82	497	313, 479, 437, 169	169, 215	Globulusin B: monoterpene	1,52 ± 0,39
8	20,73	593	285	267, 257, 241, 223, 199, 151	Kaempferol-*O*-rutinoside	0,94 ± 0,02
9	21,86	461	285	(285) 256, 241, 242, 201, 198	Luteolin-*O*-glucoronide	8,17 ± 1,32
10	22,42	447	301, 315, 343	(301) 271, 257,228,179, 151	isorhamnetin-*O*-pentoside	1,77 ± 0,39
11	23,52	649	497	313, 437, 331, 169	gallic acid monoterpene glycoside derivative	0,48 ± 0,07
12	23,52	447	315, 301, 286	(315) 300, 271	isorhamnetin-*O*-pentoside	0,82 ± 0,06
13	24,6	461	315, 299, 285	(315) 300	isorhamnetin-*O*-rhaminoside	0,69 ± 0,00
14	30,34	629	463, 445, 301	(463) 301, 283, 169	Cypellogin A or B	0,09 ± 0,04
15	30,99	629	463, 313, 301	(463) 301, 273	Cypellogin A or B	0,13 ± 0,10

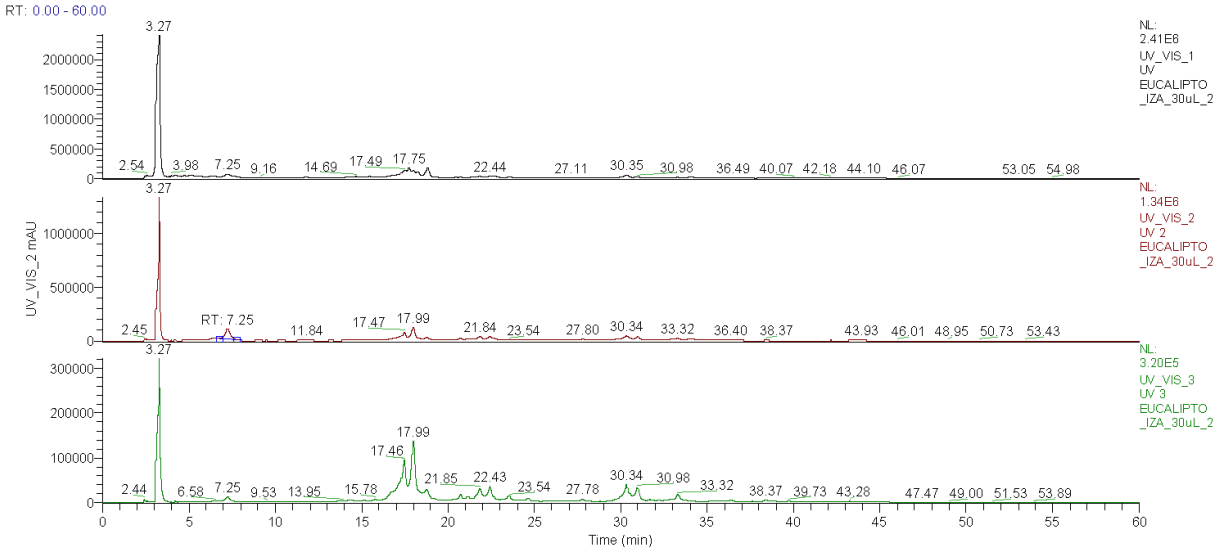


Figure 19: Chromatographic profile of Eucalyptus phenolic extract.

7.2. Bioactivities

7.2.1. Antioxidant activity

Tables 9 and 10 show the bioactivities for the sample combinations. They are divided by extraction type, being the top section for maceration extraction and lower section for heated water bath extraction. Namely antioxidant activity (TBARS) and cellular antioxidant assay (CAA) (table 9), cytotoxicity (PLP2) and anti-inflammatory activity (RAW 267.4) (table 10). The heated water bath extracts had the highest antioxidant activity, displaying the lowest EC_{50} values (a value that inhibits 50% of cell oxidation). The observed outcomes are consistent with other publications highlighting the high antioxidant potential of eucalyptus, rosemary, and propolis, which may be attributed to the amount of phenolic compounds they contain, particularly phenolic acid and flavonoids (Agatonovic-Kustrin et al., 2021; Gullon et al., 2020; Oses et al., 2020).

The results of various combinations in the antioxidant activity were equivalent. Thus, eucalyptus and propolis 50/50 (*m/m*) had the highest antioxidant properties, followed by eucalyptus and rosemary 40/60 (*m/m*) and eucalyptus and propolis 60/40 (*m/m*). Although eucalyptus seems to be the main antioxidant extract in the TBARS testing, its effectiveness is significantly reduced when it is combined with rosemary and propolis. The extract with the lowest antioxidant activity was propolis on its own.

Regarding the cellular antioxidant assay (CAA), except for the propolis extract from the maceration, all of the studied extracts demonstrated their potential to inhibit cellular oxidation in this assay. The antioxidant activity in the propolis extracts from the maceration revealed no activity at the maximum tested concentration of 2000 µg/ml. In general, all the extracts obtained by the two extraction techniques had a comparable capacity to decrease cellular oxidation, presenting very similar values. These findings agree with the TBARS findings.

Table 9: Antioxidant, expressed in µg/mL for the extracts obtained through maceration.

	TBARS	**CAA**
Maceration		
Eucalyptus	14.67 ± 0.08^{e}	86 ± 7
Rosemary	$7.61\pm0.05^{c, d}$	87 ± 1
Propolis	66 ± 1^{j}	>2000
Eucalyptus+ Rosemary 50/50	52 ± 1^{i}	84 ± 1
Eucalyptus+ Rosemary 60/40	16.8 ± 0.3^{f}	86 ± 3
Eucalyptus+ Rosemary 40/60	6.1 ± 0.1^{b}	85 ± 4
Eucalyptus+ Propolis 50/50	3.79 ± 0.01^{a}	87 ± 6
Eucalyptus+ Propolis 60/40	$6.59\pm0.01^{b, c}$	79 ± 7
Eucalyptus+ Propolis 40/60	8.81 ± 0.07^{d}	85 ± 2
Rosemary+ Propolis 50/50	$15.6\pm0.1^{e, f}$	82 ± 2
Rosemary+ Propolis 60/40	29.5 ± 0.2^{h}	85 ± 3
Rosemary+ Propolis 40/60	27.0 ± 0.3^{g}	76 ± 4
Heated water bath		
Eucalyptus	1.91 ± 0.02^{a}	84 ± 1
Rosemary	2.35 ± 0.05^{a}	83 ± 2
Propolis	110.7 ± 0.8^{f}	74 ± 7
Eucalyptus+ Rosemary 50/50	3.45 ± 0.03^{c}	92 ± 6
Eucalyptus+ Rosemary 60/40	6.372 ± 0.007^{c}	87 ± 4
Eucalyptus+ Rosemary 40/60	3.33 ± 0.014^{b}	86 ± 7
Eucalyptus+ Propolis 50/50	10.62 ± 0.02^{e}	81 ± 1
Eucalyptus+ Propolis 60/40	$7.04\pm.0.2^{c}$	86 ± 9
Eucalyptus+ Propolis 40/60	6.4 ± 0.3^{c}	80 ± 2
Rosemary+ Propolis 50/50	6.4 ± 0.3^{c}	84 ± 4
Rosemary+ Propolis 60/40	10.1 ± 0.1^{e}	80 ± 11
Rosemary+ Propolis 40/60	8.8 ± 0.1^{d}	80 ± 8

Different letters in each column mean statistically significant differences with a p-value of 0.05.

