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# Development of a liquid soap with antimicrobial potential using propolis from the Montesinho Natural Park

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**I dedicate this to my family,**

**my support and encouragement throughout this journey.**

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## ABSTRACT

Propolis is a resinous and balsamic substance produced by bees with a complex chemical composition. The benefits of propolis, including healing, anti-inflammatory, and antimicrobial properties, mainly come from botanical sources, providing a unique quantity of bioactive compounds. In this context, the Montesinho Natural Park stands out for its plant biodiversity, singular in Europe. Therefore, this project aims to valorize propolis from the Montesinho Natural Park by investigating its potential as a natural source of bioactive compounds with antiseptic properties in liquid soaps. To this end, two extraction techniques, namely maceration and ultrasound-assisted extraction, were applied and compared by total phenolic content (TPC), to evaluate their efficiency in recovering bioactive constituents. The most promising extracts were chemically characterized and incorporated into liquid soap formulations to assess their stability and antimicrobial activity against pathogenic microorganisms. Spectrophotometric analysis revealed that propolis maceration with monopropylene glycol (MMPG) was more efficient than maceration with ethanol 70% (ME70), yielding higher TPC (65.9 vs. 63.1 mg GAE/mL), total flavonoids (23.5 vs. 18.4 mg QE/mL), and antioxidant activity (DPPH: 88.4 vs. 78.1 mg AAE/mL and ABTS: 86.1 vs. 75.4 mg AAE/mL), respectively. The UHPLC-DAD-ESI/MS qualitative analysis showed similar chemical profiles between both extracts, composed of 44 compounds, predominantly flavonoids (chrysin, pinocembrin, galangin, pinobanksin) and phenolic acids (caffeic acid, *p*-coumaric acid, ferulic acid) and their methyl esterified derivatives. Antimicrobial assays revealed high inhibitory, bactericidal and antifungal potential of both propolis extracts. ME70 was slightly stronger against *Staphylococcus aureus*, *Salmonella enterica* and *Candida albicans*, while MMPG stood out for its potent bactericidal action against *Pseudomonas aeruginosa*, *S. aureus* and *Cutibacterium acnes*. The incorporation at 5% into a liquid soap, the extracts seems to corroborate with the stability of the pH (6.0) and viscosity stability, especially at 5 ℃. Although high temperatures (45 ℃) triggered slight oxidation, the final product showed promising potential against *Escherichia coli*, *S. enterica*, *S. aureus*, *C. acnes* and *C. albicans*. Overall, propolis showed promising potential as an ingredient in the cosmetics industry.

Keywords: Antimicrobial; Montesinho Natural Park; Propolis; Soap.

## RESUMO

A própolis é uma substância resinosa e balsâmica produzida pelas abelhas, com uma composição química complexa. Os benefícios da própolis, incluindo propriedades cicatrizantes, anti-inflamatórias e antimicrobianas, provêm principalmente de fontes botânicas, que fornecem uma quantidade única de compostos bioativos. Neste contexto, o Parque Natural do Montesinho destaca-se pela sua biodiversidade vegetal, singular na Europa. Portanto, este projeto visa valorizar a própolis do Parque Natural do Montesinho, investigando o seu potencial como fonte natural de compostos bioativos com propriedades antissépticas em sabonetes líquidos. Para tal, foram aplicadas e comparadas duas técnicas de extração, nomeadamente maceração e extração assistida por ultrassom, quanto ao teor de fenólicos totais (TPC), para avaliar a sua eficiência na recuperação de constituintes bioativos. Os extratos mais promissores foram caracterizados quimicamente e incorporados em formulações de sabonete líquido para avaliar a sua estabilidade e atividade antimicrobiana contra microrganismos patogénicos. A análise espectrofotométrica revelou que a maceração da própolis com monopropilenoglicol (MMPG) foi mais eficiente do que com etanol 70% (ME70), resultando em mais TPC (65,9 vs. 63,1 mg GAE/mL), flavonoides totais (23,5 vs. 18,4 mg QE/mL) e atividade antioxidante (DPPH: 88,4 vs. 78,1 mg AAE/mL e ABTS: 86,1 vs. 75,4 mg AAE/mL), respectivamente. A análise qualitativa por UHPLC-DAD-ESI/MS mostrou perfis químicos semelhantes entre os dois solventes, compostos por 44 compostos, predominantemente flavonoides (crisina, pinocembrina, galangina, pinobanksina) e ácidos fenólicos (ácido cafeico, ácido *p*-cumárico, ácido ferúlico) e seus derivados metil-esterificados. Os ensaios antimicrobianos revelaram alto potencial inibitório, bactericida e antifúngico dos extratos de própolis. O ME70 apresentou desempenho ligeiramente superior contra *Staphylococcus aureus, Salmonella enterica* e *Candida albicans*, enquanto o MMPG destacou-se pela sua potente ação bactericida contra *Pseudomonas aeruginosa*, *S. aureus* e *Cutibacterium acnes*. A incorporação a 5% em um sabonete líquido confirmou a estabilidade do pH (6,0) e da viscosidade dos extratos, especialmente a 5 °C. Embora as altas temperaturas (45 °C) tenham provocado uma ligeira oxidação, o produto final demonstrou potencial promissor contra *Escherichia coli*, *S. enterica*, *S. aureus*, *C. acnes* e *C. albicans*. De modo geral, a própolis demonstra um potencial promissor como ingrediente na indústria cosmética.



Palavras-chave: Antimicrobiano; Parque Natural de Montesinho; Própolis; Sabonete.

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| | |
|---|---|
| ABTS | 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid |
| AAE | Ascorbic Acid equivalents |
| AMR | Antimicrobial resistance |
| ATCC | American Type Culture Collection |
| BRC | Brazilian Commercial Sample |
| CB | Cocamidopropyl betaine |
| CECT | Spanish Collection of Type Cultures |
| CFU | Colony Forming Unit |
| CIMO | Centro de Investigação de Montanha |
| CLSI | Clinical and Laboratory Standards Institute |
| DB | Department of Biology of the University of Minho |
| DPPH | 2,2-difenil-1-picril-hidrazila |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DPE | Dry propolis extract |
| DW | Dry weight |
| ESA | Escola Superior Agrária de Bragança |
| EUC | European Commercial Sample |
| GAE | Gallic acid equivalents |
| INT | *p*-iodonitrotetrazolium chloride |
| IRPA | Imipenem-Resistant *Pseudomonas aeruginosa* |
| LD | Loss on drying |
| MBC | Minimal bactericidal concentration |

| | |
|---|---|
| MFC | Minimum fungicidal concentration |
| MIC | Minimal inhibitory concentration |
| MPG | Monopropylene glycol |
| MRSA | Methicillin-Resistant *Staphylococcus aureus* |
| MSSA | Methicillin-Susceptible *Staphylococcus aureus* |
| NUTS II | Nomenclature of Territorial Units for Statistics (second level) |
| PED | Propolis Extract Disk |
| QE | Quercetin equivalents |
| RP | Raw Propolis |
| SCD | Solvent Control Disk |
| SLES | Sodium lauryl ether sulfate |
| 3GC-R-Kp | Third-generation cephalosporin-resistant *Klebsiella pneumoniae* |
| TFC | Total Flavonoids Content |
| TPC | Total Phenolic Content |
| TSB | Tryptic Soy Broth |
| UAE | Ultrasound-Assisted Extraction |
| UNGA | United Nations General Assembly |
| UHPLC-DAD-ESI/MS | Ultra-High Performance Liquid Chromatography, Diode Array Detection and Electrospray Ionization Mass Spectrometry |
| WHO | World Health Organization |

# 1.INTRODUCTION

## 1.1 Montesinho Natural Park

According to the Ministry for Environment, Spatial Planning and Energy, Portugal has an area of 92 212 km², which are divided in seven regions: North, Center, Lisbon, Alentejo, Algarve, Autonomous Region of Madeira and Autonomous Region of Azores, based in the Nomenclature of Territorial Units for Statistical Purposes (NUTS II) system (Instituto Nacional de Estatística, 2012). Only the North region has 21,286 km² and it is composed of major mountain systems such as National Park of Peneda-Gerês, with Larouco and Gerês as high mountain peak, measuring 1,527 m and 1,525 m respectively, and Natural Park of Montesinho (Figure 1) with 1,492 meters of maximum altitude. (INFC, National System of Classified Areas, 2017 - 2025; Instituto Nacional de Estatística, 2012).

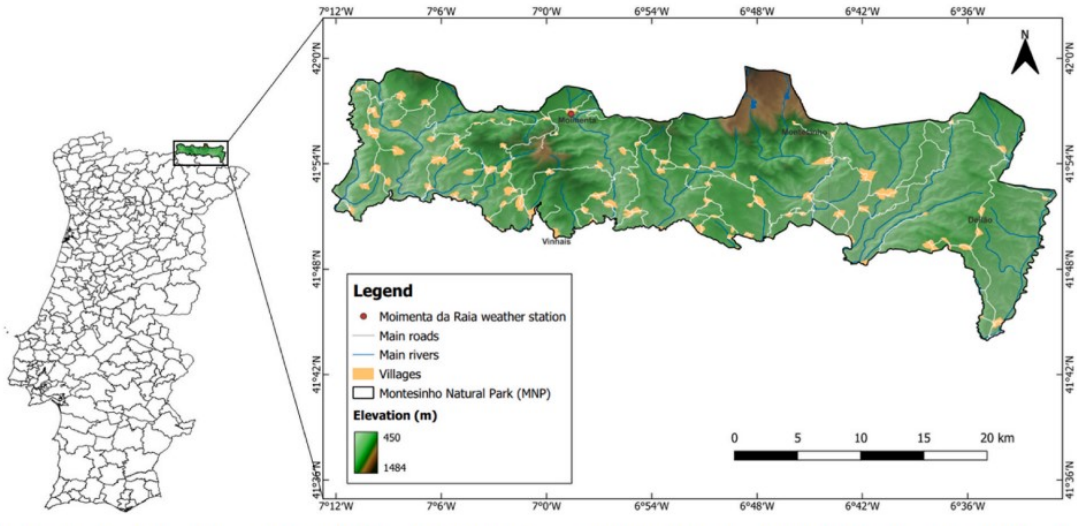



**Figure 1.** *Digital model of the Montesinho Natural Park in the District of Bragança*. Source: De Almeida, C. R., Garcia, N., Campos, J. C., Alírio, J., Arenas-Castro, S., Gonçalves, A., Sillero, N. & Teodoro, A. C. (2023). Time-series analyses of land surface temperature changes with Google Earth Engine in a mountainous region. Heliyon, 9(8).

Covering around 742 km² of the northern area, the Montesinho Natural Park (41°43' 47'' to 41° 59' 24'' N latitude and 6° 30' 53'' to 7° 12' 9'' W longitude), in the district of Bragança, northern region of the municipalities of Bragança and Vinhais, is one of the representative areas for the preservation of Iberian and European fauna and

flora (De Almeida *et al*., 2023; INFC, Natural Park of Montesinho, 2017 - 2025; Instituto Nacional de Estatística, 2012). The denomination of natural park regions is according to the biodiversity, necessarily natural or semi-natural, where preservation depends on the reduction of human activity to verify the development of the environment. In 1979, the importance of the Montesinho Natural Park ecosystem was recognised and the classification of natural park was granted. Its protection contributes to the European natural heritage, due to the great number of rare and endemic species, and to the regional and national development of natural products and services associated with the park (Campos *et al.*, 2024; INFC, National System of Classified Areas, 2017 - 2025; INFC, Natural Park of Montesinho, 2017 - 2025).

In the context of biodiversity, Montesinho Natural Park stands out from the vegetation of Portugal, the Iberian Peninsula and Europe. The specific climatic and orographic conditions of the area provide a huge flora diversity, characterized mainly by the famous chestnut groves (*Castanea sativa*). Other species are found in the region too, such as oak, sardoal and riparian forests, broom, heather, cistus, marshes, etc. The park is located in the region named Trás-os-Montes, or cold lands, with average annual temperatures between 8.5℃ and 12.8℃, with minimum of -12℃ in winters and maximum of 40℃ in summers, therefore, the fauna and flora need to be adapted (Campos *et al.*, 2024; INFC, Natural Park of Montesinho, 2017 - 2025). The bees adapted to these environmental conditions are the subspecies *Apis mellifera iberiensis*, hybrid bees between the African and European lineages, probably resulting from colonization, extinction and environmental factors. Samples from more than 3,300 colonies of the species present haplotypes of both lineages in their mitochondrial DNA, providing valuable insights into the evolution of Iberian bees (Cánovas *et al.*, 2011; Chávez-Galarza *et al.*,2017; Utzeri, V. J., Ribani, A., & Fontanesi, L., 2018).

**1.2 Honeybees**

Regarded as the most important and effective pollinators, bees are responsible for preserving, diversifying and extending the life of several plants (Almeida-Dias *et al.*, 2025; Silva *et al.*, 2025). Around 70% of global food production is dependent on cross-

pollination by pollinators, so bees are not only essential for the environment, but for agriculture and economy (Almeida-Dias *et al.*, 2025; Chakraborty & Basu, 2025).

The pollination process occurs in parallel with the collection of nectar and pollen by the bees. These two essential resources provide the main nutrients for their development and metabolism. Nectar is responsible for carbohydrates, such as sucrose, glucose, and fructose, while pollen serves as a source of proteins, minerals, and lipids (Almeida-Dias *et al.*, 2025; Amsalem *et al.*, 2025). Nourished, bees can produce beeswax, royal jelly, honey and propolis, with medicinal properties, including healing, anti-inflammatory and antimicrobial (Rocha *et al.*, 2022; Simsek *et al.*, 2021).

Bees belong to the *Apidae* family (*Hymenoptera* order) and are classified by their honey production. The genus *Apis*, from the family *Apidae*, has four main species: *Apis cerana, Apis dorsata, Apis florea*, and *Apis mellifera* (Silva *et al.*, 2025; Utzeri, V. J., Ribani, A., & Fontanesi, L., 2018). The *A. mellifera* species is the most important bee pollinator and common honeybee. Genetically, more than 30 subspecies and regional varieties have been described, which are grouped based on their evolutionary lineages: Africa, the Middle East, Western, Northern and Central Europe. In terms of economic impact, the subspecies *A. mellifera iberiensis*, originally from the Iberian Peninsula, is remarkable in Europe, considering the high honey production of Portugal and Spain (Alonso-Prados *et al.*, 2020; Utzeri, V. J., Ribani, A., & Fontanesi, L., 2018).

**Figure 2.** *A. mellifera iberiensis in the hive*. Source: Author.

### 1.2.1 Propolis

Propolis is a resinous and balsamic material produced by honeybees, composed by mixing salivary secretions with plant-based natural materials, such as exudates from leaves, shoots buds, wounds, sap flowers and pollen. Essentially, this material is composed by 40-70% resin and/or vegetable balsam, 10-30% beeswax, 5-10% aromatic and essential compounds, 5-10% pollen, and other organic components (Falcão *et al.*, 2023; Salatino, 2022; Pant *et al*., 2024; Freitas *et al*., 2022c). The term "propolis" is representative for its function in the hive, where "pro" means "in front of" and "polis" means "city" from the Greek language. So, the word can be translated as "defense of the community" (Wieczorek *et al*., 2022; Rojczyk *et al*., 2020).

In the inner walls of hives, propolis is employed as building, sealing and defensive material, so bees use it to repair damage, avoid sudden changes in temperature and protect the hive against predators and microorganisms' infections. For humans, the use of propolis as natural medicine, named Apitherapy, dates back 9,000 years, in which not only bee products are used to prevent or treat diseases, but even bee venom. Egyptians, Persians, Greeks, and Romans applied it as a therapeutic product to treatment of wounds, cuts, ulcers and other dermatological problems and as disinfectant agent. Even if propolis is not considered a therapeutic agent in conventional medicine, it is one of the few natural products that maintained popularity over time (Pant *et al*., 2024; Freitas *et al.*, 2022a; Rocha *et al.*, 2022; Salatino, 2022; Barreto *et al.*, 2020; Wieczorek *et al*., 2022; Rojczyk *et al.*, 2020; Silva-Carvalho, R., Baltazar, F., & Almeida-Aguiar, C., 2015).

Therefore, studies of propolis have gradually spread. Manifold investigations have supported the uses of propolis in cosmetic products and food industries as a natural preservative, antioxidant and functional food ingredient. In the pharmaceutical area, the incorporation of propolis extract in cosmetics and hygiene products has been growing, due to meeting market demand for natural products. In medicine perspective, its applications were suggested for anticancer treatment against breast, renal and prostate cancer cells and in dentistry therapy to prevent and treat caries, gingivitis and periodontitis (Pant *et al*., 2024; Freitas *et al.*, 2022a; Rocha *et al.*, 2022; Salatino, 2022; Barreto *et al.*, 2020; Wieczorek *et al*., 2022; Rojczyk *et al.*, 2020; Silva-Carvalho, R.,

Baltazar, F., & Almeida-Aguiar, C., 2015). However, the chemical constitution of propolis is strongly associated with the geographic, climatic, and vegetation conditions surrounding the hive, also time of collection, bee species, illumination, altitude and season of the year. So, it is crucial to know the origin of the propolis, to realize the best application of each one (Falcão *et al.*, 2023; Rodriguez-Canales *et al*., 2023; Rojczyk *et al.*, 2020; Galeotti *et al*., 2018; Falcão *et al*., 2013a).