7.2.2. Cytotoxic activity in PLP2 cells, and anti-inflammatory activity

Furthermore, several research publications in the last ten years have reported that eucalyptus, rosemary, and propolis extracts are effective anticancer agents against various types of tumor cells (Dezmirean et al., 2020; Gullon et al., 2020). All extract combinations showed effects for the PLP2 cell line with a GI50 <400 g/mL except for the maceration extracts

(eucalyptus, rosemary, and propolis) tested separately, as well as eucalyptus/rosemary 50/50 combinations. They exhibited no toxicity to this cell line at the highest tested concentration of 400 ug/ml. The two combinations with the highest activity were eucalyptus/rosemary and eucalyptus/propolis 60/40 (maceration), with GI50 values of 72.3 ±4.8 and 80±32 g/ml, respectively. While the Rosemary/Propolis 40/60 (maceration) combination has the lowest cytotoxicity, it has a GI50 of 337.5 ± 25.3 g/ml. As for the extraction method with heated water bath, only two combinations of extracts showed no toxicity, namely the Eucalyptus/Propolis (40/60 *m/m*) and Rosemary/Propolis (60/40 *m/m*). Although some of the combinations revealed toxicity for normal cells, those are not the ideal model to evaluate the toxicity of a cosmetic formulation. Therefore, the toxicity presented by these samples will be much lower, taking into account that from the skin, these levels will not reach the liver cells.

According to literature, flavonoids included in plant extracts (eucalyptus and rosemary) and propolis extracts allow them to have anti-inflammatory properties (Anjum et al., 2019). However, neither maceration nor heated water bath extracts demonstrated anti-inflammatory activity for murine macrophage cells (RAW 267.4) in this investigation, even at the highest tested dose of 400 ug/ml.

Table 10: Cytotoxic activity in PLP2 cells, and anti-inflammatory activity, expressed in IC50 µg/ml for the extracts obtained through maceration.

	Cytotoxicity PLP2	**Anti-inflammatory**
Maceration (µg/mL)		
Eucalyptus	>400^{f}	>400
Rosemary	>400^{f}	>400
Propolis	>400^{f}	>400
Eucalyptus+ Rosemary 50/50	>400^{f}	>400
Eucalyptus+ Rosemary 60/40	72.3 ± 4.8^{a}	>400
Eucalyptus+ Rosemary 40/60	166.21 ± 1.74^{b}	>400
Eucalyptus+ Propolis 50/50	218.3 ± 5.84^{c,d}	>400
Eucalyptus+ Propolis 60/40	80.2 ± 2.32^{a}	>400
Eucalyptus+ Propolis 40/60	190.5 ± 3.50^{b,c}	>400
Rosemary+ Propolis 50/50	226.8 ± 12.0^{d}	>400
Rosemary+ Propolis 60/40	221.7 ± 14.7^{d}	>400
Rosemary+ Propolis 40/60	337.5 ± 25.3^{e}	>400

	Heated water bath	
Eucalyptus	203.35 ± 15.90^{d,e,f}	>400
Rosemary	145.27 ± 3.31^{b}	>400
Propolis	204.34 ± 12.11^{d,e,f}	>400
Eucalyptus+ Rosemary 50/50	213.70 ± 16.51^{e,f,g}	>400
Eucalyptus+ Rosemary 60/40	236.70 ± 6.40^{g}	>400
Eucalyptus+ Rosemary 40/60	194.35 ± 7.57^{c,d,e}	>400
Eucalyptus+ Propolis 50/50	233.01 ± 14.14^{f,g}	>400
Eucalyptus+ Propolis 60/40	169.67 ± 9.44^{b,c}	>400
Eucalyptus+ Propolis 40/60	>400^{h}	>400
Rosemary+ Propolis 50/50	175.57 ± 13.01^{c,d}	>400
Rosemary+ Propolis 60/40	>400^{h}	>400
Rosemary+ Propolis 40/60	109 ± 3^{a}	>400

*Primary culture of pig liver cells (PLP2). Positive control: 2.29 mg/ml

7.2.3. Antimicrobial activity:

Eight bacteria were tested against the plant extracts of eucalyptus, rosemary, and propolis from both extraction methods (Tables 11-12). The extraction procedure had an impact on the inhibitory concentrations (Aleksic Sabo & Knezevic, 2019). In comparison to the maceration extracts, the three samples from heated water bath extracts showed greater antibacterial activity. Eucalyptus, rosemary, and propolis extracts had minimal inhibitory concentrations that were within the range of 0.07–5 mg/mL, 1.25–10 mg/mL, and 5–10 mg/mL for maceration extraction, respectively. The heated water bath extracts, on the other hand, showed relatively strong activity against the majority of the bacteria throughout a range of 0.3 mg/mL to 5 mg/mL for rosemary extracts, and the same range for eucalyptus was in the 2.5-10 mg/ml range. Gram-positive bacteria were more susceptible than gram-negative bacteria, which was supported by this current study and previous literature studies (Aleksic Sabo & Knezevic, 2019; Almuhayawi, 2020; Dezmirean et al., 2020; Przybylek & Karpinski, 2019).

Table 11: Antibacterial activity of the Maceration extracts.

Samples	Proportion		Gram-negative bacteria (mg/mL)					Gram--positive bacteria (mg/mL)		
			Escherichia coli	***Klebsiella pneumoniae***	***Morganella morganii***	***Proteus mirabilis***	***Pseudomonas aeruginosa***	***Enterococcus faecalis***	***Listeria monocytogenes***	**MRSA**
Eucalyptus	**100%**	**MIC/MBC**	5/>10	5/>10	2.5/>10	2.5/>10	2.5/>10	0.07/>10	2.5/>10	0.07/>10
Rosemary	**100%**	**MIC/MBC**	5/>10	2.5/>10	2.5/>10	5/>10	10/>10	2.5/>10	1.25/>10	2.5/>10
Propolis	**100%**	**MIC/MBC**	10/>10	>10/>10	10/>10	>10/>10	>10/>10	10/>10	10/>10	5/>10
Eucalyptus/Rosemary	**50/50**	**MIC/MBC**	5/>10	>10/>10	2.5/>10	2.5/>10	2.5/>10	0.3/>10	2.5/>10	0.15/>10
Eucalyptus/Rosemary	**60/40**	**MIC/MBC**	5/>10	>10/>10	2.5/>10	2.5/>10	2.5/>10	0.15/>10	1.25/>10	0.6/>10
Eucalyptus/Rosemary	**40/60**	**MIC/MBC**	5/>10	5/>10	2.5/>10	2.5/>10	5/>10	0.07/>10	2.5/>10	0.07/>10
Eucalyptus/Propolis	**50/50**	**MIC/MBC**	2.5/>10	10/>10	2.5/>10	2.5/>10	10/>10	0.07/>10	2.5/>10	0.15/>10
Eucalyptus/Propolis	**60/40**	**MIC/MBC**	5/>10	10/>10	2.5/>10	5/>10	5/>10	0.3/>10	0.6/>10	0.3/>10
Eucalyptus/Propolis	**40/60**	**MIC/MBC**	2.5/>10	10/>10	2.5/>10	2.5/>10	10/>10	0.07/>10	1.25/>10	0.3/>10
Rosemary/Propolis	**50/50**	**MIC/MBC**	10/>10	10/>10	2.5/>10	5/>10	10/>10	1.25/>10	1.25/>10	0.6/>10
Rosemary/Propolis	**60/40**	**MIC/MBC**	5/>10	>10/>10	2.5/>10	5/>10	>10/>10	10/>10	2.5/>10	0.6/>10
Rosemary/Propolis	**40/60**	**MIC/MBC**	10/>10	5/>10	5/>10	5/>10	10/>10	10/>10	5/>10	2.5/>10
Positive Control										
Ampicillin (20 mg/mL		**MIC/MBC**	<0.15/<0.15	10/>10	>10/>10	<0.15/<0.15	>10/>10	<0.15/<0.15	<0.15/<0.15	<0.15/<0.15
Imipenem (1 mg/mL)		**MIC/MBC**	<0.0078/<0.0078	<0.0078/<0.0078	<0.0078/<0.0078	<0.0078/<0.0078	0.5/>1	n.t./n.t.	<0.0078/<0.0078	n.t./n.t.
Vancomycin (1 mg/mL)		**MIC/MBC**	n.t./n.t.	n.t./n.t.	n.t./n.t.	n.t./n.t.	n.t./n.t.	<0.0078/<0.0078	n.t./n.t.	0.25/0.5

***MIC- minimal inhibitory concentration; MBC – minimal bactericidal concentration.**

Table 12: Antibacterial activity of the heated water bath extracts.

Samples	Proportion		Gram-negative bacteria (mg/mL)					Gram--positive bacteria (mg/mL)		
			Escherichia coli	***Klebsiella pneumoniae***	***Morganella morganii***	***Proteus mirabilis***	***Pseudomonas aeruginosa***	***Enterococcus faecalis***	***Listeria monocytogenes***	**MRSA**
Eucalyptus	**100%**	**MIC/MBC**	5/>10	5/>10	2.5/>10	2.5/>10	2.5/>10	0.07/>10	1.25/>10	0.07/>10
Rosemary	**100%**	**MIC/MBC**	2.5/>10	0.3/>10	1.25/>10	5/>10	5/>10	0.3/>10	0.6/>10	0.3/>10
Propolis	**100%**	**MIC/MBC**	10/>10	>10/>10	10/>10	>10/>10	>10/>10	10/>10	5/>10	2.5/>10
Eucalyptus/Rosemary	**50/50**	**MIC/MBC**	5/>10	10/>10	2.5/>10	2.5/>10	2.5/>10	0.15/>10	1.25/>10	0.15/>10
Eucalyptus/Rosemary	**60/40**	**MIC/MBC**	5/>10	10/>10	2.5/>10	2.5/>10	5/>10	0.07/>10	2.5/>10	0.07/>10
Eucalyptus/Rosemary	**40/60**	**MIC/MBC**	5/>10	10/>10	2.5/>10	2.5/>10	5/>10	0.07/>10	2.5/>10	0.15/>10
Eucalyptus/Propolis	**50/50**	**MIC/MBC**	5/>10	10/>10	2.5/>10	2.5/>10	5/>10	0.07/>10	1.25/>10	0.15/>10
Eucalyptus/Propolis	**60/40**	**MIC/MBC**	5/>10	10/>10	5/>10	2.5/>10	5/>10	0.3/>10	1.25/>10	0.6/>10
Eucalyptus/Propolis	**40/60**	**MIC/MBC**	5/>10	10/>10	2.5/>10	5/>10	5/>10	2.5/>10	2.5/>10	0.6/>10
Rosemary/Propolis	**50/50**	**MIC/MBC**	5/>10	10/>10	2.5/>10	2.5/>10	10/>10	1.25/>10	2.5/>10	0.15/>10
Rosemary/Propolis	**60/40**	**MIC/MBC**	5/>10	5/>10	1.25/>10	5/>10	5/>10	2.5/>10	2.5/>10	0.3/>10
Rosemary/Propolis	**40/60**	**MIC/MBC**	5/>10	5/>10	1.25/>10	5/>10	10/>10	2.5/>10	2.5/>10	0.15/>10
Positive Control										
Ampicillin (20 mg/mL		**MIC/MBC**	<0.15/<0.15	10/>10	>10/>10	<0.15/<0.15	>10/>10	<0.15/<0.15	<0.15/<0.15	<0.15/<0.15
Imipenem (1 mg/mL)		**MIC/MBC**	<0.0078/<0.0078	<0.0078/<0.0078	<0.0078/<0.0078	<0.0078/<0.0078	0.5/>1	n.t./n.t.	<0.0078/<0.0078	n.t./n.t.
Vancomycin (1 mg/mL)		**MIC/MBC**	n.t./n.t.	n.t./n.t.	n.t./n.t.	n.t./n.t.	n.t./n.t.	<0.0078/<0.0078	n.t./n.t.	0.25/0.5

***MIC- minimal inhibitory concentration; MBC – minimal bactericidal concentration.**

The extracts showed effective antibacterial activity against *E. faecalis* and MRSA by exhibiting the lowest MIC values (0.07 mg/mL) for eucalyptus and 0.3 mg/mL for rosemary. Propolis extract showed the lowest MIC against MRSA at 2.5 mg/ml. These results weren't surprising since several studies reported the role of our samples as antimicrobial agents. According to literature studies, propolis has a significant antimicrobial effect on gram-positive bacteria over gram-negative.

Different combinations of the three matrices in different proportions were evaluated for their antimicrobial properties. It was confirmed that extracts obtained from heated water bath have higher antimicrobial potential than the other extracts. Considering Table 12, the two combinations of *eucalyptus/rosemary* (60:40) and *eucalyptus/propolis* (50:50) extracts showed the most promising results, with inhibition capacity of all the bacteria. The MIC values were lower than those obtained with ampicillin except for *E. coli, K. pneumoniae, P. mirabilis,* and *L. monocytogenes.*

Table 13: Antifungal activity of the different extracts.