Based on botanical sources, color and main composition, propolis is organized in groups too, known as Poplar, Birch, Green, Red, Brown, Aspen, Clusia Pacific, Mediterranean, and others. The tropical types (Green, Red, Brown, Caribbean, and Pacific-type) are mainly found in tropical regions and are distinguished from others because of their constitution of sesquiterpenoid compounds such as germacrene D, ledol, and spathulenol. Specifically, the green Brazilian one, originated from the plant *Baccharis dracunculifolia*, stands out because of their abundance in artepillin C (Balasubramaniam *et al*., 2025; Pant *et al*., 2024; Falcão *et al*., 2023).

The Portuguese propolis are classified as Poplar propolis because they are produced by the Iberian bees (bees from Iberian Peninsula), in temperate zone and their botanical origin is obtained from *Populus spp*. (Trusheva *et al*., 2024; Zhang *et al*., 2023). Unlike other propolis types, Portuguese propolis is characterized by elevated levels of phenolic compounds, such as quercetin, apigenin, pinobanksin and kaempferol derivatives (Freitas *et al*., 2022a; Falcão *et al*., 2013b).

### 1.3 Antimicrobial resistance

Antimicrobial resistance (AMR) is the most health global challenge of the 21$^{st}$ century. In 2019, the AMR was classified as one of the top 10 global public health threats facing humanity by the World Health Organization (WHO) and in 2022, the Commission and the Member States, identified AMR as one of the top three priority health threats (Sinha & Upadhyay, 2025; European Commission, 2023). It is estimated that AMR will be the main cause of death and a giant source of expenses globally by 2050, if preventive measures are not implemented. In 2024, the second meeting of the United Nations General Assembly (UNGA) was focused on the significant threat posed by AMR, so the member countries committed to reduce by 10% the number of deaths globally

associated with bacterial AMR by 2030 (Sinha & Upadhyay, 2025; Medioli *et al.*, 2025; European Commission, 2023).

The primary factors contributing to antimicrobial resistance include the significant increase in antimicrobial use over recent decades, poor patient adherence to prescribed therapeutic guidelines, and the limited development of new medications to replace those that are becoming ineffective due to microbial resistance. The most difficult threats due to AMR are caused by the *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, *Staphylococcus aureus*, *Enterobacter*, *Klebsiella pneumoniae* and *Enterococcus faecium*, of which *S. aureus* are resistant to Methicillin antibiotic (MRSA) and *K. pneumoniae* are resistant to third-generation cephalosporin (3GC-R-Kp) (Sinha & Upadhyay, 2025; ECDC; 2022). *S. aureus*, *Klebsiella spp.* and *Enterococcus spp.* are already identified as the main bacteria found on the hands of hospital professionals, in addition to *Escherichia coli*, *Candida albicans* and *Staphylococcus epidermidis* (Larios-Fracarolli *et al.*, 2019).

It is estimated that half of the currently available medications are obtained from natural or related compounds, therefore, various natural matrices have been studied and evaluated as an alternative source of functional activities against microbial resistance. In the search for new pharmaceutical compounds, propolis appears to have great potential, mainly due to its high levels of phenolic compounds and flavonoids, compounds associated with antimicrobial action (Silva *et al.*, 2025; Deng *et al.*, 2025; Oliveira, Araújo & Almeida-Aguiar, 2024; Salatino, 2022; Silva-Carvalho, R., Baltazar, F., & Almeida-Aguiar, C., 2015).

### 1.4 Antimicrobial activity of poplar propolis

The growing emergence of antibiotic-resistant pathogens has prompted extensive research into the antimicrobial properties of propolis as a promising alternative to conventional antimicrobial therapies (Silva-Carvalho, R., Baltazar, F., & Almeida-Aguiar, C., 2015). In this field, Portuguese propolis, which is highly rich in phenolic compounds, particular flavones and flavonols, has been shown to exhibit antibacterial activity against different strains of *Listeria monocytogenes*. For example,

propolis from Gerês (Portugal) was demonstrated to have greater antimicrobial potential than the antibiotics erythromycin, vancomycin and amoxicillin/clavulanic acid, against MRSA and Gram-negative bacteria, such as *E. coli* while propolis from Bragança was reported as inhibitor of *S. aureus* and *P. aeruginosa* (Deng *et al.*, 2025; Oliveira, Araújo & Almeida-Aguiar, 2024; De Carli *et al.*, 2022; Freitas *et al.*, 2022a; Silva-Carvalho, R., Baltazar, F., & Almeida-Aguiar, C., 2015; Silva *et al*., 2012). In contrast, Chinese propolis extract was suggested as an alternative to antibiotics against *Clostridium perfringens* due the action of caffeic acid phenethyl ester, while luteolin and quercetin, a flavone and flavonoid, respectively exhibit significant minimum inhibitory concentration against *S. aureus* as well as kaempferide, a flavonol component of Brazilian green propolis, was pointed to the treatment of skin infections caused by the same bacteria (Pratami *et al.*, 2024; Salatino, 2022). Therefore, a correct understanding of propolis leads to its correct application, since different types of propolis demonstrate different antimicrobial results. Table 1 summarizes some of the studies addressing the antimicrobial activity of Portuguese propolis.

Table 1 - Portuguese propolis antimicrobial activity

| **Microorganism** | **Result of inhibition** | **Reference** |
|---|---|---|
| **Propolis from Peneda-Gerês National Park** | | |
| *Bacillus subtilis* DB 48886 | Positive | Oliveira, R. D., Araújo, C., & Almeida-Aguiar, C. (2024).<br>Freitas *et al*., (2022b).<br>Freitas *et al*., (2022c). |
| *Staphylococcus aureus* ATCC 6538 (MSSA) | Positive | Oliveira, R. D., Araújo, C., & Almeida-Aguiar, C. (2024).<br>Freitas *et al*., (2022b).<br>Freitas *et al*., (2022c). |

| | | |
|---|---|---|
| *Staphylococcus aureus* M746665 (MRSA) | Positive | Oliveira, R. D., Araújo, C., & Almeida-Aguiar, C. (2024).<br>Freitas *et al*., (2022b).<br>Freitas *et al*., (2022c). |
| *Escherichia coli* CECT 423 | Positive | Oliveira, R. D., Araújo, C., & Almeida-Aguiar, C. (2024).<br>Freitas *et al*., (2022b).<br>Freitas *et al*., (2022c). |
| *Candida albicans* DB 53B | Negative | Oliveira, R. D., Araújo, C., & Almeida-Aguiar, C. (2024). |
| *Saccharomyces cerevisiae* DB BY4741 | Negative | Oliveira, R. D., Araújo, C., & Almeida-Aguiar, C. (2024). |
| *Bacillus cereus* ATCC 7064 | Positive | Freitas *et al*., (2022b).<br>Freitas *et al*., (2022c). |
| *Bacillus megaterium* DB 932 (methicillin-sensitive) | Positive | Freitas *et al*., (2022b).<br>Freitas *et al*., (2022c). |
| *Propionibacterium acnes* DB H60803 | Positive | Freitas *et al*., (2022b). |

**Propolis from Bragança**

| | | |
|---|---|---|
| *Arthrobacter sp.* CIMO 21MS005 | Positive | De Carli *et al.,* (2022). |
| *S. aureus* | Positive | De Carli *et al.,* (2022). |
| *S. aureus* ATCC 6538™ | Positive | Silva *et al.*, (2012). |
| *Staphylococcus aureus* ESA (175, 159) (MRSA) | Positive | Silva *et al.*, (2012). |

| | | |
|---|---|---|
| *Staphylococcus hominis* CIMO 21MS001 | Positive | De Carli *et al.,* (2022). |
| *Pantoea sp.* CIMO 21MS004 | Negative | De Carli *et al.,* (2022). |
| *Erwinia sp.* CIMO 21MS006 | Negative | De Carli *et al.,* (2022). |
| *Bacillus cereus* | Negative | De Carli *et al.,* (2022). |
| *Escherichia coli* | Negative | De Carli *et al.,* (2022). |
| *Escherichia coli* ATCC 29998 | Positive | Silva *et al.*, (2012). |
| *Metschnikowia rancensis* CIMO 21MS010 | Positive | De Carli *et al.,* (2022). |
| *Cephalosporins-resistant E.coli* ESA (37, 54) | Positive | Silva *et al.*, (2012). |
| *Cladosporium sp.* CIMO 21MS014 | Negative | De Carli *et al.,* (2022). |
| *Penicillium brevicompactum* CIMO 21MS015 | Negative | De Carli *et al.,* (2022). |
| *Botrytis cinerea* CIMO 21MS019 | Negative | De Carli *et al.,* (2022). |
| *Alternaria sp.* CIMO 21MS020 | Negative | De Carli *et al.,* (2022). |
| *P. aeruginosa* ATCC 15442™ | Positive | Silva *et al.*, (2012). |
| *P. aeruginosa* (IRPA) ESA (22, 23) | Positive | Silva *et al.*, (2012). |
| *C. albicans* ATCC 10231™ | Positive | Silva *et al.*, (2012). |
| *Fluconazol-resistant C. albicans* ESA (500, 504) | Positive | Silva *et al.*, (2012). |

**Propolis from Montesinho Natural Park**

| | | |
|---|---|---|
| *C. albicans* ATCC 90028 and ATCC 64550 | Positive | Silva *et al.*, (2024). |
| *C. glabrata* ATCC 2001 | Positive | Silva *et al.*, (2024). |
| *C. parapsilosis* ATCC 22019 | Positive | Silva *et al.*, (2024). |
| *C. krusei* ATCC 6258 | Positive | Silva *et al.*, (2024). |
| *C. tropicalis* ATCC 750 | Positive | Silva *et al.*, (2024). |
| **Propolis from Beja and Coimbra** | | |
| *P. aeruginosa* ATCC 15442™ | Positive | Silva *et al.*, (2012). |
| *P. aeruginosa* (IRPA) ESA (22, 23) | Positive | Silva *et al.*, (2012). |
| *C. albicans* ATCC 10231™ | Positive | Silva *et al.*, (2012). |
| *Fluconazol-resistant C. albicans* ESA (500, 504) | Positive | Silva *et al.*, (2012). |
| *Cephalosporins-resistant E.coli* ESA (37, 54) | Positive | Silva *et al.*, (2012). |
| *Escherichia coli* ATCC 29998 | Positive | Silva *et al.*, (2012). |
| *S. aureus* ATCC 6538™ | Positive | Silva *et al.*, (2012). |
| *Staphylococcus aureus* ESA (175, 159) (MRSA) | Positive | Silva *et al.*, (2012). |

ATCC (American Type Culture Collection); ESA (Escola Superior Agrária de Bragança); IRPA (Imipenem-resistant *Pseudomonas aeruginosa*); MRSA (methicillin-resistant *Staphylococcus aureus*); MSSA (methicillin-susceptible *Staphylococcus aureus*).

The propolis from the Montesinho Natural Park is recently well produced and commercialized, but it is not yet widely studied. Nevertheless, some searches exhibit a rich phenolic profile of the Montesinho's Park propolis, mainly characterized by the

presence of ferulic acid, vanillic acid, *p*-coumaric acid and myricetin (Silva *et al*., 2024; Trusheva *et al*., 2024; De Carli *et al*, 2022).

### 1.5 Propolis extraction

Propolis has a diverse chemical composition of polar and nonpolar compounds. Most of them are specialized hydrophobic plant metabolites, therefore, the choice of solvent for extraction influences the yield and composition of the extracts. The lipophilic characteristics of the matrix directed for a traditional extraction tincture made with ethanol or ethanol/water 70% aiming extracts richer in phenolic compounds, which are closely associated with antimicrobial activity (Trusheva *et al.*, 2024; Oliveira, Araújo & Almeida-Aguiar, 2024; Pereira, Cunha & Almeida-Aguiar, 2022).

The technique of extraction determines its effectiveness to leach out bioactive compounds from raw propolis as well as the influence of factors such as solvent type, concentration and temperature. Each method offers unique advantages that contribute to enhancing propolis quality in terms of phenolics, flavonoids and antioxidant capacity. Therefore, the optimization and combination of extraction parameters have been studied to improve extraction efficiency using different solvents, with the aim of reducing the growth of microorganisms, among other applications. Beyond ethanol, solvents such as monopropylene glycol and water have been analyzed, as well, to extract phenolics from propolis, majorly for food and pharmaceutical applications, using alternative extraction methods such as high hydrostatic pressure, microwave-assisted, ultrasound-assisted, supercritical fluid, and pressurized liquid extraction (Pant *et al.*, 2024; Lazović *et al.*, 2024; Mahmad *et al*., 2023).

#### 1.5.1 Maceration

Maceration is a traditional, conventional process, one of the simplest, popular and inexpensive extraction techniques, used for the extraction of different bioactive compounds from plant material. The method involves immersing the sample, grinded into smaller particles to increase the surface area, in a solvent for a set amount of time while stirring occasionally. The process makes it simpler for the solvent to permeate the

cell wall and reach the cell cavity holding the active ingredient, which exposes the chemical constituents to react with the solvent and helps in removal of different plant components (Amalia *et al.*, 2025; Chacon *et al.*, 2025; Saqib Farooq *et al.*, 2022).

The efficiency for the removal of bioactive compounds from the plant material depends on the type of solvent, their polarity and type of plant material, in addition to time-temperature combinations. This method is extensively used for the extraction of different types of bioactive compounds at laboratory scale, research and industry due to its simplicity and ability to produce stable extracts when optimized for time and solvent composition. Furthermore, the method has several advantages, including simple equipment and work technique, low operational costs, and the ability to extract thermolabile compounds, even if have long processing time of extraction and low efficiency as disadvantages (Amalia *et al.*, 2025; Chacon *et al.*, 2025; Saqib Farooq *et al.*, 2022).

### 1.5.2 Ultrasound-assisted

Ultrasound-assisted extraction is an economic and green alternative to conventional techniques. The method is based on cavitation, where ultrasound waves cause bubbles, disrupting the cellular structures and allowing the solvent into the interior of the particles, which promotes greater penetration and highest yield in bioactive compounds. In general, it shows better percentage extraction of phenolic and flavonoids than the maceration process (De Santana *et al.*, 2024; Silva *et al.*, 2024)

The technique is defined as a non-thermal technology that is an alternative for pasteurization in the food industry, because of its capacity for microbial inactivation. Although the technique can be associated with temperature to optimize extraction, authors report a beneficial effect of low temperature (below 30℃), due the degradation of thermolabile compounds in nature matrices (Akhan *et al.*, 2025; Pant *et al.*, 2024; Chemat *et al.*, 2017; García-Pérez, Mulet Pons & Carcel Carrión, 2011). For propolis extraction, ultrasound facilitates the extraction of structures with antioxidant potential even at room temperature, thus searching indicates 25℃ as a promisor temperature to propolis extraction (De Santana *et al.*, 2024; Silva *et al.*, 2024).

## 2.OBJECTIVES

Despite the growing interest in natural bioactive products, the valorisation of propolis from specific geographical regions, such as the Montesinho Natural Park, remains underexplored, particularly regarding its chemical composition, extraction efficiency, and potential applications in antimicrobial formulations. In this context, this project aim to valorize propolis from the Montesinho Natural Park by investigating its potential as a natural source of bioactive compounds with antiseptic properties in liquid soaps. To this end, two extraction techniques, namely maceration and ultrasound-assisted extraction, were applied and compared, to evaluate their efficiency in recovering bioactive constituents. The resulting extracts were chemically characterized, and the most promising samples were subsequently incorporated into liquid soap formulations to assess their antimicrobial activity against pathogenic microorganisms. This approach aimed to explore the potential of propolis as a sustainable antiseptic ingredient for cosmetic and pharmaceutical applications.

To attain this objective, the work was divided into the following stages:

I. To characterize raw propolis material;
II. To obtain propolis using maceration and ultrasound-assisted techniques and compare their efficiency based on extraction kinetics of total phenolic compounds;
III. To select the most promising extracts and evaluate their total flavonoid content, antioxidant activity, antimicrobial activity and phenolic profile by UHPLC-DAD-ESI/MS;
IV. To formulate liquid soaps incorporating the selected propolis extracts as ingredients;
V. To evaluate the antimicrobial activity and stability of the propolis-enriched liquid soap formulations through physicochemical tests and stress conditions, including light exposure, extreme temperatures, and freeze-thaw cycles.

## 3.MATERIALS AND METHODS

For monitoring the methods, a methodology diagram (Figure 3) was created for better visualization of the steps.

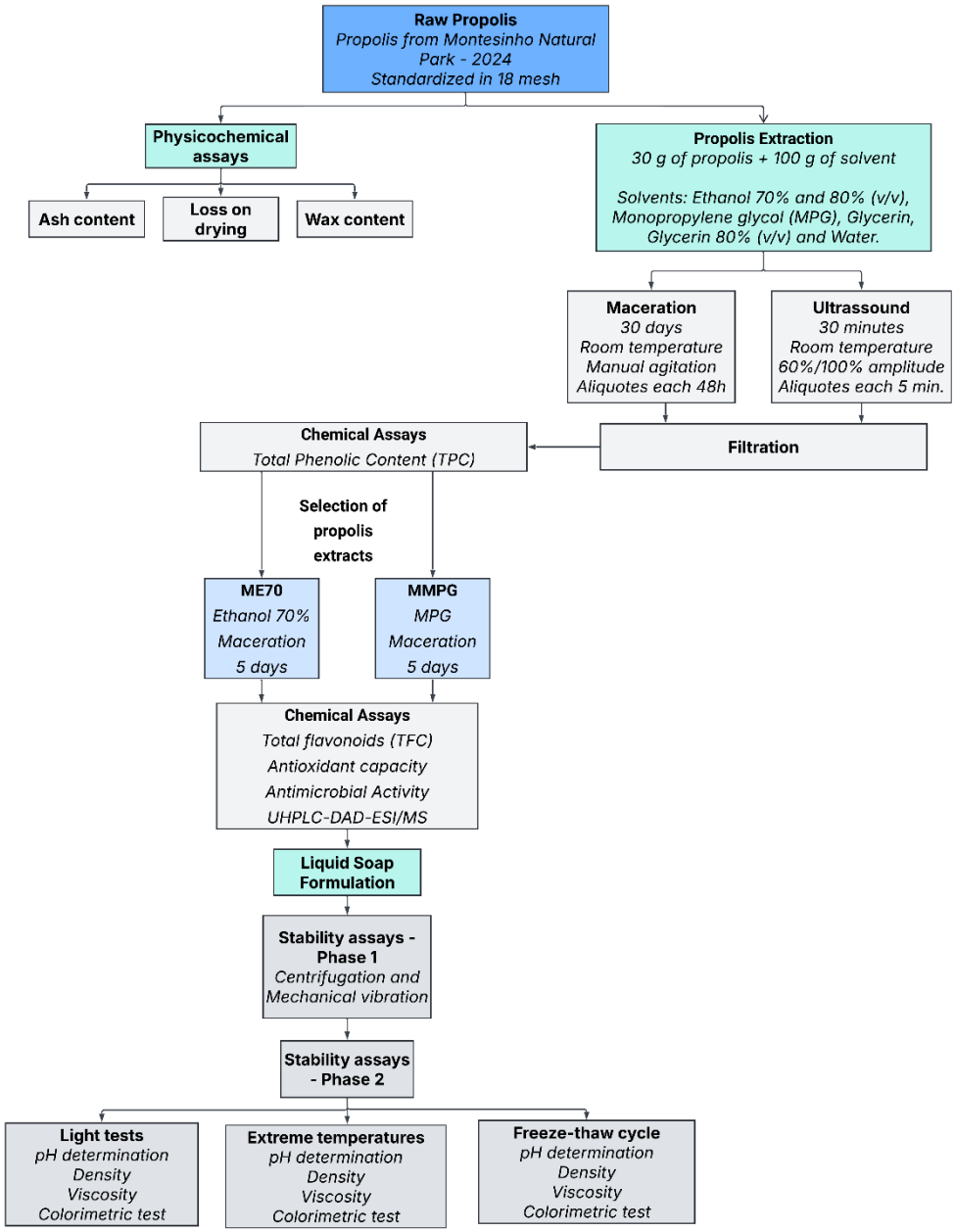



**Figure 3.** *Methodology Diagram*. Source: Author.

### 3.1 Samples

Propolis was collected from *Apis mellifera iberiensis* bee hives from five apiaries located in the Montesinho Natural Park in 2024, supplied by Sandra Isabel Barbosa Unip. Lda - Montesino. All samples were ground, granulometrically standardized in 18 mesh (approximately 1 mm × 1mm) and mixed proportionally by the quantity of each one. The mixed propolis were stored at −20 ℃ until further analysis.

**Figure 4.** *Mixed propolis from Montesinho Natural Park granulometrically standardized.* Source: Author.

### 3.2 Raw propolis physicochemical assays

#### 3.2.1 Ash content

To quantify the non-combustible component of raw propolis, an ash content assay was performed. For the method, 2 g of raw propolis ($w_2$) was placed in a dried crucible, previously desiccated and weighted ($w_1$), in a muffle furnace at 300 ℃ for 30 minutes (min). After the sample was carbonized, the temperature increased at 600 ℃ during 3 hours (h). In sequence, the ashed sample was cooled, desiccated and weighed ($w_3$) (Barreto *et al.*, 2020). The content of ash (Y) in the sample was calculated by Equation 1 (E1), expressed in percentage.

$$Y = \frac{w_3 - w_1}{w_2} \qquad \text{(E1)}$$

### 3.2.2 Loss on drying determination

The amount of all volatile substances, including moisture, present in the propolis was determined in terms of loss on drying. The gravimetric method was made with 2 g of raw propolis ($m_i$) placed in a dried capsule, previously desiccated and weighted ($m_C$), in an oven at 105 ℃ for 1 h. The capsule was transferred to a desiccator, until it cools, and the weight was measured ($m_f$). The content of loss on drying ($L_D$) was expressed in percentage of dry weight (DW) by Equation 2 (E2) (Barreto *et al.*, 2020).

$$L_D = 100 - \left[\frac{m_f - m_C}{m_i} \times 100\right] \quad \text{(E2)}$$

### 3.2.3 Wax content

The content of wax was determined using *n*-hexane, which removes waxes and some other non-polar compounds. The method was performed using 2 g of raw propolis ($w_4$) in a cellulose cartridge and extracted with *n*-hexane in a Soxhlet apparatus for 6 h. The extract was evaporated to dryness under reduced pressure at maximum 50 ℃. Subsequently, the dried extract was treated with 120 mL of hot methanol and heated until a clear supernatant was obtained, with a small oily residue remaining at the bottom of the flask. The hot methanolic phase was then carefully transferred to a previously weighted 150 mL flask. This apparatus was then cooled to 0 °C to promote wax precipitation and filtered again using a pre-weighed filter paper. The flask and the wax-precipitated filter paper were then oven-dried at 40 ℃ and placed in a desiccator until a constant mass ($w_5$) (Barreto *et al.*, 2020). The content of wax ($Z_1$) was expressed in percentage of mass by Equation 3 (E3).

$$Z_1 = \frac{w_5}{w_4} \times 100 \quad \text{(E3)}$$

### 3.3 Propolis extraction

The propolis extraction was conducted by maceration and ultrasound-assisted extraction (UAE), using 30 g of grounded propolis and 100 g of solvent, resulting in an

extraction at 23.1% (w/w). The solvents used were ethanol/water 70% and 80% (v/v), monopropylene glycol, glycerin, glycerin/water 80% (v/v) and water.

For the maceration process, extraction was carried out for 30 days at room temperature in the dark, with periodic manual agitation. UAE was performed using an Ultrasonic Sonicator (CY-500, Optic Ivymen Systems™, COMECTA®) for 30 min, at sonication amplitudes of 60% and 100%, maintained at 25 ℃. During both processes, the extraction kinetics were monitored by periodically determining the total amount of phenolic compounds. Accordingly, aliquots were collected every 48 h during maceration and every 5 min during UAE. Afterwards, the samples were filtered, analyzed for total phenolic content by the Folin-Ciocalteu method (see details in Section 3.4.1) and stored at -20 ℃ until further analysis.

To determine extract concentration, the samples were dried at 105 ℃ (ethanol 70% and 80%) and 180 ℃ (monopropylene glycol) in order to evaporate the solvent and calculate the extract concentration (in g per mL), being hereafter referred to as dry propolis extract (DPE). For reference in all subsequent analyses and discussions, a summary of the sample codes of each extraction method and solvent is presented in Table 2.

Table 2 - Summary of the sample codes.

| **Maceration** | **Ultrasound** | **Solvent** |
|---|---|---|
| ME70 | UE70 | Ethanol/water 70% (v/v) |
| ME80 | UE80 | Ethanol/water 80% (v/v) |
| MMPG | UMPG | Monopropylene glycol |
| MGA | UGA | Glycerin |
| MGA80 | UGA80 | Glycerin/water 80% (v/v) |
| MA | UA | Water |

Source: Author.

### 3.4 Characterization and Bioactivity Assays of Propolis Extracts

#### 3.4.1 Total Phenolic Content

The Total Phenolic Content (TPC) of all propolis was determined spectrophotometrically following the Folin-Ciocalteu method (Silva *et al.*, 2024), with slight modifications. The methodology was performed in a 96-well microplate and consisted of sample, Folin-Ciocalteu reagent, and $Na_2CO_3$ solution (3.5%, w/v). The microplate was incubated for 1 h, at room temperature, in the dark. The absorbance was read at 750 nm in a Synergy H1 Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). The gallic acid standard curve was prepared with ethanol 99.5% (v/v) and used as a reference (linearity range = 0.015-0.225 mg/mL, $R^2$=0.992). The results were calculated considering the dilution factor and expressed as milligrams of gallic acid equivalents (GAE) per milliliter of propolis extract (mg GAE/mL), per gram of dry propolis extract (DPE) (mg GAE/g DPE), and per gram of raw propolis (RP) (mg GAE/g RP). The assay was carried out in triplicate.

For comparison, TPC analysis was also performed in four commercial extracts: two originated from Europe namely REFRILIEF (EUC 1) and Biover (EUC 2) and two from Brazil, Natz (BRC 1) a green propolis extract and a handcrafted extract from Santo Antonio da Platina - Paraná, which mixes propolis and geopropolis (BRC 2).

#### 3.4.2 Total Flavonoids

The Total Flavonoids Content (TFC) of the selected propolis extracts was measured following the method of Woisky & Salatino (1998), with minor modification. This method is based on the formation of stable acid–complexes between aluminium chloride ($AlCl_3$) and the hydroxyl groups of flavonoids. A 2% (w/v) solution of $AlCl_3$ was prepared, as well as a quercetin solution standard curve used as a reference (linearity range = 2.50-40.0 µg/mL, $R^2$=0.998). Then, 100 µL of each sample followed by 100 µL of a 2% $AlCl_3$ solution were added to the microplate, incubated for 10 min at 25 °C in the dark, and the absorbance was measured at 415 nm. The same was made with the blank (ethanol 99.5% (v/v)) and the calibration curve. At the end, the flavonoid content was calculated considering the dilution factor and expressed as milligrams of quercetin

equivalents (QE) per mL of extract (mg QE/mL), per g of dry propolis extract (mg QE/g DPE), and per g of raw propolis (mg QE/g RP). The assay was carried out in triplicate, at two different microplates.

### 3.4.3 Antioxidant activity

The antioxidant capacity of selected extracts was determined using two assays: 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) (Re *et al*., 1999). The DPPH assay is based on the reduction of the stable free radical $DPPH^{\bullet}$ by antioxidant compounds, resulting in a color change from purple to yellow. Briefly, a DPPH solution (3.4 mg in 100 mL) and an ascorbic acid standard curve (0.488-15.625 µg/mL) were prepared. After, 50 µL of sample or standard and 250 µL of DPPH solution were added to the microplate. After incubation for 30 min in the dark at 25 ℃, the absorbance was measured at 517 nm.

For the ABTS assay, the radical cation $ABTS^{\bullet+}$ was generated by the oxidation of the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) with potassium persulfate ($K_2S_2O_8$), resulting in a dark green solution that becomes decolorized in the presence of antioxidant compounds. Briefly, an ABTS solution (3.8 mg/mL) was made in parallel with the ascorbic acid standard curve (0.488-15.625 µg/mL). Then, 50 µL of sample or standard and 250 µL of the ABTS solution were added to the microplate. After incubation for 20 min in the dark at 25 ℃, the absorbance was measured at 734 nm. The antioxidant activity, estimated by DPPH and ABTS radical scavenging assays, was calculated considering the dilution factor and expressed as milligrams of ascorbic acid equivalents (AAE) per mL of extract (mg AAE/mL), per g of dry propolis extract (mg AAE/g DPE), and per g of raw propolis (mg AAE/g RP). Both assays were carried out in triplicate, at two different microplates.

### 3.4.4 Antimicrobial activity

For antimicrobial susceptibility testing, the selected extracts were evaluated against the following Gram-negative bacteria: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica* (ATCC 13076); and the

following Gram-positive bacteria: *Staphylococcus aureus* (ATCC 25923), *Cutibacterium acnes* (ATCC 11827), *Staphylococcus epidermidis* (ATCC 35983). Finally, the extracts were also screened against fungi *Aspergillus fumigatus* (ATCC 204305) and *Aspergillus brasiliensis* (ATCC 16404) and the yeast *Candida albicans* (ATCC 90028). All microorganisms were purchased from Frilabo, Porto, Portugal.

The microorganisms were cultivated in specific culture medium and growth conditions: *S. aureus, C. acnes, S. epidermidis, S. enterica* and *C. albicans* were cultivated for 24 h at 37 ℃ on Columbia agar supplemented with 5% sheep blood, while *E. coli* and *P. aeruginosa* were cultivated on MacConkey agar at same conditions. The fungi were grown on malt agar for 72 h at 25 ℃.

The agar disk diffusion method followed the M02-A11 standard of the Clinical and Laboratory Standards Institute (CLSI). For the procedure, the concentration of each microorganism species suspension was normalized to an optical density of 0.5 point on the McFarland scale, corresponding to approximately $1 \times 10^8$ cells/mL, by measuring the absorbance at 600 nm, according to M7-A9 standard (CLSI). The microbial suspension was uniformly seeded on malt agar for fungi and Mueller–Hinton agar for others with a sterile swab. After complete absorption, disks soaked with the propolis extracts (PED) were placed in the middle of the plate. The same was performed with ethanol/water 70% (v/v) and monopropylene glycol as solvents controls (SCD). All samples were filtered with 0.22 µm nylon microfilter in order to minimize microorganisms contamination. The plates were incubated at 37 °C for a period of 24–48 h. Antimicrobial activity was evaluated by measuring the inhibition halos, in millimeters, of each microorganism.

In addition to the disk diffusion method, the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) was conducted using colorimetric assay with all microorganisms, according to M100 standard (CLSI). For the bacteria and yeast, the extracts ME70 23.1% (260 mg/mL) and MMPG 23.1% (288.5 mg/mL) and solvents CE70 70% and CMPG 100% samples were filtered with 0.22 µm nylon microfilter, in order to minimize microorganisms contamination. 90 µL of samples were added in the first well (96-well microplate) in duplicate with 100 µL of Tryptic Soy Broth (TSB). In the remaining wells 90 µL of TSB medium were added. Then the samples were

serial diluted to obtain the concentration ranges. After, 10 µL of inoculum (standardized at 1 × $10^5$ Colony Forming Unit (CFU) /mL) was added at all the wells assuring the presence of 1 × $10^5$ CFU. Two negative controls were prepared, one with TSB and another one with the extract. Three positive controls were prepared with TSB and each inoculum and another with medium, antibiotics, and bacteria. Ampicillin (20 mg/mL), an aminopenicillin, was used for all bacteria, while Streptomycin (1 mg/mL), an aminoglycoside, was used mainly for Gram-negative bacteria and Methicillin (1 mg/mL), a penicillin, for *Staphylococcus spp*. The microplates were incubated at 37 ℃ for 24 h. The MIC of samples was detected following addition (40 µL) of 0.2 mg/mL *p*-iodonitrotetrazolium chloride (INT) and incubation at 37 ℃ for 30 min. MIC was defined as the lowest concentration that inhibits the visible bacterial growth by changing the coloration from yellow to pink if the microorganisms are viable. For the determination of MBC, 10 µL of liquid from each well that showed no change in color was plated on solid medium, Blood agar (7% sheep blood) and incubated at 37 ℃ for 24 h; exception to *C. acnes*, *S. epidermidis* and *C. albicans* were incubated at 37 ℃ for 48 h. The lowest concentration that yielded no growth determined the MIC. MBC was defined as the lowest concentration required to kill bacteria.

For the antifungal activity assay, fungal spores were washed from the surface of agar plates with 0.85% sterile serum containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0 x $10^5$ CFU to a final volume of 100 µL per well. Next, 10 µL of each extract and solvents samples was added in duplicate to the first well (96-well microplate), followed by 190 µL of malt extract medium. 90 µL of malt extract medium was added to the remaining wells. Extracts and solvents samples were then serial diluted to obtain a concentration range. MIC were determined by serial dilution in 96-well microplates. Minimum fungicidal concentrations (MFCs) were determined by serial subculturing 2 µL of the extracts dissolved in the medium and inoculating them into microplates containing 100 µL of MEB per well for 72 hours, followed by incubation at 26 ℃ for 72 h. The MFC indicate 99.5% kill of the original inoculum. The commercial fungicide ketoconazole (Frilabo, Porto, Portugal) was used as a positive control.

### 3.4.5 UHPLC-DAD-ESI/MS analysis

In addition to the analyzes described before, the selected extracts (ME70 and MMPG) were filtered through a 0.22 µm nylon membrane (Whatman) and analyzed by Ultra-High Performance Liquid Chromatography coupled to Diode Array Detection and Electrospray Ionization Mass Spectrometry. The UHPLC-DAD-ESI/MS analysis was performed on an Ultimate 3000 (Dionex Co., Sunnyvale, CA, USA) apparatus equipped with an autosampler/injector, a binary pump, a column compartment, and an ultimate 3000 Diode Array Detector (Dionex Co.) coupled to a mass spectrometer LTQ XL Linear Ion Trap equipped with an ESI source. The separation was conducted on a Hypersil Gold (Thermo Scientific, Waltham, MA, USA) C18 column (100 mm length; 2.1 mm i.d.; 1.9 m particle diameter, end-capped) and its temperature was maintained at 30℃. The mobile phase was composed of (A) 0.1% of formic acid (v/v) and acetonitrile (B). The solvent gradient started with 20% of solvent (B), reaching 40% at 25 min, 60% at 35 min, and 90% at 50 min, followed by the return to the initial conditions. The flow rate was 0.2 mL/min and the UV-Vis spectral data for all peaks were accumulated in the range of 200-700 nm while the chromatographic profiles were recorded at 280 nm. Control and data acquisition were carried out with the Thermo Xcalibur Qual Browser data system (Thermo Scientific). Nitrogen above 99% purity was used, and the gas pressure was 520 kPa (75 psi). The instrument was operated in negative-ion mode with ESI needle voltage set at 5.00 kV and an ESI capillary temperature of 275℃. The full scan covered the mass range from m/z 100 to 2000. All solvents used were of LC-MS grade (Caetano *et al*., 2023).