			Maceration		Heated Water bath	
Samples	**Proportion**		*A. brasiliensis*	*A.fumigatus*	*A.brasiliensis*	*A. fumigatus*
Eucalyptus	**100%**	**MIC/MBC**	>10/>10	10/>10	>10/>10	10/>10
Rosemary	**100%**	**MIC/MBC**	>10/>10	10/>10	>10/>10	10/>10
Propolis	**100%**	**MIC/MBC**	>10/>10	10/>10	10/>10	10/>10
Eucalyptus/Rosemary	**50/50**	**MIC/MBC**	>10/>10	10/>10	>10/>10	10/>10
Eucalyptus/Rosemary	**60/40**	**MIC/MBC**	>10/>10	10/>10	>10/>10	10/>10
Eucalyptus/Rosemary	**40/60**	**MIC/MBC**	>10/>10	10/>10	>10/>10	10/>10
Eucalyptus/Propolis	**50/50**	**MIC/MBC**	>10/>10	10/>10	>10/>10	10/>10
Eucalyptus/Propolis	**60/40**	**MIC/MBC**	>10/>10	10/>10	>10/>10	10/>10
Eucalyptus/Propolis	**40/60**	**MIC/MBC**	>10/>10	10/>10	>10/>10	10/>10
Rosemary/Propolis	**50/50**	**MIC/MBC**	10/>10	10/>10	>10/>10	10/>10
Rosemary/Propolis	**60/40**	**MIC/MBC**	10/>10	10/>10	>10/>10	10/>10
Rosemary/Propolis	**40/60**	**MIC/MBC**	>10/>10	10/>10	>10/>10	10/>10
Positive Control						
Ketoconazol		**MIC/MBC**	0.06/1.25	0.5/1		

On the other hand, the obtained extracts and different combinations showed antifungal activity against *A. fumigatus* with MIC values of 10 mg/ml (table 13).

7.2.4. Anti-tyrosinase activity

Propolis and the plant extracts have been shown in some studies to have the ability to reduce melanogenic activity because they contain flavonoids like caffeic acid phenethyl ester, which has been shown to be able to inhibit mushroom tyrosinase by the chelation of active sites, making them suitable candidates for the cosmetic goal of whitening face skin (El-Guendouz et al., 2016; Huang et al., 2015; Özer et al., 2008). In order to determine this, the two extract mixtures of eucalyptus and propolis (50:50) and eucalyptus and rosemary (60:40) were examined for their ability to inhibit tyrosinase. The results are shown in table 14. The sample *Eucalyptus/Propolis* was the most effective at inhibiting mushroom tyrosinase, with rates of 26% at 10 mg/ml and 20% at 10 mg/ml, respectively. Both mixtures presented a similar activity.

Table 14: Anti-tyrosinase activity of the extracts

Samples	**EC_{50}**
Eucalyptus/Propolis (50:50 *m/m*)	26% at 10mg/mL
Eucalyptus/Rosemary (60:40 *m/m*)	20% at 10mg/mL
Positive control (Butylresorcinol)	369 µg/mL

NA: No activity; Samples where the EC_{50} could not be determined up to the maximum tested concentration are presented as % tyrosinase inhibition.

7.3. Incorporation effects

The best combinations were *eucalyptus/rosemary* and *eucalyptus/propolis*, 60:40 and 50:50, respectively. These two blends were combined to create two separate formulas: soap (figure 20) and cosmetic serum (figure 21). The antibacterial, anti-tyrosinase, and antioxidant properties of the new products, as well as their physical aspects and their stability throughout time, were studied.

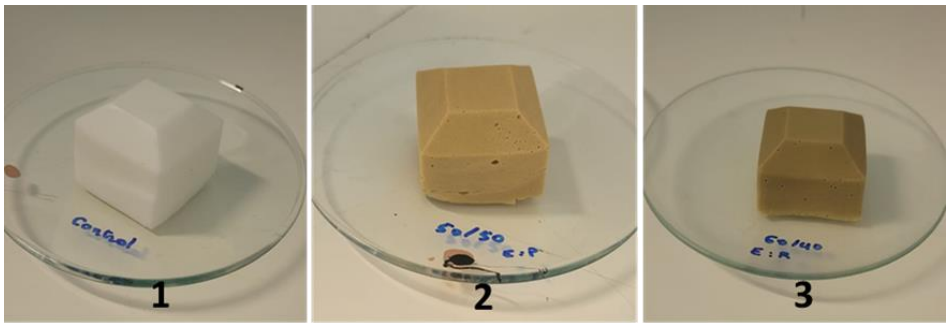


Figure 20: Formulated soaps 1: glycerin (control), 2: (*Eucalyptus/Propolis* 50/50) 3: (*Eucalyptus/ Rosemary* 60/40)

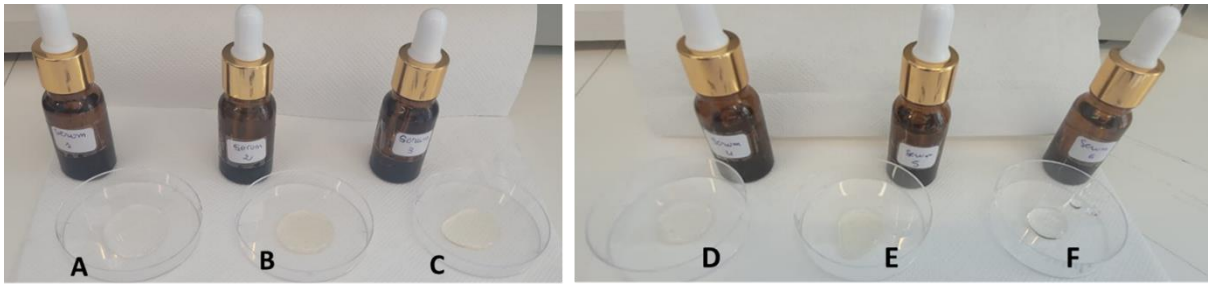


Figure 21: Serum formulations; **A:** (*Eucalyptus/Propolis*) +conservatives serum; **B**: (*Eucalyptus/Rosemary*) +conservatives serum; **C**: ascorbic acid+ conservatives serum; **D**: (*Eucalyptus/Propolis*) serum; E: (*Eucalyptus/Rosemary*) serum; **F**: Ascorbic acid serum.

7.3.1. Soap

Table 15 pertains to the analysis of the soaps and is divided into two sections; the upper represents the storage time (ST) and the lower the incorporation type (I); but, included in each day are all different types of preservatives; and, at the bottom, for each type of preservative extract, the two analysis times are also included. When each of the factors can be analyzed independently (p-value ST × I > 0.05), the classification is made using post-hoc tests; a Student's T test for ST, a Tukey's test for homoscedastic samples, and Tahmane T2 for non-homoscedastic ones in the case of I. However, if the p-value ST ×I < 0.05, no classification can be performed and, in some cases, only general trends can be obtained using estimated marginal means plots (EMM).

Table 15: 2-way analysis of variance for the factors, namely storage time and incorporation type for the soap samples.

		L*	a*	b*	pH	TBARS
Storage Time	0 Days	76±12	0.0±0.7	25±17	8.6±0.2*	133±7
(ST)	60 Days	66±17	3±3	17±11	8.0±0.3	193±31
***p*-value (n=3)**	Student's T-test	<0.001	<0.001	<0.001	<0.001	<0.001
	Glycerin	91±2	-1.0±0.1	2.6±0.7	8.4±0.45[a, b]	184±55
Incorporation (I)	Eucalyptus+ Propolis	61±5	3±3	29±4	8.5±0.2[b]	152±27
	Eucalyptus+ Rosemary	61±7	2±2	32±8	8.1±0.2[a]	154±18
***p*-value (n=6)**	Tukey's test	<0.001	<0.001	<0.001	0.031	<0.001
ST×I (n=18)	*p*-value	<0.001	<0.001	<0.001	0.101	<0.001

For the storage time (ST), an asterisk (*) means statistically significant differences with a p- value of 0.05 while for the incorporation (I), different letters represent statistically significant differences using the same p-value. The SD were calculated from results obtained in different conditions, and thus should not be considered as precision measurements, but rather an interval of values. TBARS expressed as μg/ml.