### 3.5 Liquid soap formulation

Before preparing the propolis soaps, a preliminary neutral sample was prepared beforehand, considering the indicative of Borković *et al.* (2024), with slight modifications. By formulation, the resulting volume was 60 mL, because of the amount of water and reagents.

Subsequently, five different formulations were prepared: two formulations with propolis extracts (PE5 and PG5), two with the solvents (E5 and G5) and a neutral (E0).

PE5 and PG5 were prepared with 5% ethanolic and glycolic propolis extract, respectively, while E5 was formulated with 5% of 70% ethanol and G5 with 5% MPG. E5 and G5 were used as control for the propolis samples, while E0 was a control for the solvent samples. The percentages of propolis extract in the soap were considered in relation to the total volume of the preliminary formulation. The soap preparation was conducted by mixing the SLES, CB and water, using a magnetic agitation, followed by the propolis extract/solvent and sodium chloride. At the end, the pH was standardized at 6.0 ± 0.1 using a pH meter, directly applied to the final formulation. The pH adjustment was performed using citric acid solutions to acidify, and sodium hydroxide to basicize. The Table 3 presents the components used in each formulation.

Table 3 - Formulation of liquid soap preparations.

| Materials | E0 | E5 (5%) | G5 (5%) | PE5 (5%) | PG5 (5%) |
|---|---|---|---|---|---|
| Sodium lauryl ether sulfate (SLES) (28%) | 17 g | 17 g | 17 g | 17 g | 17 g |
| Cocamidopropyl betaine (CB) | 2.5 g | 2.5 g | 2.5 g | 2.5 g | 2.5 g |
| Ethanolic propolis extract | - | - | - | 3 mL | - |
| Glycolic propolis extract | - | - | - | - | 3 mL |
| Ethanol 70% | - | 3 mL | - | - | - |
| Monopropylene glycol | - | - | 3 mL | - | - |
| Sodium chloride | 2g | 2 g | 2 g | 2 g | 2 g |
| Citric Acid (50%) | 0.2 mL | 0.15 mL | 0.15 mL | - | - |
| Sodium hydroxide (10%) | - | - | - | 1.25 mL | 1.35 mL |
| Distilled water | 45 mL | 39 mL | 39 mL | 39 mL | 39 mL |

Source: Author. Note: E0: Neutral formulation; E5: Formulation with 5% of Ethanol 70% (v/v); G5: Formulation with 5% MPG; PE5: Formulation with 5% of ME70; PG5: Formulation with 5% of MMPG; - Ingredient not used.

SLES, an anionic surfactant, CB, an amphoteric surfactant, and sodium chloride were responsible to form the basic composition of the liquid soap and give the thickening, foam reinforcement and emulsification characteristics (Borković *et al.*, 2024). Citric acid and sodium hydroxide were pH regulators, distilled water was the diluent, and propolis extract was a possible natural antiseptic agent. The incorporation of propolis extract as a common ingredient in the labelling of cosmetic products was supported by Decision (EU) 2022/677 concerning Regulation (EC) No 1223/2009.

### 3.6 Antimicrobial activity of the liquid soap

The samples were left to rest for 24 hours in the dark, at room temperature, after the day of formulation. So, the antimicrobial susceptibility of samples was assessed by the same method described in topic 3.4.4, aiming to evaluate the comparative efficiency between propolis extract and the final product with propolis extract incorporated. This resting period was important so that the gas incorporated into the formulation was released gradually and did not interfere with the analyses.

### 3.7 Stability assays of the liquid soap formulations

According to Good Manufacturing Practices (GMP), ISO 22716, and Regulation (EC) No 1223/2009 on cosmetic products, the safety and quality of cosmetic formulations must be ensured through appropriate testing procedures capable of demonstrating compliance with established acceptance criteria. Accordingly, the liquid soap formulations were subjected to a two-phase stability evaluation protocol, with all tests performed and analyzed in duplicate.

Phase 1 consisted of preliminary physical stability tests, including centrifugation and mechanical vibration, to evaluate formulation homogeneity and resistance to phase separation. Only formulations that successfully passed these initial tests proceeded to Phase 2. Phase 2 involved accelerated stability testing under different stress conditions for 15 days, including exposure to light, extreme temperatures, and freeze–thaw cycles. During these tests, the formulations were periodically evaluated through physicochemical analyses, namely pH, density, viscosity, and colorimetric

measurements. These conditions were selected to simulate potential transport and storage environments that could compromise product stability and integrity (ANVISA, 2004; Borković et al., 2024; Idawati et al., 2023).

For each formulation, 1 L of product was prepared using the formulation in Table 3 and distributed into 50 mL plastic bottles. Packaging conditions followed the recommendations established by ANVISA (2004), including the maintenance of approximately one-third headspace in each bottle to allow possible gas exchange, the use of properly sealed containers, and storage in opaque bottles, except for samples intended for light exposure testing. Independent bottles were assigned to each evaluation day, ensuring that samples designated for later analyses (e.g., day 15) remained undisturbed under their respective test conditions throughout the entire experimental period.

### 3.7.1 Centrifugation test

To verify the formulation stability, 1 g of soap formulation was submitted to a cycle of 3,000 rpm for 30 min at room temperature. At the end of the centrifugation period, the samples were examined for phase separation, precipitation, caking or coalescence, which is an indication of cosmetic formulation instability. The assay was conducted in triplicate.

### 3.7.2 Mechanical vibration test

To simulate transport conditions, 1 g of samples were subjected to vibration in a vortex mixer for 10 seconds at 2500 rpm. The samples were then examined to check for phase separation.

### 3.7.3 Light test

To evaluate the appearance, transparency and color of the formulation, samples were placed in transparent plastic containers and exposed to artificial daylight lamps under a photoperiodic system (16 hours of light and 8 hours of darkness) for 15 days. Every five days, a sample was taken for physicochemical analysis.

### 3.7.4 Extreme temperatures test

All formulations were subjected to extreme temperatures tests of 45 ± 2 ℃ and 5 ± 2 ℃ for 15 days to verify the stability, without humidity standard. Every five days, samples were taken from extreme conditions and physicochemical analysis was performed at room temperature (ANVISA, 2004).

### 3.7.5 Freeze-thaw cycle test

For the freeze-thaw cycle test, all samples were freeze at temperatures of -20 ℃ ± 2 ℃ for 24 h and thawed at room temperature for 24 h. After the 48-hour test period, a cycle was completed and samples were collected and analyzed at room temperature (ANVISA, 2004).

### 3.7.6 pH determination

The pH values were measured by diluting the liquid soap in distilled water at a concentration of 10% (w/v) and stored for 1 h (ANVISA, 2004). The assay was conducted in a duplicate and average values were calculated.

### 3.7.7 Density

The density of the samples was measured at room temperature, in terms of grams per milliliters, using the Equation (E2):

$$\rho = \frac{m}{V} \qquad \text{(E2)}$$

The analysis was made in triplicate, using 2 g of sample and a graduated cylinder. The average values were calculated.

### 3.7.8 Viscosity

The viscosity of the formulations was measured in a Rotational Viscometer, using around 25 mL of sample and L4 spindle with different rotations speeds (6, 10, 12, 20, 30, 50, 60, 100 and 200 rpm) in duplicate. The temperature was controlled at 25 ± 2 ℃. This parameter determines if a product has the appropriate consistency or fluidity, ease of application, the feel to the touch and it can indicate if the stability is adequate (Oliveira, 2025; ANVISA, 2004).

### 3.7.9 Colorimetric test

The color of the formulations was followed by a scanning spectrophotometric analysis, in the visible region (300 nm - 700 nm), using a scan step of 10.0 nm. Their spectrum were compared to control sample spectrums at the same conditions of stability tests. Variation on the intensity or wavelength of the absorption bands was considered as formulation instability, since that indicates modifications in intensity of the color or coloring material (ANVISA, 2004).

## 3.8 Statistical analysis

Results are shown as arithmetic mean values ± standard deviation. In each assay, the differences results in triplicate were analyzed using Dixon's Q Test, to identify and reject outliers, followed by analyzes of variance (ANOVA) and Tukey's HSD between different samples. *p*-values less than 0.05 were considered statistically significant. This treatment was conducted using SASM-Agri v. 8.2 program (Canteri, *et al.*, 2001).

# 4.RESULTS AND DISCUSSION

## 4.1 Raw propolis characterization

The botanical diversity of propolis implies variations in the physical and chemical characteristics of the raw matrix, which can also be influenced by environmental conditions and bee species. Furthermore, the process of collecting and handling propolis alters its quality and, consequently, its commercial value. Therefore, several analyzes are used to control the standard of raw propolis and its extracts (Rodriguez-Canales *et al*., 2023; Barreto *et al.*, 2020). Table 4 presents the values of loss on drying, ash and wax content, which are three of the physicochemical parameters requested to class the raw propolis by quality. As standard comparation, the Embrapa (Brazilian Agricultural Research Corporation) values were taken from due to the great importance of Brazilian propolis in terms of quantity, variety and quality (Barreto *et al.*, 2020), and also due to the lack of specific regulations for Portuguese propolis.

Table 4 - Physicochemical analyses results of raw propolis.

| Parameter | Propolis from Montesinho Natural Park | Embrapa (2020) |
|---|---|---|
| Loss on drying (%) | 5.4 ± 0.1 | < 8.0 |
| Ash content (% DW) | 0.8 ± 0.0 | < 5.0 |
| Wax content (% DW) | 7.3 ± 0.7 | < 25.0 |

Source: Author. Note: DW: Raw propolis dry weight. Embrapa: Brazilian Agricultural Research Corporation.

The loss on drying determines the humidity and high values of it would benefit the growth of fungus, so the accepted limit is less than 8% of humidity in the raw matrix. Ash content indicates the presence of mineral components, which high levels could signify adulteration or contamination in the collection process, thus only less than 5% are accepted by Barreto *et al.*(2020).

Beeswax is naturally produced by bees and incorporated into propolis. High levels of wax imply low levels of resins and bioactive components, since bees have had to prioritize wax production due to the scarcity of plant resins in nature. Therefore,

lower percentages of beeswax in propolis are desirable and tolerated up to 25% (Barreto *et al.*, 2020).

The humidity, ash and wax content determined for the propolis from Montesinho Natural Park were in agreement with the parameter established for propolis control quality, resulting in 5.4 %, 0.8 % DW and 7.3 % DW, respectively.

### 4.2 Extraction kinetics

Polyphenols dominate the bioactive fraction present in propolis and are critically linked to its biological activities. Due to the rich polyphenolic content of propolis that exhibits significant antimicrobial activity (Balasubramaniam *et al*., 2025), the present work determined total phenolic content as a key parameter to extraction kinetics. Different methods and solvents were tested, due their significant influence in bioactive extraction (Balasubramaniam *et al*., 2025; Freitas *et al.*, 2019).

Figure 5 shows the extraction kinetics of propolis obtained by the maceration method using five different solvents. Aliquots were collected at each 48 h throughout the extraction process and analyzed for total phenolic content to monitor the extraction progress and construct the kinetic curves.

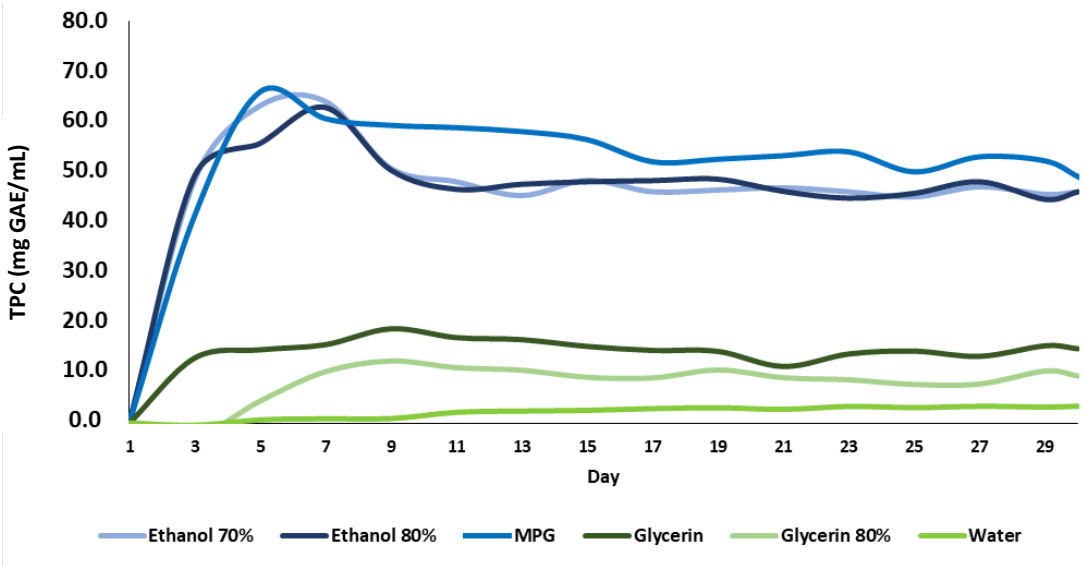



**Figure 5.** *Extraction kinetics of Total Phenolic Content by maceration method.*

Source: Author. Note: MPG: Monopropylene glycol; GAE: Gallic acid equivalents.

Higher concentrations of phenolics were extracted at least until the ninth day for all solvents. The maximum value obtained for each condition is described in Table 5. To determine it, statistical analysis was made between the triplicated values of its extraction day. In sequence, significant differences were observed among extracts ($p < 0.05$).

Table 5 – Maximum values of Total Phenolic Content by Maceration.

| Sample | Time | TPC (mg GAE/g RP) | TPC (mg GAE/mL) | TPC (mg GAE/g DPE) |
|---|---|---|---|---|
| ME70 | 5 days | 237.329 ± 18.573[a] | 63.064 ± 4.935[a] | 476.575 ± 37.090[a] |
| ME80 | 7 days | 243.319 ± 38.756[a] | 62.746 ± 9.994[a] | 409.210 ± 65.179[b] |
| MMPG | 5 days | 211.142 ± 18.525[a] | 65.876 ± 5.780[a] | 433.396 ± 14.952[ab] |
| MGA | 9 days | 48.810 ± 10.238[b] | 18.743 ± 3.931[b] | - |
| MGA80 | 9 days | 32.589 ± 2.092[bc] | 12.319 ± 0.791[bc] | - |
| MA | 30 days | 11.433 ± 0.735[c] | 3.419 ± 0.220[c] | 0.0 ± 0.0[c] |

Source: Author. Note: Different lowercase letters between lines indicate significant differences ($p < 0.05$); - Not determined; RP: Raw Propolis; DPE: Dry Propolis Extract; GAE: Gallic acid equivalents.

The TPC results were expressed on three distinct bases, namely in volumetric (mg GAE/mL), raw propolis terms (mg GAE/g RP) and dry propolis extract (mg GAE/g DPE). The evaluation in terms of raw matrix was chosen in order to evaluate the efficiency of extraction, because as higher the TPC values per gram of propolis were obtained, less raw material would be used, thus optimizing the process. In maceration, ethanol 70% (ME70), ethanol 80% (ME80) and MPG (MMPG) were the most promising solvents, since the greater comparative efficiency of extraction (ranging between 211.142 ± 18.525 and 243.319 ± 38.756 mg GAE/g RP). Even if ME70, ME80 and MMPG did not present statistical difference, values of ethanolic extracts were better than the glycolic.

Nevertheless, results expressed per unit of dry propolis extract provide an indication of richness, with higher values reflecting a greater concentration of total

phenolic compounds (TPC). Due, ME70 was the purest extract, reaching 476.575 ± 37.090 mg GAE/g DPE, with no statistical similar results. Therefore, ME70 results suggested that ethanol 70% was the solvent with the higher extraction efficiency of TPC from propolis, as well as was the solvent which demonstrated the higher richness in TPC when compared to the other solvents.

In parallel with maceration, the extraction kinetics of the propolis by ultrasound method was made, aiming to verify if percentage extraction of phenolic would be better (De Santana *et al.*, 2024; Silva *et al.*, 2024). The results were plotted in Figure 6. All samples were analyzed by the same phenolic assay as maceration to construct the curve, with the exception of glycerin, which could not be sonified due to its viscosity.

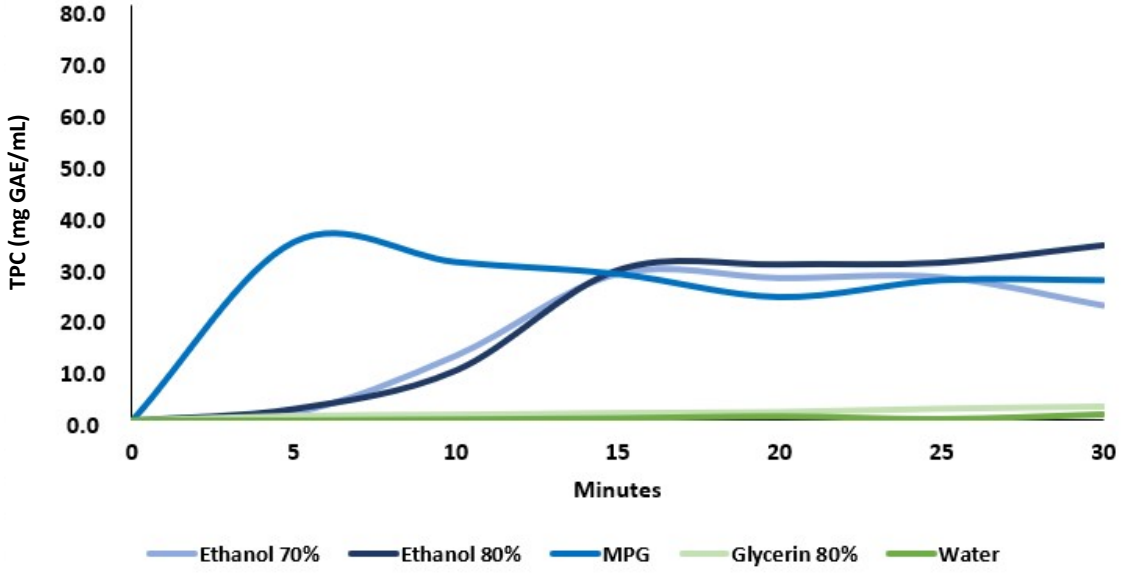



**Figure 6.** *Extraction kinetics of Total Phenolic Content by ultrasound method.*

Source: Author. Note: MPG: Monopropylene glycol; GAE: Gallic acid equivalents.

The maximum TPC recovered from the extraction kinetic curves for ultrasound-assisted extraction (UAE) is presented in Table 6, and statistical analyzes were conducted using the same protocol as in Table 5. From Figure 6, it is possible to verify that phenolic extraction with MPG (UMPG), was the fastest, reaching the peak in 5 minutes, while 70% (UE70) and 80% ethanol (UE80) reached the peak 10 minutes later.

In the UAE, the most promising extracts were UE70, UE80 and UMPG, with results ranging from 105.928 ± 22.481 to 112.066 ± 21.734 mg GAE/g RP. However, the

efficiency achieved in maceration extraction, for the same solvents, was almost double that of the UAE, possibly due to the extraction method mechanism, which facilitates solvent permeability through the cell wall and removal of active ingredients (Amalia et al., 2025; Chacon et al., 2025; Saqib Farooq et al., 2022), which points to the traditional method as the most promising. In terms of purity, sample UE70 showed the best result, with 234.563 ± 49.782 mg GAE/g DPE, although UE80 was more efficient (112.066 ± 21.734 mg GAE/g RP).

Table 6 - Maximum values of Total Phenolic Content by Ultrasound.

| Sample | Time | TPC (mg GAE/g RP) | TPC (mg GAE/mL) | TPC (mg GAE/g DPE) |
|---|---|---|---|---|
| UE70 | 15 min. | 105.928 ± 22.481[a] | 28.148 ± 5.974[a] | 234.563 ± 49.782[a] |
| UE80 | 15 min. | 112.066 ± 21.734[a] | 28.899 ± 5.605[a] | 154.815 ± 30.024[b] |
| UMPG | 5 min. | 109.776 ± 5.830[a] | 34.250 ± 1.819[a] | - |
| UGA | - | - | - | - |
| UGA80 | 30 min. | 7.321 ± 0.392[b] | 2.767 ± 0.148[b] | - |
| UA | 30 min. | 3.549 ± 0.298[b] | 1.061 ± 0.089[b] | 23.763 ± 1.994[c] |

Source: Author. Note: Different lowercase letters between lines indicate significant differences ($p < 0.05$); - Not determined; RP: Raw Propolis; DPE: Dry Propolis Extract; GAE: Gallic acid equivalents.

A comparison of propolis extract obtained using the two extraction methods revealed that, for the same solvent system, maceration generally yielded higher total phenolic content (mg GAE/mL) than ultrasound-assisted extraction (UAE)(Figure 7). De Santana *et al.* (2024) and Silva *et al.* (2024) indicate the opposite was expected, since greater penetration and better extraction of bioactive compounds by the UAE method. This discrepancy may indicate that the chosen extraction parameters may not have been the most suitable, in terms of sonication amplitude, concentration, temperature, or even extraction time. In future, lower concentrations and ranges between 70% and 80% could be assessed, since Akhan *et al.* (2025) obtained better TPC extraction yields in less

time. Thus, based on the extraction kinetics curves of the two methods, the most promising solvents for TPC extraction were established as ethanol 70% by maceration (237.329 ± 18.573 mg GAE/g RP), ethanol 80% (243.319 ± 38.756 mg GAE/g RP) and MPG (211.142 ± 18.525 mg GAE/g RP).

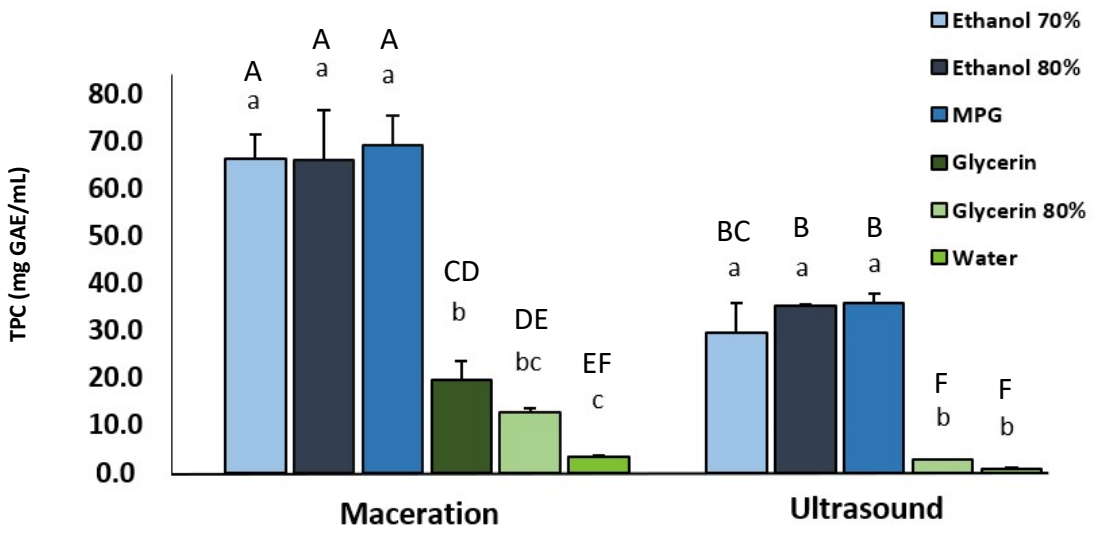



**Figure 7.** *Total Phenolic Content by extraction methods.* Source: Author. Note: Different capital letters between solvents indicate significant differences ($p < 0.05$); Different lowercase letters indicate statistical differences between solvents within the same extraction process ($p < 0.05$); MPG: Monopropylene glycol; GAE: Gallic acid equivalents.

Overall, the maximum value obtained was 243.319 ± 38.756 mg GAE/g RP (for ME80). However, Silva *et al.* (2012) reported superior values for propolis from Bragança (277.17 mg GAE/g of RP), using 80% of ethanol at 70 °C. Their work compared phenolic compound extraction of Portuguese poplar propolis from Beja, Bragança and Coimbra and achieved values of 277.17 mg GAE/g of RP for propolis from Bragança, followed by Coimbra and Beja, with 157.31 mg GAE/g of RP and 87.15 mg GAE/g of RP, respectively. In a global perspective, the TPC results obtained in this study were distinguished, since Western Australian propolis had results ranging from 9.26 to 59.3 mg GAE/g RP, Azerbaijan ranging from 10.94 to 79.86 mg GAE/g RP and Malaysia with 28.09 mg GAE/g RP (Achenbach *et al.*, 2024). However, the values obtained were lower than those reported for Brazilian propolis (approximately 324 mg GAE/ g RP), which is among the

most extensively studied type of propolis worldwide (De Santana *et al.*, 2024; Reis *et al*., 2019).

Although Silva *et al.* (2024) reported TPC values of approximately 217.7 mg GAE/g DPE for propolis from Bragança, with UAE extraction in water at 25 ℃, and Paula *et al.* (2023) reported 210 ± 133 mg GAE/g DPE for propolis from Montesinho Natural Park, using absolute ethanol in agitation overnight, the ME70 and ME80 extracts obtained in this study exhibited markedly higher phenolic contents (476.575 ± 37.090 and 409.210 ± 65.179mg GAE/g DPE, respectively). The variation in total phenolic compound (TPC) content can be attributed to differences in the extraction method, parameters (and their combinations), such as solvents, temperature, and concentration ratio since specific combinations of extraction parameters can sometimes have adverse effects. Another possibility could be the seasonal changes and collection time, which provokes variation in the bioactivity of propolis (Balasubramaniam *et al*., 2025).

Considering that ME70, ME80 and MMPG were the best profiles and were statistically equivalent in terms of raw propolis (mg GAE/g of RP) and milliliters (mg GAE/mL), for the liquid soap formulation, only ME70 and MMPG extracts were selected as the most promising propolis extracts. The exclusion of sample ME80 was justified by the equivalence of the results and its ethanol concentration for extraction, since the greater the presence of solvent, the higher the associated costs and, possibly, greater difficulties in achieving cosmetic stability.

### 4.3 Comparison with commercial extracts

The market for natural products grows every year, and the use of propolis extracts has increased in the pharmaceutical, cosmetic and personal hygiene industries. In this scenario, meeting the demands of a highly competitive global market has been a constant necessity, with Brazilian propolis standing out as a powerful essential bioactive natural matrix (Embrapa, 2020). Thus, to contextualize the performance of the extracts obtained in this study, commercially available propolis extracts, both Brazilian (BRC1 and BRC2) and European (EUC1 and EUC2), were acquired, and their total phenolic compound (TPC) content was measured and compared (Table 7).

BRC 1 was a Brazilian green propolis sample, which is currently highly valued for its phenolic compounds, especially its richness in artepillin C (Zelca *et al.*, 2022). Therefore, considering its concentrations, MMPG and ME70 had high-ranking content of TPC when compared to commercial samples selected. This suggests that the propolis extracts obtained in this study could be strong competitors in the international propolis market.

Table 7 - Total Phenolic Content in MMPG and ME70 and commercial extracts.

| Samples | Extract concentration (mg/mL) | Extraction solvent | TPC (mg GAE/mL) |
|---|---|---|---|
| MMPG | 288.5 | MPG | $65.876 \pm 5.780^{a}$ |
| ME70 | 260.0 | Ethanol 70% | $63.064 \pm 4.935^{a}$ |
| BRC 1 | 110.0 | Ethanol 96% | $20.530 \pm 1.284^{b}$ |
| BRC 2 | - | - | $10.856 \pm 0.100^{c}$ |
| EUC 1 | 333.3 | - | $2.273 \pm 0.191^{d}$ |
| EUC 2 | 620.0 | - | $0.471 \pm 0.029^{d}$ |

Source: Author. Note: Different lowercase letters between lines indicate significant differences ($p < 0.05$) EUC: European commercial sample; BRC: Brazilian commercial sample; GAE: Gallic acid equivalents; - Not indicated in label.

### 4.4 Chemical and bioactivity characterization of the selected propolis extracts

#### 4.4.1 Total flavonoids and antioxidant activity

To use propolis as a functional ingredient, it must first undergo several cleaning processes to remove waxes and impurities, and subsequently extract the polyphenols (Pant *et al.*, 2024; Zhang *et al*., 2023). So, for liquid soap formulation, the propolis was extracted a second time, at the same parameters verified in kinetics (5 days, maceration extraction), and the phenolics content were confirmed. In addition, total flavonoids concentration and antioxidant activity was quantified (Table 8).

Table 8 - Chemical characterization of selected propolis extracts.

| Analyzes | ME70 | MMPG |
|---|---|---|
| **Total Flavonoids** | 18.419 ± 0.787$^{B}$ mg QE/mL | 23.497 ± 1.288$^{A}$ mg QE/mL |
| | 69.318 ± 2.962$^{B}$ mg QE/g RP | 75.310 ± 4.127$^{A}$ mg QE/g RP |
| | 111.161 ± 4.750$^{B}$ mg QE/g DPE | 154.923 ± 8.490$^{A}$ mg QE/g DPE |
| **DPPH scavenging activity** | 78.100 ± 2.650$^{B}$ mg AAE/mL | 88.418 ± 1.748$^{A}$ mg AAE/mL |
| | 293.917 ± 9.974$^{A}$ mg AAE/g RP | 283.390 ± 5.604$^{B}$ mg AAE/g RP |
| | 417.335 ± 15.995$^{B}$ mg AAE/g DPE | 582.974 ± 11.528$^{A}$ mg AAE/g DPE |
| **ABTS scavenging activity** | 75.443 ± 3.892$^{B}$ mg AAE/mL | 86.092 ± 2.362$^{A}$ mg AAE/mL |
| | 283.919 ± 14.647$^{A}$ mg AAE/g RP | 275.937 ± 7.572$^{B}$ mg AAE/g RP |
| | 455.302 ± 23.489$^{B}$ mg AAE/g DPE | 567.641 ± 15.577$^{A}$ mg AAE/g DPE |

Source: Author. Note: Different capital letters between columns indicate significant differences ($p < 0.05$); RP: Raw Propolis; DPE: Dry Propolis Extract; GAE: Gallic acid equivalents; AA: Ascorbic Acid equivalents; QE: Quercetin equivalents; ME70: Propolis Maceration Ethanol 70% (v/v); MMPG: Propolis Maceration Monopropylene glycol.

Propolis is particularly rich in flavonoids, which is one of the polyphenols closely associated with pharmacological properties, including antimicrobial capacity, thus, the flavonoids and antioxidant activity were examined. Antioxidant activity was measured by two methods, DPPH and ABTS, to confirm the veracity of results. So, as expected, DPPH and ABTS assays showed similar values for both extracts.

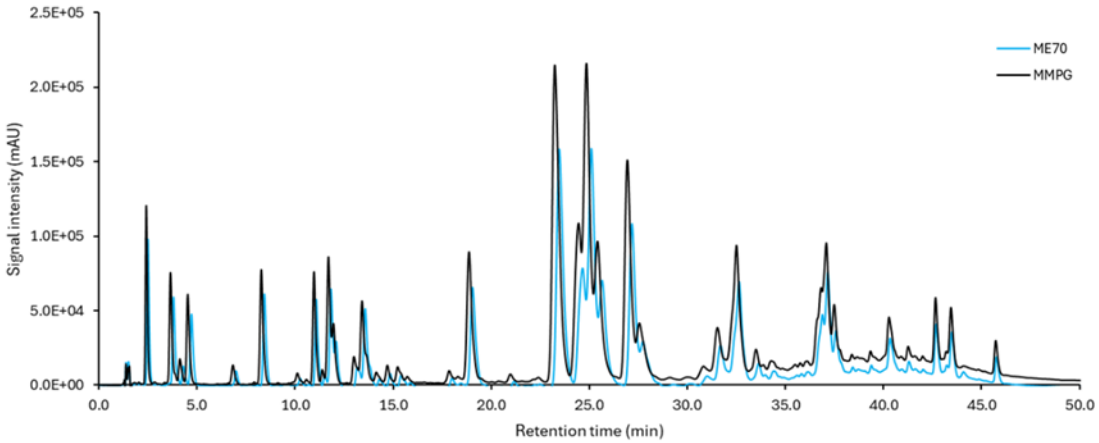
Even though the total polyphenol and flavonoid content obtained stands out in relation to propolis from Poland, Turkey, Uruguay and Romania, which ranged between 85 and 155 mg GAE/g DPE and from 7 to 60 mg QE/g DPE (Kurek-Górecka *et al.*, 2022), Falcão *et al.* (2013a) obtained higher flavonoid values for propolis originating from the northern region of Portugal, ranging from 87 to 460 mg QE/g DPE. However, the propolis extract from Gerês showed similar results to the samples in terms of total phenolic and flavonoid content (Balasubramaniam *et al.*, 2025; Oliveira, R. D., Araújo, C., & Almeida-Aguiar, C., 2024).

The Portuguese propolis is a powerful antioxidant agent, due its important source of total phenols, flavones, and flavonols (Silva-Carvalho, R., Baltazar, F., & Almeida-Aguiar, C., 2015). The antioxidant activity of ME70 and MMPG was superior to that of Chinese propolis, which presented approximately 270 mg of AAE/DPE (Zhu *et al.*, 2025; Hwang, S. J., & Lee, J. H., 2023), however were inferior when compared to propolis from Gerês and Bornes (Freitas *et al.*, 2019; Moreira, L., Dias, L. G., Pereira, J. A., & Estevinho, L., 2008).

The MMPG extract showed greater statistical significance for almost all parameters analyzed, indicating that monopropylene glycol was the best solvent in terms of extraction of total phenolics and total flavonoids, as well as antioxidant activity. Although MMPG was a distinct extract, the ME70 extract was also incorporated into the liquid soap, since the stability of the samples could vary in the formulation.

### 4.4.2 UHPLC-DAD-ESI/MS analysis

The geographical origin of propolis determines the type of phenolic compounds present, due to the plant resin collected by bees. Polyphenols are secondary plant metabolites, and flavonoids and phenolic acids dominate the chemical composition of propolis and are critically linked to its biological activities. Therefore, the determination of phenolic composition of each propolis extract was necessary (Balasubramaniam *et al.*, 2025; Kurek-Górecka *et al.*, 2022).