Considering the exterior color of the soaps, a significant interaction was found for all color coordinates, and therefore only general tendencies for L* and a* were determined. This suggests that both the storage time and the incorporation had a considerable impact on the color change, and the particular contribution of each could not be quantified. The EMM (Figure 22) shows that using glycerin had a significant change in color regardless of ST for L* (lightness), whereas for a* (red- greenness) (Figure 23), 60 days of storage seemed to induce a more intense hue, especially for Eucalyptus+ Propolis, while glycerin incorporated soap showed higher greenness.

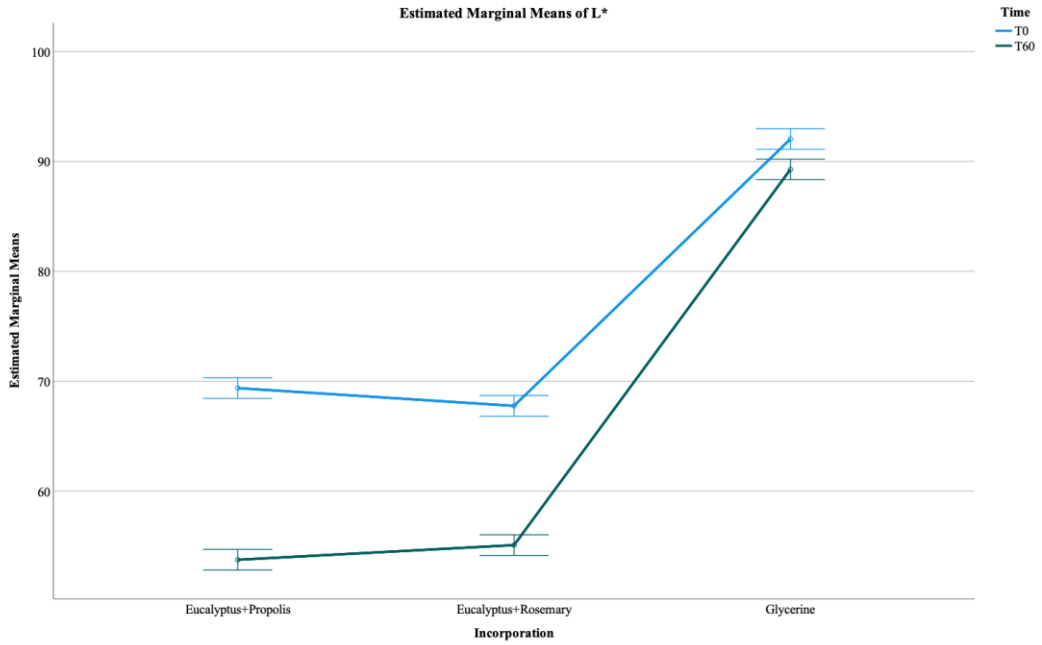


Figure 22: EMM plot of L* for the soap samples.

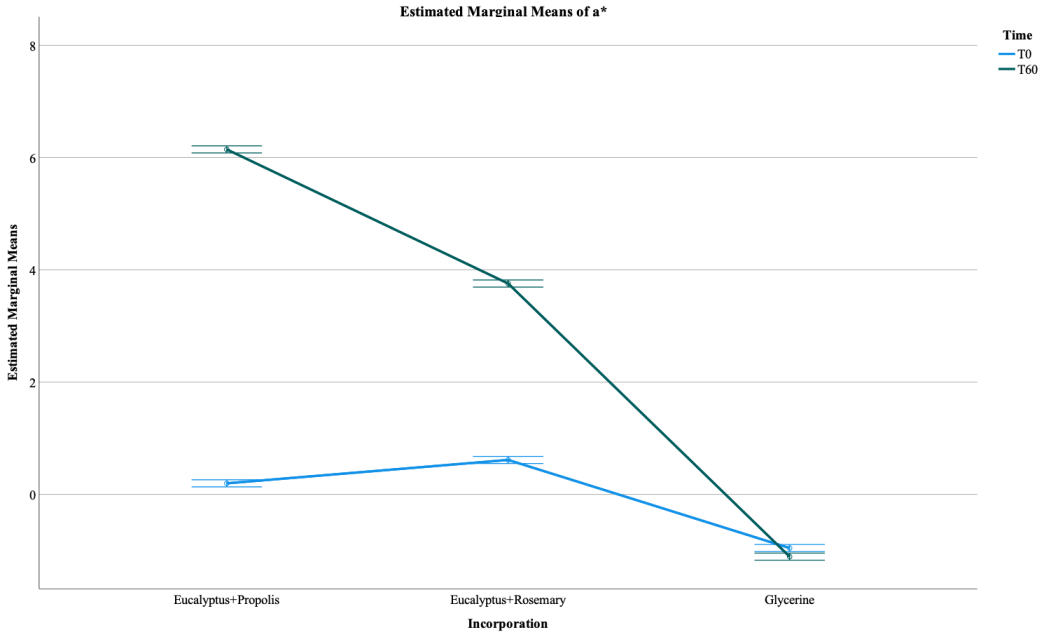


Figure 23: EMM plots of a* for the soaps.

The overall colors from the three coordinates (L*, a*, and b*) are shown in Figure 24, where the color variations are rather noticeable for the samples with glycerin while being extremely minor for the samples with rosemary and propolis.

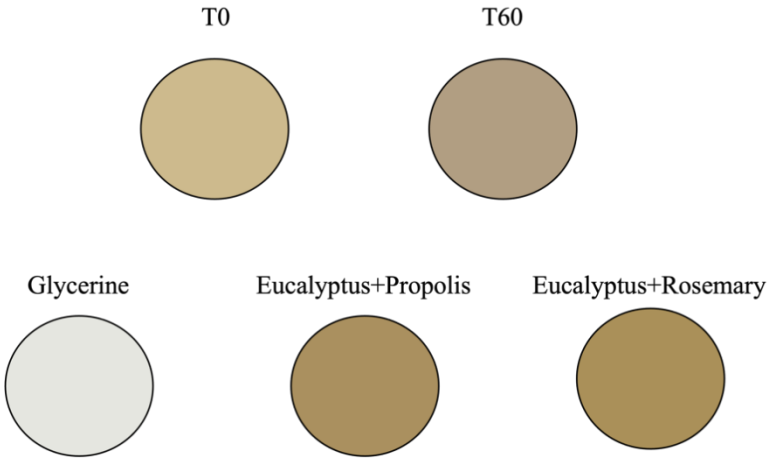


Figure 24: Color of the different samples shown in Table 15

In terms of pH, a notable acidification was observed, with a considerable difference between T0 and T60. Additionally, although the pH amplitude difference was not particularly large, there was a noticeable difference between soaps containing propolis and rosemary extracts. Numerous factors, including temperature, inadequate storage, and active agent instability carried on by oxidation, might influence pH variation. The preparation must have a certain pH value since a low pH produces skin irritation, whilst a high pH creates dry, scaly skin (Septiyanti et al., 2019).