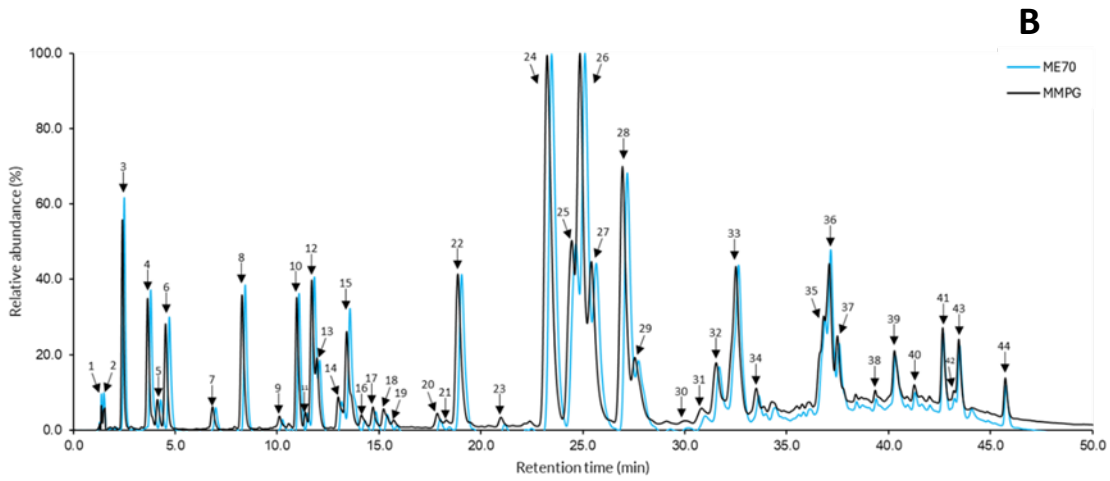



**Figure 8.** *Chromatographic profiles of propolis.* Source: Author. Note: A - UHPLC-DAD-ESI/MS chromatogram (signal intensity); B - UV profile expressed in relative abundance (%); Peaks marked with numbers correspond to the tentatively identified compounds presented in Table 9.

The UHPLC-DAD-ESI/MS chromatographic profiles of the most promising propolis extracts, namely the ethanolic (ME70) and monopropylene glycol (MMPG) extracts, are presented in Figure 8. The signal intensity chromatograms (Figure 8-A) display a complex peak distribution typical of phenolic-rich matrices in both extracts. Similarly, the UV profiles expressed as relative abundance (Figure 8-B) reveal a highly comparable qualitative composition, with no significant variation in compound distribution.

A total of 44 compounds were tentatively identified in both extracts (Table 9), reflecting the chemical complexity of the samples studied. In general, the bioactive fraction of both extracts was predominantly composed of flavonoids and phenolic acids, including several methylated and esterified derivatives. This composition is consistent with the typical profile of propolis from temperate regions, commonly associated with *Populus* species as the main botanical source (Balasubramaniam *et al.*, 2025; Freitas *et al.*, 2022a).

A detailed analysis of the chromatographic data in Table 9, allowed the identification of several phenolic acids, including caffeic acid (Compound 3), p-coumaric acid (Compound 4), ferulic acid (Compound 6), and cinnamic acid (Compound 12), as well as their corresponding derivatives, such as p-coumaric acid methyl ester

(Compound 13). These compounds are widely reported as characteristic constituents of European propolis and are known to contribute to its biological properties, particularly antioxidant and antimicrobial activity (Balasubramaniam *et al.*, 2025; De Carli *et al.*, 2022; Kurek-Górecka *et al.*, 2022; Rojczyk *et al.*, 2020).

In addition, a wide range of flavonoids was identified, including flavones such as apigenin (Compound 14) and chrysin (Compound 24), flavanones such as naringenin (Compound 15) and pinocembrin (Compound 26), and flavonols such as quercetin (Compound 9) and galangin (Compound 27). Several methylated and esterified derivatives were also detected, particularly related to pinobanksin (Compounds 10 and 21) and pinocembrin derivatives (Compounds 17 and 30). These compounds are characteristic of "poplar-type" propolis commonly found in temperate regions and are widely reported in European samples, being strongly associated with plant exudates from *Populus* species (Freitas *et al.*, 2022a; Kurek-Górecka *et al.*, 2022; Falcão *et al.*, 2013a; Falcão *et al*., 2013b; Kurek-Górecka *et al.*, 2013). The identified phenolic acids and flavonoids are well known for their antioxidant properties, which agrees with the results obtained in the spectrophotometric assays. Although the chromatograms revealed a highly similar qualitative composition between ME70 and MMPG extracts, some differences in absolute signal intensity were observed, particularly for the major peaks detected between 23 and 28 min, which tended to be more intense in the MMPG extract (Figure 8-A). These differences may reflect variations in extraction yield or compound concentration. However, after normalization of peak areas, the relative abundance profiles (Figure 8-B) remained highly comparable, indicating that both solvents extracted essentially the same set of phenolic compounds and preserved the overall phenolic fingerprint of the propolis sample.

Overall, these results demonstrate that propolis from Montesinho Natural Park exhibits a phenolic profile characteristic of European "poplar-type" propolis, with the extraction solvent having a limited impact on the qualitative composition of the extracts. This suggests that the choice of solvent may be guided primarily by practical considerations, such as extraction efficiency or intended application, rather than by selectivity towards specific phenolic compounds. Furthermore, the consistent chemical

profile observed across both extracts reinforces the robustness of the identified phenolic fingerprint as a representative signature of this propolis sample.

Table 9 - Tentative assignment of propolis extracts by UHPLC-DAD-ESI/MS.

| Peak number | Retention time (min) | $UV_{max}$ (nm) | $[M-H]^-$ (m/z) | Probable Compound |
|---|---|---|---|---|
| **1** | 1.3 | 281 | 133 | Malic acid |
| **2** | 1.5 | 253 | 137 | 4-hydroxybenzoic acid |
| **3** | 2.4 | 233, 295, 323 | 179 | Caffeic acid |
| **4** | 3.6 | 309 | 163 | *p*-coumaric |
| **5** | 4.0 | 294, 322 | 477 | Calceolarioside A/B |
| **6** | 4.4 | 298, 323 | 193 | Ferulic acid |
| **7** | 6.7 | 229 | 121 | Benzoic acid |
| **8** | 8.2 | 237, 292, 321 | 207 | 3,4-dimethyl-caffeic acid |
| **9** | 10.0 | 254, 368 | 301 | Quercetin |
| **10** | 10.9 | 287 | 285 | Pinobanksin-5-methyl-ether |
| **11** | 11.3 | 253, 343 | 315 | Quercetin-3-methyl-ether |
| **12** | 11.6 | 277 | 147 | Cinnamic acid |
| **13** | 11.8 | 307 | 177 | *p*-coumaric acid methyl ester |
| **14** | 12.9 | 267, 336 | 269 | Apigenin |
| **15** | 13.3 | 291 | 271 | Naringenin |
| **16** | 14.0 | 255, 368 | 315 | Isorhamnetin |
| **17** | 14.6 | 233, 286 | 269 | Pinocembrin-5-methyl ether |
| **18** | 15.1 | 266, 351 | 299 | Luteolin-5-methyl ether |
| **19** | 15.6 | 253, 354 | 329 | Quercetin-dimethyl ether (isomer) |
| **20** | 17.7 | 261, 300, 352 | 283 | Galangin-5-methyl ether |
| **21** | 18.2 | 288 | 327 | Pinobanksin-5-methyl-ether-3- |

| | | | | |
|---|---|---|---|---|
| | | | | *O*-acetate |
| **22** | 18.7 | 311 | 299 | *p*-coumaric acid derivative |
| **23** | 20.8 | 256, 357 | 329 | Quercetin-dimethyl ether (isomer) |
| **24** | 23.1 | 267, 314 | 253 | Chrysin |
| **25** | 24.3 | 240, 297, 326 | 247 | Caffeic acid isoprenyl ester |
| **26** | 24.7 | 290 | 255 | Pinocembrin |
| **27** | 25.2 | 266, 290, 357 | 269 | Galangin |
| **28** | 26.8 | 293 | 313 | Pinobanksin-3-*O*-acetate |
| **29** | 27.4 | 242, 300, 325 | 283 | Caffeic acid phenylethyl ester |
| **30** | 30.7 | 310 | 433 | Pinocembrin-5-*O*-3-hydroxyl-4-methoxyphenylpropionate |
| **31** | 31.4 | 313 | 253 | *p*-coumaric acid phenyl ester |
| **32** | 32.4 | 246, 294, 325 | 295 | Caffeic acid cinnamyl ester (isomer) |
| **33** | 33.4 | 292 | 327 | Pinobanskin-3-*O*-propionate |
| **34** | 33.8 | 299 | 341 | Pinobanskin-3-*O*-butanoate |
| **35** | 36.6 | 268, 303 | 387 | Trihydroxyflavone |
| **36** | 37.0 | 292, 322 | 417 | Methylated pinobanksin-3-*O*-phenylpropionate |
| **37** | 37.4 | 292 | 341 | Pinobanksin-3-*O*-butyrate or isobutyrate |
| **38** | 39.3 | 288, 339 | 565 | *p*-coumaric acid-4-hydroxyphenylethyl ester dimer |
| **39** | 40.3 | 292 | 355 | Pinobanksin-3-*O*-pentenoate or 2 methylbutyrate |
| **40** | 41.2 | 292 | 369 | Pinobanksin-3-*O*-hexanoate |
| **41** | 42.6 | 281 | 323 | Caffeic acid derivative |

| | | | | |
|---|---|---|---|---|
| **42** | 43.2 | 280 | 293 | *p*-Methoxy-cinnamic acid cinnamyl ester (isomer) |
| **43** | 43.4 | 275 | 429 | Unknown |
| **44** | 45.7 | 310 | 473 | *p*-coumaric acid derivative |

Source: Author.

### 4.4.3 Antimicrobial activity

The antimicrobial activity of propolis extract was evaluated by two methods, disk diffusion and microdilution. A scanning of microorganisms was selected for both assays, aiming to understand the possible action of the extracts as an antiseptic agent. Gram-negative, Gram-positive bacteria and yeast were chosen due to their presence on the hands of hospital professionals and as threats to antimicrobial resistance (Sinha & Upadhyay, 2025; ECDC, 2022; Larios-Fracarolli*et al.*, 2019). The fungi, in turn, are commonly found in soil, decaying vegetation, seeds, and grains, and are opportunistic pathogens in humans (Mousavi *et al*., 2016). Figure 9 shows an example of halos formed by propolis extracts against *Candida albicans* and *Salmonella enterica*.

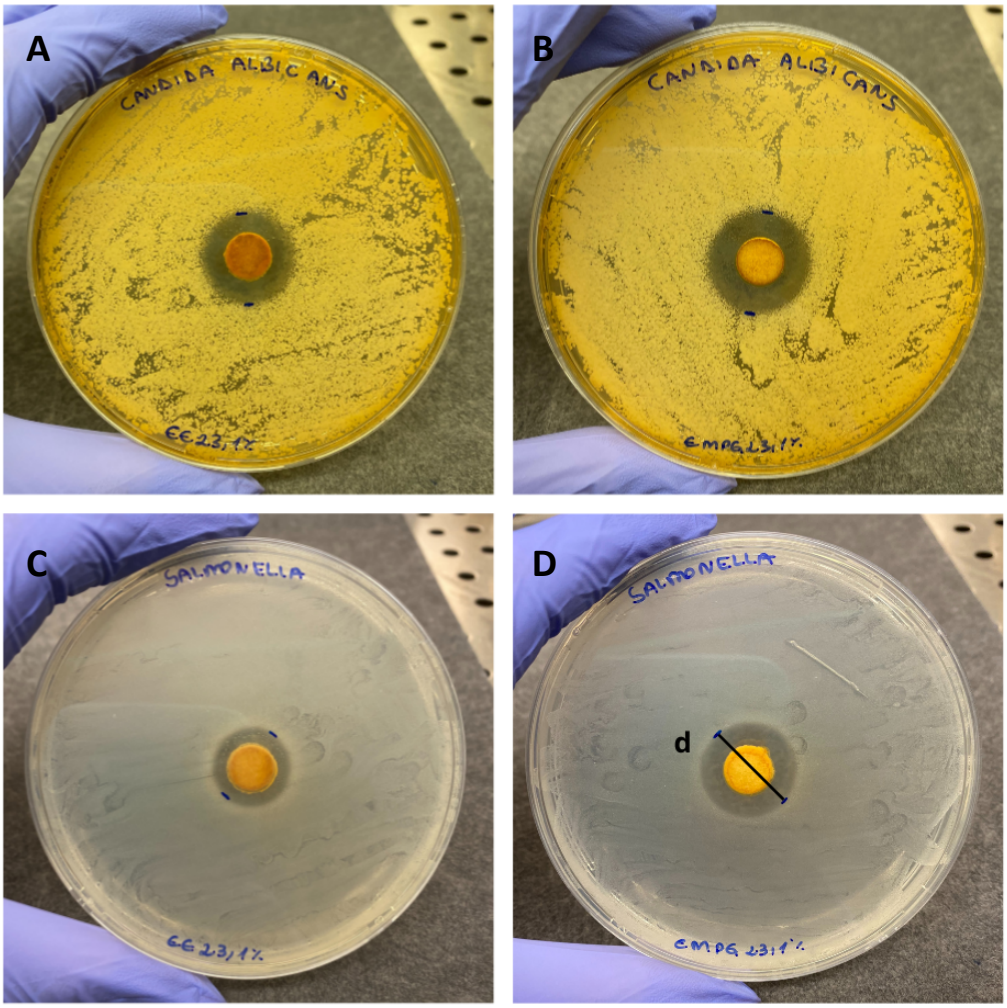



**Figure 9.** *Chromatographic profiles of propolis.* Source: Author. Note: A - *Candida albicans* with ME70; B - *Candida albicans* with MMPG; C - *Salmonella enterica (ATCC 13076)* with ME70; D - *Salmonella enterica* (ATCC 13076) with MMPG; "d" indicates the diameter of inhibition halo.

For the method, the discs were soaked in propolis extracts (23.1% w/w) and solvents. The halo results for each microorganism are organized in Table 10, in mm. Ethanol 70% and monopropylene glycol were tested as negative controls to distinguish the inhibitory effect of propolis from that of the solvents. Considering a disk diameter of 11 mm, when the halo measurement resulted in 0 mm, there was microorganism growth on the disk, therefore, there was no inhibition. However, when the result was 11 mm, the microorganisms were inhibited on the disk but did not form an inhibition halo. Thus, inhibition halos were only considered for values greater than 11 mm.

Table 10 - Disk diffusion results.

| Microorganisms | Diameter of inhibition halo (mm) | | | |
|---|---|---|---|---|
| | CE70 | CMPG | ME70 | MMPG |
| **Gram-negative bacteria** | | | | |
| *Escherichia coli* | 14.5 ± 3.54$^{A}$ | 12.0 ± 0.0$^{A}$ | 13.0 ± 0.0$^{A}$ | 11.0 ± 0.0$^{A}$ |
| *Pseudomonas aeruginosa* | 10.0 ± 0.0$^{B}$ | 15.0 ± 0.0$^{A}$ | 10.0 ± 0.0$^{B}$ | 0.0 ± 0.0$^{B}$ |
| *Salmonella enterica* | 0.0 ± 0.0$^{C}$ | 11.0 ± 0.0$^{B}$ | 16.5 ± 2.12$^{A}$ | 18.0 ± 0.0$^{B}$ |
| **Gram-positive bacteria** | | | | |
| *Staphylococcus aureus* | 13.0 ± 1.41$^{BC}$ | 12.0 ± 0.0$^{C}$ | 18.0 ± 1.41$^{A}$ | 16.0 ± 0.0$^{AB}$ |
| *Cutibacterium acnes* | 0.0 ± 0.0$^{C}$ | 15.0 ± 0.0$^{A}$ | 13.0 ± 0.0$^{B}$ | 13.0 ± 0.0$^{B}$ |
| *Staphylococcus epidermidis* | 0.0 ± 0.0$^{D}$ | 15.5 ± 0.71$^{A}$ | 11.0 ± 0.0$^{C}$ | 13.0 ± 0.0$^{B}$ |
| **Yeast** | | | | |
| *Candida Albicans* | 0.0 ± 0.0$^{D}$ | 11.0 ± 0.0$^{C}$ | 18.0 ± 0.0$^{B}$ | 20.0 ± 0.0$^{A}$ |
| **Fungi** | | | | |
| *Aspergillus brasiliensis* | 0.0 ± 0.0$^{C}$ | 0.0 ± 0.0$^{C}$ | 25.0 ± 0.0$^{B}$ | 30.0 ± 0.0$^{A}$ |
| *Aspergillus fumigatus* | 0.0 ± 0.0$^{C}$ | 0.0 ± 0.0$^{C}$ | 11.0 ± 0.0$^{B}$ | 30.0 ± 0.0$^{A}$ |

Source: Author. Note: Different capital letters between columns indicate significant differences ($p < 0.05$); CE70: Control of Ethanol 70%; CMPG: Control of Monopropylene glycol; ME70: Propolis Maceration Ethanol 70% (v/v); MMPG: Propolis Maceration Monopropylene glycol.

To assess effective inhibition, the halo of the solvent was subtracted from the halo of the propolis, and the results were categorized as follows: “S” when the halo of the control disks with solvent (SCD) was larger than the halo of the disk with propolis extract (PED); “P” when the halo of the PED was larger than the halo of the SCD, indicating an expressed difference between inhibition by propolis extract and inhibition by solvent. The categories were shown in Table 11.

Table 11 - Disk diffusion effective results for propolis extract.

| Microorganisms | Effective Inhibition (mm) | | Inhibition Category | |
|---|---|---|---|---|
| | ME70 | MMPG | ME70 | MMPG |
| **Gram-negative bacteria** | | | | |
| *Escherichia coli* | 0 | 0 | S | S |
| *Pseudomonas aeruginosa* | 0 | 0 | S | S |
| *Salmonella enterica* | $16.5 \pm 2.12^{A}$ | $7.0 \pm 0.0^{B}$ | P | P |
| **Gram-positive bacteria** | | | | |
| *Staphylococcus aureus* | $5.0 \pm 1.41^{A}$ | $4.0 \pm 0.0^{B}$ | P | P |
| *Cutibacterium acnes* | $13.0 \pm 0.0^{A}$ | 0 | P | S |
| *Staphylococcus epidermidis* | $11.0 \pm 0.0^{A}$ | 0 | P | S |
| **Yeast** | | | | |
| *Candida Albicans* | $18.0 \pm 0.0^{A}$ | $9.0 \pm 0.0^{B}$ | P | P |
| **Fungi** | | | | |
| *Aspergillus brasiliensis* | $25.0 \pm 0.0^{B}$ | $30.0 \pm 0.0^{A}$ | P | P |
| *Aspergillus fumigatus* | $11.0 \pm 0.0^{B}$ | $30.0 \pm 0.0^{A}$ | P | P |

Source: Author. Note: Different capital letters between columns indicate significant differences ($p < 0.05$); ME70: Propolis Maceration Ethanol 70% (v/v); MMPG: Propolis Maceration Monopropylene glycol.

Disk diffusion analysis showed that *S. enterica*, *S. aureus*, *C. acnes*, *S. epidermidis*, *C. albicans*, *A. brasiliensis,* and *A. fumigatus* had significant inhibition when propolis extract was applied, while *E. coli* and *P. aeruginosa* had no inhibition effect in the same conditions. The results obtained support the view of Deng *et al.* (2025), who indicated

that propolis is more effective against Gram-positive bacteria than against Gram-negative bacteria. Overall, bacteria and yeast presented higher statistical significance halo for ME70 and the fungus had for MMPG.

Microorganisms in the "P" category indicate that the presence of propolis in solution maximizes the inhibition potential of solvent. Even if a positive control of zone inhibition could not be performed with antibiotic disks, the European Commission on Antimicrobial Susceptibility Testing (EUCAST) guided a document with breakpoint tables for interpretation of minimum inhibitory concentrations (MICs) and zone diameters. From that, the susceptible standard dosing was defined by a high likelihood of therapeutic success using a specific agent. For *S. aureus,* zones ≥ 26 mm are susceptible for Benzylpenicillin (1 μg) antibiotic and *Salmonella spp.* had not susceptible zones for Ciprofloxacin (5 μg). In disagreement with this work, *E. coli* showed zone inhibition ≥ 50 mm for Temocillin (30 μg) and ≥ 50 mm for Piperacillin (30 μg) against *P. aeruginosa*.

Ethanolic extracts of propolis from Gerês have been reported to be effective against *E. coli*, with inhibition zones ranging between 15.33 ± 1.61 and 22.17 ± 2.16 mm, but showing no activity against *C. albicans* (Oliveira, R. D., Araújo, C., & Almeida-Aguiar, C., 2024). Alternatively, Brazilian red propolis extract presented inhibition zones of 13 mm for *S. aureus* and 11.4 mm for *Candida spp.* at a concentration of 100 mg/mL (Silva *et al.*, 2018).

In summary, it is crucial to highlight that the ME70 extract showed almost 70% equivalent efficacy against *S. aureus* when compared to its antibiotic, while MMPG showed approximately 62%. Furthermore, both propolis extracts inhibited *S. enterica*, contrary to what was expected by EUCAST (2026). Thus, considering that *S. epidermidis*, a natural component of the human skin microbiota, was not affected by the glycolic extract, the MMPG extract appears to be the best option for incorporation into liquid soap formulations.

The second antimicrobial assay performed was the microdilution method, which indicated the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs). This method is more sensitive than the previous one since it indicates the approximate concentration of extract at which the microorganism is

inactivated (MIC) and killed (MBC). The results of microdilution may guide the amount of propolis extract in a final cosmetic formulation when the objective is the antiseptic application. Figure 10 shows microorganisms inhibition by the microdilution method.

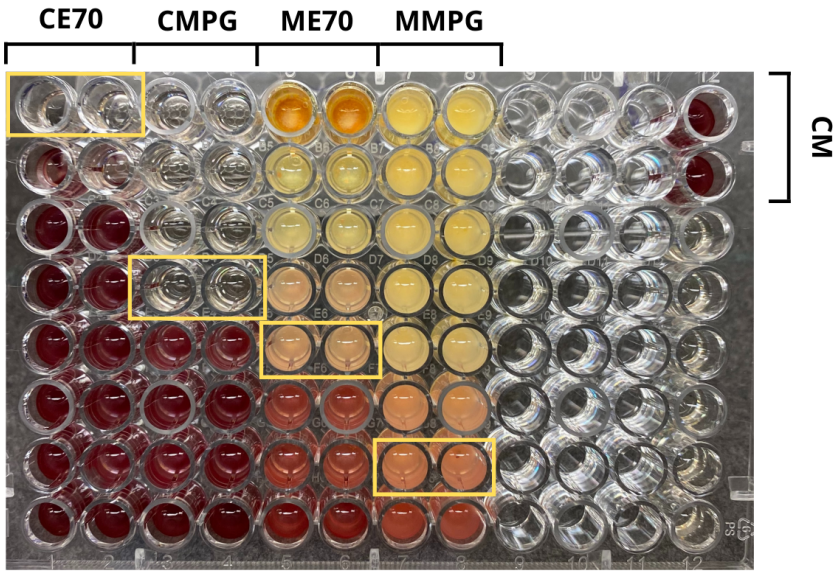



**Figure 10.** *Microorganisms inhibition by microdilution method.* Source: Author. Note: CE70: Control of Ethanol 70%; CMPG: Control of Monopropylene glycol; CM: Control of microorganism growth; Yellow rectangle indicates the inhibition by the minimum concentration of extract.

MIC and MBC values are presented in Table 12. Solvents (CE70 and CMPG) were assessed as negative controls in order to understand the influence of propolis on antimicrobial activity. In parallel, three categories of antibiotics were tested as positive controls. Streptomycin 0.1% is an antibiotic (aminoglycoside class) used against Gram-negative bacteria, methicillin 0.1% (penicillin class) applied against *Staphylococcus*, and ampicillin 2% (penicillin class) used against Gram-positive and some Gram-negative bacteria (Fernandez-Llimos, F., 2022). Due to differences in concentration ranges, results for samples and positive controls were expressed in percentage and milligrams per mL. To convert 23.1% (w/w), the density of each solvent was used, resulting in 260 mg/mL for ME70 and 288.5 mg/mL for MMPG. Negative controls were expressed only in percentage.

The microdilution results showed better MICs for the samples when compared to the negative control, indicating that the propolis extract showed significant inhibition against all microorganisms evaluated. However, for MBC values, *C. acnes*, *E. coli* and *P. aeruginosa* did not show differences between MMPG and the control with monopropylene glycol, as well as *S. enterica* against ME70 and *C. albicans* against ME70 and MMPG. Overall, ME70 was slightly stronger against *S. aureus*, *S. enterica* and *C. albicans*, due the minimum inhibitory concentrations of 2.03 mg/mL of propolis extract. In contrast, MMPG stood out for its potent bactericidal action against *P. aeruginosa*, *S.aureus* and *C. acnes*, with MICs of 36.09 mg/mL. Also, the antifungal potential of ME70 and MMPG were remarkable, with MICs of 2.03 vs. 2.25 mg/mL respectively, for *A. brasiliensis* and *A. fumigatus*.

According to Silva *et al*. (2024), De Carli *et al*. (2022) and Silva *et al*. (2012), positive inhibition was expected from propolis of Bragança region against *S. aureus*, *P. aeruginosa* and *C. albicans*. However, the authors showed that each strain of *E. coli* presents divergent results for propolis inhibition. In agreement with De Carli *et al.* (2022), the *E. coli* ATCC 25922 strain tested in this study showed no inhibition in the disk diffusion assay but was susceptible in the microdilution protocol. Even *C. albicans* did not have inhibition or bactericidal results for the positive controls, EUCAST (2025) indicate a MIC of ≤0.06 mg/mL against the antifungal agent Posaconazole.

The differences observed between the two methods can be attributed to variations in inoculum concentration. In microdilution assays, the lower microbial cell density facilitates the action of propolis extracts, resulting in lower MIC values and an apparent higher antimicrobial efficacy. In contrast, the higher cell density in disk diffusion assays may limit the diffusion and effectiveness of bioactive compounds present in propolis, leading to reduced or absent inhibition zones. In general, based on the MICs results, more than 9 mg/mL and less than 16.25 mg/mL of propolis extract would be sufficient to inhibit most of the pathogenic strains tested, without inhibiting *S. epidermidis*, a skin-protecting bacterium.

Table 12 - Microdilution significance results for propolis extract.

| **Microorganisms** | **Samples** | | | | | | | | **Positive Control** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CE70 70% | | CMPG 100% | | ME70 23.1% [260 mg/mL] | | MMPG 23.1% [288.5 mg/mL] | | Streptomycin 0.1% [1 mg/mL] | | Methicillin 0.1% [1 mg/mL] | | Ampicillin 2% [20 mg/mL] | |
| | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| **Gram-negative bacteria** | | | | | | | | | | | | | | |
| *Escherichia coli* | 35 | 35 | 6.25 | 6.25 | 2.89 [32.5] | 5.775 [65.0] | 1.44 [17.98] | 5.775 [72.13] | 0.0015 [0.01] | 0.0015 [0.01] | n.t | n.t | 0.015 [0.15] | 0.015 [0.15] |
| *Pseudomonas aeruginosa* | 70 | 70 | 12.5 | 12.5 | 0.72 [8.125] | 5.775 [65.0] | 0.18 [2.25] | 2.89 [36.09] | 0.006 [0.06] | 0.006 [0.06] | n.t | n.t | 0.063 [0.63] | 0.063 [0.63] |
| *Salmonella enterica* | 17.5 | 35 | 12.5 | 12.5 | 0.18 [2.03] | 11.55 [130.0] | 0.18 [2.25] | 1.44 [17.98] | 0.0007 [0.007] | 0.0007 [0.007] | n.t | n.t | 0.015 [0.15] | 0.015 [0.15] |
| **Gram-positive bacteria** | | | | | | | | | | | | | | |
| *Staphylococcus aureus* | 35 | 70 | 12.5 | 25 | 0.18 [2.03] | 11.55 [130.0] | 0.18 [2.25] | 2.89 [36.09] | 0.0007 [0.007] | 0.0007 [0.007] | 0.0007 [0.007] | 0.0007 [0.007] | 0.015 [0.15] | 0.015 [0.15] |
| *Cutibacterium acnes* | 17.5 | 35 | 6.25 | 12.5 | 0.72 [8.125] | 5.775 [65.0] | 1.44 [17.98] | 2.89 [36.09] | 0.0007 [0.007] | 0.0007 [0.007] | 0.0007 [0.007] | 0.0007 [0.007] | 0.015 [0.15] | 0.015 [0.15] |
| *Staphylococcus epidermidis* | 70 | 70 | 12.5 | 25 | 1.44 [16.25] | 5.775 [65.0] | 0.36 [4.5] | 2.89 [36.09] | n.t | n.t | n.t | n.t | n.t | n.t |

| Microorganisms | Samples | | | | | | | | Positive Control | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CE70 70% | | CMPG 100% | | ME70 23.1% [260 mg/mL] | | MMPG 23.1% [288.5 mg/mL] | | Streptomycin 0.1% [1 mg/mL] | | Methicillin 0.1% [1 mg/mL] | | Ampicillin 2% [20 mg/mL] | |
| **Yeast** | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| *Candida albicans* | 17.5 | >70 | 6.25 | >100 | 0.18 [2.03] | >23.1 [260.0] | 0.72 [8.99] | >23.1 [288.5] | n.t | n.t | n.t | n.t | n.t | n.t |

| Microorganisms | Samples | | | | | | | | Positive Control | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CE70 70% | | CMPG 100% | | ME70 23.1% [260.0 mg/mL] | | MMPG 23.1% [288.5 mg/mL] | | Ketoconazole 0.1% [1 mg/mL] | |
| **Fungi** | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| *Aspergillus brasiliensis* | >70 | >70 | 12.5 | 12.5 | 0.18 [2.03] | 0.18 [2.03] | 0.18 [2.25] | 0.18 [2.25] | 0.006 [0.06] | 0.0125 [0.125] |
| *Aspergillus fumigatus* | >70 | >70 | 6.25 | 6.25 | 0.18 [2.03] | 0.18 [2.03] | 0.18 [2.25] | 0.18 [2.25] | 0.05 [0.5] | 0.1 [1] |

Source: Author. Note: [ ] values expressed in mg/ml; CE70: Control of Ethanol 70%; CMPG: Control of Monopropylene glycol; ME70: Propolis Maceration Ethanol 70% (v/v); MMPG: Propolis Maceration Monopropylene glycol; MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration; n.t: Not Tested or Not Determined.

### 4.5 Liquid soap formulations

In the medical field, antiseptic agents are particularly used to decontaminate the skin of the hands and to control the risk of infections. Nonetheless, after the pandemic, the general public increasingly uses them for fear of microbial contamination in homes and workplaces to reduce the spread of infections. Usually, a commercial antiseptic liquid soap, when used frequently for a long time, could cause side effects and skin irritation due the complex soap-base ingredients and chemicals agents (Idawati *et al.*, 2023; Babalska, Z. Ł., Korbecka-Paczkowska, M. & Karpiński, T. M., 2021). Thus, this work chose a clean label approach, with less ingredients and a potential natural antiseptic agent, namely propolis extract, for its formulation.

The evaluation of propolis extract as antiseptic agent was organized in verify the antimicrobial activity of the final product and its stability at different conditions. So, five different formulations were prepared (PE5, PG5, E5, G5, and E0). Samples PE5 and PG5 were prepared with 5% ethanolic (ME70) and glycolic propolis extract (MMPG), respectively. As a control, samples without propolis were prepared: E5 with 5% ethanol 70%, G5 with 5% MPG, and E0 as neutral (without solvent or propolis). Figure 11 shows the liquid soap samples being packaged and organized for stability assays.

**Figure 11.** *Samples being packaged.* Source: Author.

### 4.5.1 Antimicrobial activity

Broadly, antiseptic products are applied to the skin or other living tissues with the aim of inhibiting microbial growth (Babalska, Z. Ł., Korbecka-Paczkowska, M. & Karpiński, T. M., 2021). In the propolis example, the microbial inhibitions are provided by the phenolics content of propolis. Therefore, the final soap formulation was evaluated against the same strains already tested with propolis extract (Table 13). Due to the MIC results of Table 12, 5% of propolis extract was decided to incorporate in liquid soap, which represents 13 mg/mL for PE5 and 14.425 mg/mL for PG5.

In Table 13, MIC and MBC results were organized and expressed in milligrams per millimeter, with maximum concentration of 5% in each liquid soap. All five formulations were tested, aiming to distinguish propolis extract influence in microbial inhibition. In contrast to the results obtained for the extracts alone (Table 12), almost all strains showed no significant differences between control formulations (water and solvents) and propolis-containing soaps in terms of MIC, with exception of *S. enterica* and *C. albicans*. These similar values suggest that the antimicrobial effect was primarily driven by the base formulation components rather than by the incorporated propolis.

Unexpectedly, lower MIC values were observed for the control formulations (E0, E5 and G5) against *S. enterica*, as well as for E5 against *A. brasiliensis* and *S. aureus*, indicating that, in these cases, the presence of propolis did not enhance antimicrobial activity. However, even though MIC was not desirable, MBC values were better for both propolis-containing formulations compared to the controls for *E. coli*, *S. aureus* and *C. acnes* and for PE5 for *S. enterica*, meaning bactericidal potential. Additionally, *C. albicans* was the only microorganism showing lower MIC and MBC values in propolis formulations compared to controls.

Due to the low inhibition of the final product in most strains, it is presumed that the amount of propolis extract incorporated was not sufficient to act as a desirable antiseptic agent, since the pure propolis extract showed promising bactericidal values. Therefore, for future samples, it would be necessary to test a larger volume of incorporated propolis extract, as well as higher concentrations of the extract, considering the dilution factor of the liquid soap when used.