Although certain trends may be deduced from the EMM plots, a substantial interaction was seen for TBARS (Figure 25). It is understandable that the antioxidant activity decreases from T0 to T60, whereas T0 shows relatively little variation in antioxidant activity in all soaps.

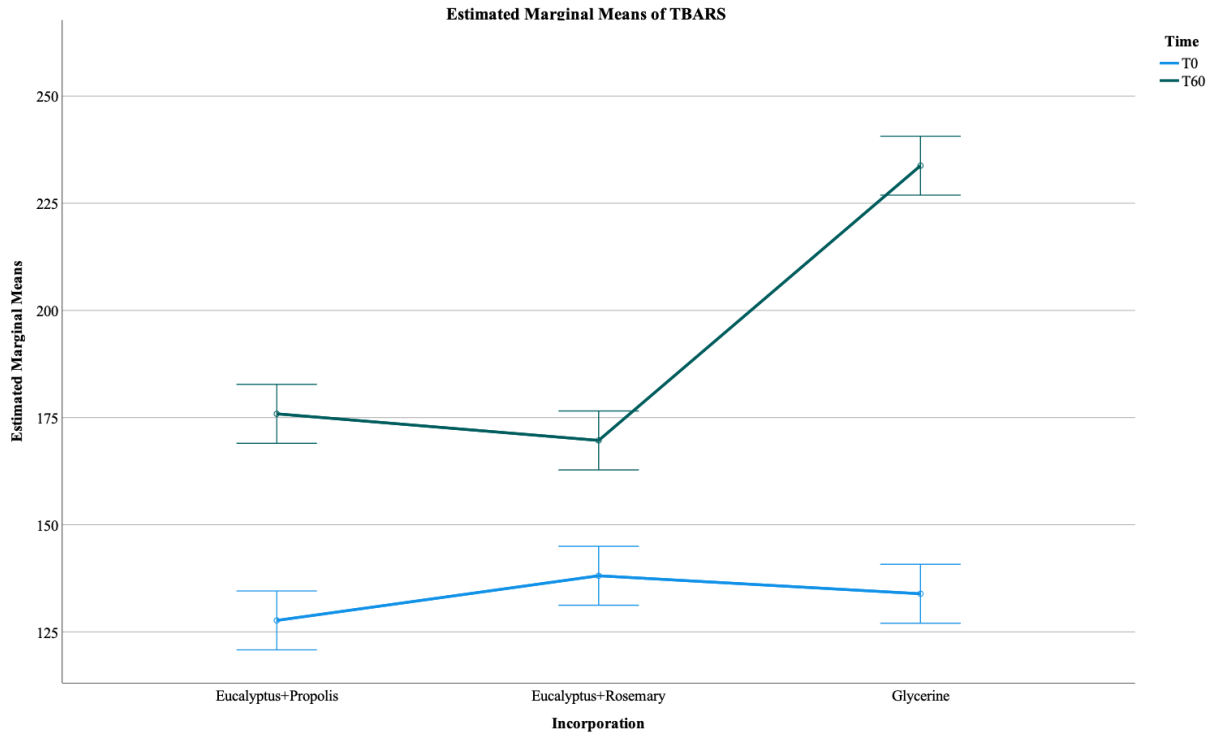


Figure 25: EMM plots of TBARS for the soap samples.

Table 16 indicates the overall hardness of the soaps just for T1, with the Eucalyptus+ Propolis soaps being the softest, while the glycerin and eucalyptus+ rosemary soaps did not exhibit any significant differences. According to the Goik research (Goik et al., 2015), cosmetic products created without propolis had the lowest fragility and the highest hardness. Those made with propolis, on the other hand, have limited elastic characteristics and are more susceptible to cracking. This study confirms the softening effect induced by propolis on the soaps.

Table 16: Hardness of soaps expressed in grams after a penetration analysis.

	Hardness (g)
Glycerin	1045±30[b]
Eucalyptus/Propolis	761±61[a]
Eucalyptus/Rosemary	1018±26[b]

Both new formulated soaps, and the control soap revealed no anti-tyrosinase activity at the maximum tested concentration of 10 mg/ mL, maybe due to some degradation of the bioactive compounds.

Table 17: Anti-tyrosinase activity of the soap formulations.

Soap samples	**EC_{50}**
Eucalyptus/Propolis 50:50	NA
Eucalyptus/Rosemary 60:40	
Base soap: glycerin	
Positive control (Butylresorcinol)	369 µg/mL

NA: No activity; Samples where the EC_{50} could not be determined up to the maximum tested concentration are presented as % tyrosinase inhibition.

Regarding the antimicrobial effectiveness against four clinical bacteria: *E. coli*, *P. aeruginosa*, *MRSA*, and *P. acnes*, this activity was measured twice, at T1 and T2, and the findings are shown in table 18. The current study found that there is no large change in antibacterial activity over time. The antimicrobial activity of all soaps against *E. coli*, *P. aeruginosa*, and *MRSA* remained stable after 2 months of storage, however the antimicrobial activity of both soaps' *Eucalyptus/Propolis* (50:50) and *Eucalyptus/Rosemary* (60:40) against *P. acnes* declined from MIC/MBC 2.5>10 and 5>10, respectively, to MIC/MBC 10>10. The results also revealed that soap *Eucalyptus/Propolis* (50:50) had the lowest concentration against *P. acnes*. This bacterium is the most common bacteria discovered on skin that causes acne (Vora et al., 2018). As a result, this soap can be used as a topical acne inhibitor.

Table 18: Antimicrobial activity of the formulated soaps.

							T1					
	Soap EP		**Soap ER**		**Glycerin**		**Ampicillin (10mg/mL)**		**Imipenem (1mg/mL)**		**Vancomycin (1mg/mL)**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative bacteria												
Escherichia coli	5	>10	2.5	>10	5	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	5	>10	5	>10	10	>10	>10	>10	0.5	1	n.t.	n.t.
Gram-positive bacteria												
MRSA	10	>10	10	>10	10	>10	<0.15	<0.15	n.t.	n.t.	0.25	0.5
Propionibacterium acnes	2.5	>10	5	>10	10	>10	n.t.	n.t.	n.t.	n.t.	0.07	5
							T2					
	Soap EP		**Soap ER**		**Glycerin**		**Ampicillin (10mg/mL)**		**Imipenem (1mg/mL)**		**Vancomycin (1mg/mL)**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative bacteria												
Escherichia coli	5	>10	2.5	>10	5	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	5	>10	5	>10	5	>10	>10	>10	0.5	1	n.t.	n.t.
Gram-positive bacteria												
MRSA	*10*	*>10*	*10*	*>10*	*10*	*>10*	*<0.15*	*<0.15*	*n.t.*	*n.t.*	*0.25*	*0.5*
Propionibacterium acnes	10	>10	10	>10	10	>10	n.t.	n.t.	n.t.	n.t.	0.07	5

***The maximum concentration tested was (10mg/mL) for all samples. T1 (July), T2(September)**

7.3.2. Serum

The serums were performed in triplicate batches of 30 mL, and all parameters for each serum replication were analyzed also in triplicate.

Table 19 shows the antioxidant activity (TBARS) and physical properties (viscosity, color, and pH) of six distinct serum formulas. The sample with the statistically significant highest viscosity was Eucalyptus/Propolis+ Preservative, whereas no statistical changes could be found for the other samples, indicating that the addition of other components did not modify the initial viscosity In terms of pH, although the differences were statistically significant, they are negligible due to the variance found between 5.75 and 5.94, with the exception of the serum containing ascorbic acid, which exhibited the lowest value of 4.9. As predicted, ascorbic acid enhanced serum acidity. Since the skin has an acidic pH of 4.1-6.7, these formulations are ideal for it (Miss. Purva et al., 2022). Concerning the colors, very few conclusions could be drawn due to the mixture of most coordinates. Figure 26 illustrates the many colors present in serums for easier understanding.

Combinations with the highest antioxidant activity (TBARS) were ascorbic acid +preservative and eucalyptus/rosemary +preservative. While the significant antioxidant activity of ascorbic acid, a very effective hydrophilic antioxidant, contributed to the sample's low EC_{50}; the natural preservatives worked in synergy to reach a very low antioxidant activity as well. The eucalyptus+ propolis was the least antioxidant of all the samples. This suggests that for the optimum antioxidant activity, rosemary should be the antioxidant with the major contribution, while the synergistic impact of natural antioxidants should not be considered out.

Table 19: Analysis of variance for the factors, namely storage time and incorporation type for the serum samples

	Viscosity	pH	L*	a*	b*	TBARS
Eucalyptus/Propolis	538±6^{a}	5.94±0.03^{d}	45.0±0.6^{b,c}	-1.43±0.07$^{a, b}$	15.1±0.6^{c}	0.124±0.001^{f}
Eucalyptus/Rosemary	503±25^{a}	5.93±0.03^{c,d}	37±1^{a}	-1.54±0.07^{a}	19.6±0.5^{d}	0.005±0.001^{c}
Ascorbic Acid	563±63^{a}	4.90±0.04^{a}	51±2^{d}	-1.0±0.1$^{a, b, c}$	5.2±0.5^{a}	0.03±0.01^{d}
Eucalyptus/Propolis +Preservative	835±123^{b}	5.85±0.02^{c}	50.0±0.8$^{c, d}$	-0.623±0.006^{c}	6.7±0.03$^{a, b}$	0.020±0.001^{e}
Eucalyptus/Rosemary +Preservative	672±56$^{a, b}$	5.85±0.03^{c}	45±4^{b}	-0.90±0.07$^{b, c}$	8.5±0.9^{b}	0.0010±0.0005a
Ascorbic Acid +Preservative	531±15^{a}	5.75±0.03^{b}	50.8±0.7^{d}	-1.2±0.5$^{a, b, c}$	5±2^{a}	0.0010±0.0003^{a}

* TBARS expressed as µg/mL.

Figure 26 represents the total colors of the three coordinates referring to table 19 (L*, a*, and b*), namely the serum samples, where the color differences are quite noticeable for the treatments Eucalyptus+ Rosemary and Eucalyptus+ Propolis, displaying a brown tint, while the other mixtures show very similar colors.

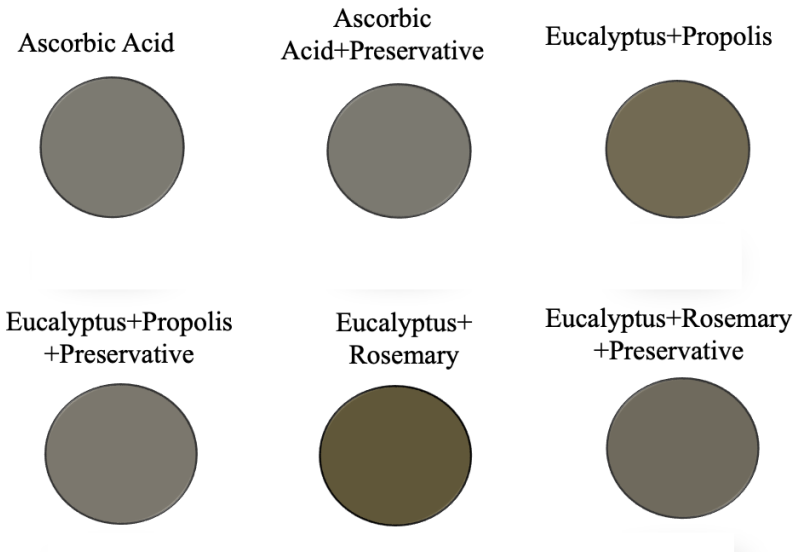


Figure 26: Color of the different samples shown in Table 18.

The antibacterial activity of the various serum formulas was tested, and the results are shown in table 20. It was observed that the artificial serum (Eucalyptus/Propolis +Preservative, Eucalyptus/Rosemary +Preservative, and Ascorbic Acid Preservative) had significant antibacterial activity against all the bacteria examined. Furthermore, the lowest activity was shown against *P.aeruginosa*, with a MIC/MBC of 1 mg/ml. The two serums, Eucalyptus/Propolis +Preservative and Eucalyptus/Rosemary +Preservative, had greater antimicrobial activity against *P.acnes* than the positive control serum Ascorbic Acid Preservative, with MIC/MBC of 0.25/>1 mg/ml compared to 1/>1 mg/ml. These results might be explained by the fact that the additional extracts are more active than ascorbic acid. However, none of the natural serum (Eucalyptus/Propolis, Eucalyptus/Rosemary, and Ascorbic Acid) shown antibacterial activity with MIC/MBC greater than the starting concentration.

Table 20: Antimicrobial activity of the formulated serums.

	Eucalyptus/ propolis +preservatives serum		Eucalyptus/rosemary +preservatives serum		Ascorbic acid +preservatives serum		Ampicillin (10mg/mL)		Imipenem (1mg/mL)		Vancomycin (1mg/mL)	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative bacteria												
Escherichia coli	0.5	>1	0.5	>1	0.5	>1	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	1	**>1**	1	>1	1	>1	>10	>10	0.5	1	n.t.	n.t.
Gram-positive bacteria												
MRSA	0.5	>1	0.5	>1	0.5	>1	<0.15	<0.15	n.t.	n.t.	0.25	0.5
Propionibacterium acnes	0.25	>1	0.25	>1	1	>1	n.t.	n.t.	n.t.	n.t.	0.07	5
	Eucalyptus/ propolis serum		**Eucalyptus/rosemary serum**		**Ascorbic acid serum**		**Ampicillin (10mg/mL)**		**Imipenem (1mg/mL)**		**Vancomycin (1mg/mL)**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative bacteria												
Escherichia coli	>1	>1	>1	>1	>1	>1	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	>1	>1	>1	>1	>1	>1	>10	>10	0.5	1	n.t.	n.t.
Gram-positive bacteria												
MRSA	>1	>1	>1	>1	>1	>1	<0.15	<0.15	n.t.	n.t.	0.25	0.5
Propionibacterium acnes	>1	>1	>1	>1	>1	>1	n.t.	n.t.	n.t.	n.t.	0.07	5

***The maximum concentration tested was (1mg/mL) for all samples.**

Only the synthetic serums (Eucalyptus/Propolis +Preservative, Eucalyptus/Rosemary +Preservative, and Ascorbic Acid +Preservative)) displayed antibacterial properties against all of the examined microorganisms, as was demonstrated in the preceding section. Due to this, the antibacterial activity of the serum's various ingredients was assessed. In table 21, the findings are displayed. The carboxymethyl cellulose (CMC) was the only ingredient that did not exhibit antimicrobial activity against any of the tested microorganisms. The least effective antimicrobial agents against P. acnes were sodium benzoate and potassium sorbate, with MIC/MBC values of 150> 300 and 300>300 mg/ml, respectively. However, with MIC/MBC values of 3.75 >60 mg/ml and 25% >50%, ascorbic acid and citric acid buffer had the strongest antibacterial efficacy against this bacterium. The extracts *Eucalyptus/Propolis* (50:50) and *Eucalyptus/Rosemary* (60:40) further shown remarkable antibacterial activity against all microorganisms. These findings highlight the significant impact of the additional excipients as antibacterial agents. They are utilized to improve the extracts' stability and enable their incorporation into other formulations. Numerous research has noted the antimicrobial properties of citric acid buffer, ascorbic acid, potassium sorbate, sodium benzoate, and others. When used as cosmetic preservatives in concentrations below 1%, they were acknowledged as safe (Nowak et al., 2021; Shaikh et al., 2016).

Table 21: Antimicrobial activity of serum reagents

	Carboxymethyl Cellulose (300mg)		Glycerin (600mg)		Extract EP (60 mg)		Extract ER (60mg)		Ampicillin (10mg/mL)		Imipenem (1mg/mL)		Vancomycin (1mg/mL)	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative bacteria														
Escherichia coli	>300	>300	600	>600	3.75	60	3.75	60	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	>300	>300	600	>600	3.75	60	3.75	60	>10	>10	0.5	1	n.t.	n.t.
Gram-positive bacteria														
MRSA	>300	>300	600	>600	3.75	60	3.75	60	<0.15	<0.15	n.t.	n.t.	0.25	0.5
Propionibacterium acnes	>300	>300	>600	>600	3.75	60	3.75	60	n.t.	n.t.	n.t.	n.t.	0.07	5
	Ascorbic Acid (60mg)		**Potassium Sorbate (300 mg)**		**Sodium Benzoate (300mg)**		**Citric acid (buffer)**		**Ampicillin (10mg/mL)**		**Imipenem (1mg/mL)**		**Vancomycin (1mg/mL)**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative bacteria														
Escherichia coli	15	60	37.5	>300	37.5	>300	50%	>50%	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	15	60	37.5	>300	37.5	>300	50%	>50%	>10	>10	0.5	1	n.t.	n.t.
Gram-positive bacteria														
MRSA	15	60	37.5	>300	37.5	>300	50%	>50%	<0.15	<0.15	n.t.	n.t.	0.25	0.5
Propionibacterium acnes	3.75	60	300	>300	150	>300	25%	>50%	n.t.	n.t.	n.t.	n.t.	0.07	5

EP: eucalyptus/propolis; **ER**: eucalyptus/rosemary

*The buffer was directly tested as it was liquid.

The anti-tyrosinase potential of the prepared serums was evaluated in the current study. Except formula 5, all serum formulas exhibited anti-tyrosinase action, as shown in table 22. This might be explained by the fact that the concentration of *Eucalyptus/Rosemary* (60:40) extracts in the analyzed serum was below the detection limit of the method used.

Furthermore, as compared to the natural formulations (Eucalyptus/Propolis, Eucalyptus/Rosemary, and Ascorbic Acid), the artificial serums (Eucalyptus/Propolis +Preservative, Eucalyptus/Rosemary +Preservative, and Ascorbic Acid +Preservative) had the highest activity. According to various research, ascorbic acid acts as an antioxidant that has the potential to inhibit melanogenesis (Lee et al., 2011). For that, Ascorbic Acid +Preservative and Ascorbic Acid serums contain ascorbic acid as an active ingredient and are utilized as positive controls with and without preservatives, respectively. When the two formulations, Eucalyptus/Propolis +Preservative and Eucalyptus/Rosemary +Preservatives serums, were compared to their control, Ascorbic Acid +Preservative serum, they showed greater activity with EC_{50} of 0.76 mg/ml and 0.67 mg/mL, respectively, compared to 0.47 mg/ml. In terms of natural formulas, Eucalyptus/Propolis +Preservative serum has the lowest activity.

Table 22: Anti-tyrosinase activity of the serum (mg/ml).

Samples	EC_{50}
Eucalyptus/Propolis +Preservative	0.76
Eucalyptus/Rosemary +Preservative	0.67
Ascorbic Acid +Preservative	0.47
Eucalyptus/Propolis	10% at 1mg/mL
Eucalyptus/Rosemary	NA
Ascorbic Acid	0.39
Positive control (Butylresorcinol)	369 µg/mL

NA: No activity; Samples where the EC_{50} could not be determined up to the maximum tested concentration are presented as % tyrosinase inhibition.

8. Conclusion

Several cosmetic and pharmaceutical items are vulnerable to microbial contamination, which leads to a degradation process and presents a possible health concern to users because contaminated products can potentially cause irritation or infection in certain cases. To prevent this type of condition, preservatives, whether natural or artificial, are frequently applied.

They act in three separate ways: as antibacterial agents; as antioxidants; and as enzyme-acting agents. The use of typical preservatives, which has been going on for a while, has actually been a major issue for the personal care industry. Cosmetic formulators are under increasing demand to use cutting-edge natural ingredients as a result of the controversy surrounding parabens and phenoxyethanol. In the current work, cosmeceuticals with natural bioactive components derived from propolis, rosemary, and eucalyptus have been developed.

Before being incorporated, various extract combinations were tested for their activity. The two heated water bath extraction combinations of eucalyptus and rosemary (60:40 *m/m*) and eucalyptus and propolis (50:50 *m/m*) were shown to have the highest bioactivities (antimicrobial and antioxidant). Separately, these two combinations were incorporated into two different cosmetic formulations: soaps and serums. The findings of the soap study revealed that they have considerable qualities. After being stored for two months, they maintained their antioxidant and antibacterial properties. Furthermore, there was little change in their physical characteristics. Even though the phenolic extract combination had anti-tyrosinase activity prior to their incorporation, both soaps didn't exhibit any such action. On the other hand, it was shown after examining the outcomes of the six serum formulas that those including artificial preservatives revealed higher antioxidant, antibacterial, and anti-tyrosinase activity than those without. Nevertheless, the mixtures analyzed revealed very strong antioxidant activity, being able to substitute the artificial preservative. A possible strategy could be the increase in the incorporation doses in order to guarantee the initial bioactivity.

Generally, it was able to demonstrate that the assessed natural extracts may serve as efficient substitutes for synthetic preservatives and can be used alone or in combination with artificial preservatives.

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