Table 13 - Microdilution results for liquid soap.

| **Microorganisms** | **Samples** | | | | | | | | | | **Positive Control** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | E0 5% | | E5 5% | | G5 5% | | PE5 5% | | PG5 5% | | Streptomycin 1 mg/mL | | Methicillin 1 mg/mL | | Ampicillin 20 mg/mL | |
| | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| **Gram-negative bacteria** | | | | | | | | | | | | | | | | |
| *Escherichia coli* | 5 | >5 | 5 | >5 | 5 | >5 | 5 | 5 | 5 | 5 | 0.01 | 0.01 | n.t | n.t | 0.15 | 0.15 |
| *Pseudomonas aeruginosa* | 5 | >5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 0.06 | 0.06 | n.t | n.t | 0.63 | 0.63 |
| *Salmonella enterica* | 0.078 | >5 | 0.078 | 5 | 0.078 | 5 | 5 | 0.63 | 0.15 | 5 | 0.007 | 0.007 | n.t | n.t | 0.15 | 0.15 |
| **Gram-positive bacteria** | | | | | | | | | | | | | | | | |
| *Staphylococcus aureus* | 0.31 | >5 | 0.15 | >5 | 0.31 | >5 | 1.25 | 5 | 0.31 | 5 | 0.007 | 0.007 | 0.007 | 0.007 | 0.15 | 0.15 |
| *Cutibacterium acnes* | 5 | >5 | 1.25 | >5 | 1.25 | >5 | 1.25 | 5 | 1.25 | 5 | 0.007 | 0.007 | 0.007 | 0.007 | 0.15 | 0.15 |
| *Staphylococcus epidermidis* | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * | * |
| **Yeast** | | | | | | | | | | | | | | | | |
| *Candida albicans* | 0.31 | 0.31 | 0.31 | 0.31 | 0.31 | 0.31 | 0.078 | 0.078 | 0.078 | 0.078 | n.t | n.t | n.t | n.t | n.t | n.t |

| Microorganisms | Samples | | | | | | | | | | Positive Control | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | E0 5% | | E5 5% | | G5 5% | | PE5 5% | | PG5 5% | | Ketoconazole 1 mg/mL | |
| **Fungi** | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
| *Aspergillus brasiliensis* | 0.039 | 0.039 | 0.078 | 0.078 | 0.039 | 0.039 | 0.039 | 0.039 | 0.039 | 0.039 | 0.06 | 0.125 |
| *Aspergillus fumigatus* | 0.039 | 0.039 | 0.039 | 0.039 | 0.039 | 0.039 | 0.039 | 0.039 | 0.039 | 0.039 | 0.5 | 1 |

Source: Author. Note: * Test contaminated; E0: Control Formulation with Water (5%); E5: Control Formulation with Ethanol 70% (5%); G5: Control Formulation with Monopropylene glycol (5%); PE5: Formulation with ME70 (5%); PG5: Formulation with MMPG (5%); MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration; n.t: Not Tested or Not Determined.

### 4.5.2 Stability tests

In Phase 1, samples were tested for centrifugation and mechanical vibration analyzing, in order to identify cosmetic formulation instability. Figure 12 shows the triplicates samples with propolis extract approved in phase 1.

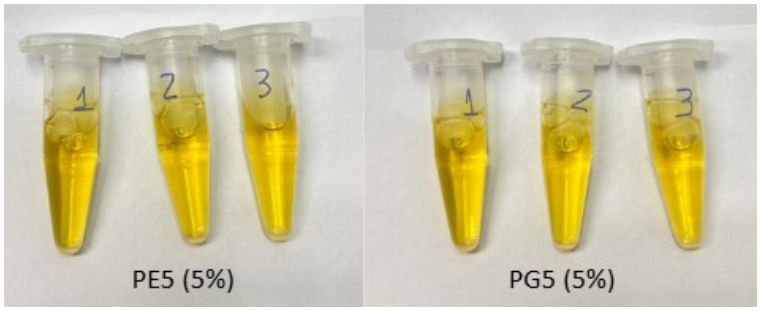



**Figure 12.** *Triplicates samples with propolis extract approved in phase 1*. Source: Author.
Note: PE5 (5%): Sample with 5% of selected ethanolic propolis extract; PG5 (5%): Sample with 5% of selected glycolic propolis extract.

By analysis, the liquid soaps stayed homogeneous (Figure 12) and were approved to Phase 2 of testing, which determined density, pH, viscosity and color of samples stored in different conditions. In the protocol, day 0 was established as 24 hours after the cosmetic formulation, so that the air incorporated during formulation can be naturally moved out from the soap to the “head space” and do not interfere with the assays.

#### 4.5.2.1 Density and pH determination

Density and pH are parameters used to verify the stability of a cosmetic and ensure its safety. The final product must have a pH range compatible with the skin, which is slightly acidic (4.6 - 5.8), therefore, a pH of 6 ± 0.1 was standardized in the final formulation (Ramos, L. C., de Lima, T. L. C., & De Souza, G. O., 2021). Over the days, pH analysis was measured by a 10 % (w/v) dilution (ANVISA, 2004), simulating the contraction of usage. For each Phase 2 test, the parameter results were obtained and organized as presented in Tables 14, 15, 16, and 17.

When evaluating the samples by day, indicated by lowercase letters in Table 14, the density remained statistically constant. However, for pH values, samples E0, PE5, and PG5

showed statistical differences on day zero and day 5 and on days 10 and 15, only the propolis samples showed differences.

Table 14 - Light assay results.

| **Light** | | **Day** | | | |
|---|---|---|---|---|---|
| Sample | Analysis | 0 | 5 | 10 | 15 |
| E0 | pH | $6.25 \pm 0.07^{b\ A}$ | $6.45 \pm 0.07^{ab\ A}$ | $6.55 \pm 0.07^{a\ A}$ | $6.45 \pm 0.07^{a\ A}$ |
| | Density | $1.02 \pm 0.03^{a\ A}$ | $1.09 \pm 0.01^{a\ A}$ | $1.08 \pm 0.01^{a\ A}$ | $1.05 \pm 0.01^{a\ A}$ |
| E5 | pH | $6.55 \pm 0.07^{a\ A}$ | $6.65 \pm 0.07^{a\ A}$ | $6.8 \pm 0.1^{a\ A}$ | $6.6 \pm 0.14^{a\ A}$ |
| | Density | $1.02 \pm 0.03^{a\ A}$ | $1.04 \pm 0.01^{a\ A}$ | $1.09 \pm 0.04^{a\ A}$ | $1.08 \pm 0.01^{a\ A}$ |
| G5 | pH | $6.5 \pm 0.1^{a\ A}$ | $6.65 \pm 0.07^{a\ A}$ | $6.8 \pm 0.14^{a\ A}$ | $6.8 \pm 0.1^{a\ A}$ |
| | Density | $1.08 \pm 0.03^{a\ A}$ | $1.08 \pm 0.03^{a\ A}$ | $1.09 \pm 0.01^{a\ A}$ | $1.08 \pm 0.01^{a\ A}$ |
| PE5 | pH | $6.25 \pm 0.07^{b\ A}$ | $6.1 \pm 0.14^{bc\ A}$ | $6.1 \pm 0.1^{b\ A}$ | $6.0 \pm 0.1^{b\ A}$ |
| | Density | $1.03 \pm 0.01^{a\ A}$ | $1.01 \pm 0.1^{a\ A}$ | $1.09 \pm 0.01^{a\ A}$ | $1.06 \pm 0.01^{a\ A}$ |
| PG5 | pH | $6.2 \pm 0.1^{b\ A}$ | $6.0 \pm 0.1^{c\ BC}$ | $6.1 \pm 0.1^{b\ AB}$ | $5.85 \pm 0.07^{b\ C}$ |
| | Density | $1.03 \pm 0.07^{a\ A}$ | $1.06 \pm 0.01^{a\ A}$ | $1.09 \pm 0.04^{a\ A}$ | $1.05 \pm 0.01^{a\ A}$ |

Source: Author. Note: Different capital letters between columns indicate significant differences ($p < 0.05$); Different lowercase letters between lines indicate significant differences ($p < 0.05$); E0: Neutral sample; E5: Sample with 5% of ethanol; G5: Sample with 5% of monopropylene glycol; PE5: Sample with 5% of ethanolic propolis extract; PG5: Sample with 5% of glycolic propolis extract.

Regarding pH, most formulations maintained values close to the initial standard, with no significant variations over time. In contrast, PG5 showed statistically significant changes, with a gradual decrease from 6.00 to 5.85, indicating a slight shift in pH under light exposure. Although the majority of formulations demonstrated good pH stability, The variations observed in PG5 and PE5 suggested that the incorporation of propolis extract may influence pH stability over time. However, all values remained within an acceptable range for topical application. To understand the density and pH behavior of the formulas at extreme

temperatures, the liquid soaps were exposed to 5 °C and 45 °C, and measurements were taken. Tables 15 and 16 present the values obtained.

Table 15 - Extreme temperature assay results at 5°C.

| **Extreme temperature** | | **Day** | | | |
|---|---|---|---|---|---|
| Sample | Analysis | 0 | 5 | 10 | 15 |
| E0 | pH | $6.3 \pm 0.1^{b\ A}$ | $6.3 \pm 0.07^{abc\ A}$ | $6.3 \pm 0.1^{bc\ A}$ | $6.3 \pm 0.1^{b\ A}$ |
| | Density | $1.0 \pm 0.01^{a\ A}$ | $1.1 \pm 0.01^{a\ A}$ | $1.1 \pm 0.04^{a\ A}$ | $1.06 \pm 0.01^{a\ A}$ |
| E5 | pH | $6.5 \pm 0.1^{a\ A}$ | $6.4 \pm 0.1^{ab\ A}$ | $6.5 \pm 0.1^{ab\ A}$ | $6.6 \pm 0.1^{a\ A}$ |
| | Density | $1.04 \pm 0.01^{a\ A}$ | $1.1 \pm 0.01^{a\ A}$ | $1.1 \pm 0.01^{a\ A}$ | $1.06 \pm 0.03^{a\ A}$ |
| G5 | pH | $6.6 \pm 0.07^{a\ A}$ | $6.55 \pm 0.07^{a\ A}$ | $6.6 \pm 0.1^{a\ A}$ | $6.55 \pm 0.07^{a\ A}$ |
| | Density | $1.05 \pm 0.01^{a\ A}$ | $1.1 \pm 0.01^{a\ A}$ | $1.1 \pm 0.01^{a\ A}$ | $1.08 \pm 0.01^{a\ A}$ |
| PE5 | pH | $6.2 \pm 0.07^{b\ A}$ | $6.2 \pm 0.07^{bc\ A}$ | $6.1 \pm 0.1^{c\ A}$ | $6.2 \pm 0.1^{bc\ A}$ |
| | Density | $1.03 \pm 0.04^{a\ A}$ | $1.1 \pm 0.04^{a\ A}$ | $1.1 \pm 0.01^{a\ A}$ | $1.04 \pm 0.03^{a\ A}$ |
| PG5 | pH | $6.2 \pm 0.1^{b\ A}$ | $6.1 \pm 0.07^{c\ A}$ | $6.1 \pm 0.1^{c\ A}$ | $6.1 \pm 0.1^{c\ A}$ |
| | Density | $1.03 \pm 0.01^{a\ A}$ | $1.1 \pm 0.04^{a\ A}$ | $1.1 \pm 0.01^{a\ A}$ | $1.04 \pm 0.01^{a\ A}$ |

Source: Author. Note: Different capital letters between columns indicate significant differences ($p < 0.05$); Different lowercase letters between lines indicate significant differences ($p < 0.05$); E0: Neutral sample; E5: Sample with 5% of ethanol; G5: Sample with 5% of monopropylene glycol; PE5: Sample with 5% of ethanolic propolis extract; PG5: Sample with 5% of glycolic propolis extract.

At 5 °C, no significant differences between samples were observed for either density or pH when comparing formulations within the same day, as indicated by the capital letters. Density remained stable throughout the storage period for all samples, with no statistically significant variations over time. Regarding pH, minor fluctuations were observed across the storage period, as indicated by lowercase letters, however, these variations were small (ranging from approximately 6.1 to 6.6) and remained within the acceptable range for topical applications. Overall, the results indicated good physicochemical stability of the formulations under low-temperature conditions, with no clear influence of propolis incorporation on pH or density behavior.

However, at high temperatures (45 ℃), samples G5, PE5, and PG5 did not exhibit similar behavior on all days, with the propolis formulations showing acidification (ranging from approximately 6.2 to 5.5). This behavior was expected, considering the extreme condition that the product was exposed to, since high temperatures reduce the bioactivity of phenolic compounds, promote their oxidation, releasing hydrogen ions into the solution and lowering the pH (Akhan *et al.*, 2025; Zelca *et al.*, 2022). However, the lack of statistical stability of the pH of PE5 and PG5 over 15 days may indicate that, if monitored for more days, the samples could continue to acidify, which was not observed in the other samples, which underwent changes but stabilized. Therefore, even if this behavior was expected, it was also expected that the pH would stabilize over the days, so that the final formulation could be adjusted accordingly, anticipating such behavior.

Table 16 - Extreme temperature assay results at 45℃.

| **Extreme temperature** | | **Day** | | | |
|---|---|---|---|---|---|
| Sample | Analysis | 0 | 5 | 10 | 15 |
| E0 | pH | $6.3 \pm 0.1^{b\ A}$ | $6.4 \pm 0.28^{ab\ A}$ | $6.35 \pm 0.07^{b\ A}$ | $6.25 \pm 0.07^{b\ A}$ |
| | Density | $1.0 \pm 0.01^{a\ A}$ | $1.1 \pm 0.01^{b\ A}$ | $1.1 \pm 0.01^{a\ A}$ | $1.05 \pm 0.01^{a\ A}$ |
| E5 | pH | $6.5 \pm 0.1^{a\ A}$ | $6.6 \pm 0.14^{a\ A}$ | $6.7 \pm 0.1^{a\ A}$ | $6.75 \pm 0.07^{a\ A}$ |
| | Density | $1.04 \pm 0.01^{a\ A}$ | $1.0 \pm 0.01^{b\ A}$ | $1.1 \pm 0.03^{a\ A}$ | $1.05 \pm 0.01^{a\ A}$ |
| G5 | pH | $6.6 \pm 0.1^{a\ AB}$ | $6.5 \pm 0.1^{ab\ B}$ | $6.7 \pm 0.1^{a\ A}$ | $6.65 \pm 0.07^{a\ AB}$ |
| | Density | $1.05 \pm 0.01^{a\ A}$ | $1.1 \pm 0.01^{a\ A}$ | $1.1 \pm 0.01^{a\ A}$ | $1.09 \pm 0.01^{a\ A}$ |
| PE5 | pH | $6.2 \pm 0.07^{b\ A}$ | $5.9 \pm 0.1^{b\ B}$ | $5.7 \pm 0.1^{c\ C}$ | $5.6 \pm 0.1^{c\ C}$ |
| | Density | $1.03 \pm 0.04^{a\ A}$ | $1.0 \pm 0.01^{b\ A}$ | $1.1 \pm 0.01^{a\ A}$ | $1.05 \pm 0.01^{a\ A}$ |
| PG5 | pH | $6.2 \pm 0.1^{b\ A}$ | $5.9 \pm 0.1^{b\ AB}$ | $5.6 \pm 0.1^{c\ BC}$ | $5.5 \pm 0.1^{c\ C}$ |
| | Density | $1.03 \pm 0.01^{a\ A}$ | $1.1 \pm 0.01^{ab\ A}$ | $1.1 \pm 0.01^{a\ A}$ | $1.08 \pm 0.01^{a\ A}$ |

Source: Author. Note Different capital letters between columns indicate significant differences ($p < 0.05$); Different lowercase letters between lines indicate significant differences ($p < 0.05$); E0: Neutral sample; E5: Sample with 5% of ethanol; G5: Sample with 5% of monopropylene glycol; PE5: Sample with 5% of ethanolic propolis extract; PG5: Sample with 5% of glycolic propolis extract.

To finalize this stage of the tests, the formulations were subjected to freeze-thaw cycles to verify the behavior of pH and density. Table 17 shows that sample E5 was the only one that did not show statistical similarity over the days. Similarly to exposure to 5 ℃, the soaps with incorporated propolis extract were those that presented a pH close to the standard value of 6 ± 0.1, indicating, once again, that low temperatures favor the stability of the main samples.

Therefore, based on the overall assessment, exposure to high temperatures are factors that interfere with pH stability due to the degradation of phenolic compounds, as previously indicated by the authors (Akhan *et al.*, 2025; Zelca *et al.*, 2022). Although light was another parameter indicated for oxidation, in this study temperature was a more significant factor than light.

Table 17 - Freeze-thaw cycles assay results.

| **Freeze-thaw cycles** | | **Day** | | | |
|---|---|---|---|---|---|
| Sample | Analysis | 0 | 2 | 4 | 6 |
| E0 | pH | $6.3 \pm 0.1^{b\,A}$ | $6.43 \pm 0.14^{ab\,A}$ | $6.25 \pm 0.07^{a\,A}$ | $6.45 \pm 0.21^{a\,A}$ |
| | Density | $1.0 \pm 0.01^{a\,A}$ | $1.03 \pm 0.01^{a\,A}$ | $1.05 \pm 0.01^{a\,A}$ | $0.98 \pm 0.03^{a\,A}$ |
| E5 | pH | $6.5 \pm 0.1^{a\,B}$ | $6.73 \pm 0.03^{a\,A}$ | $6.5 \pm 0.1^{a\,B}$ | $6.45 \pm 0.07^{a\,B}$ |
| | Density | $1.04 \pm 0.01^{a\,A}$ | $1.04 \pm 0.01^{a\,A}$ | $1.08 \pm 0.01^{a\,A}$ | $1.04 \pm 0.02^{a\,A}$ |
| G5 | pH | $6.55 \pm 0.07^{a\,A}$ | $6.62 \pm 0.06^{a\,A}$ | $6.15 \pm 0.49^{a\,A}$ | $6.55 \pm 0.07^{a\,A}$ |
| | Density | $1.05 \pm 0.01^{a\,A}$ | $1.05 \pm 0.01^{a\,A}$ | $1.1 \pm 0.08^{a\,A}$ | $1.06 \pm 0.01^{a\,A}$ |
| PE5 | pH | $6.15 \pm 0.1^{b\,A}$ | $6.22 \pm 0.03^{b\,A}$ | $5.7 \pm 0.57^{a\,A}$ | $6.15 \pm 0.07^{a\,A}$ |
| | Density | $1.03 \pm 0.04^{a\,A}$ | $1.06 \pm 0.03^{a\,A}$ | $1.1 \pm 0.01^{a\,A}$ | $1.06 \pm 0.03^{a\,A}$ |
| PG5 | pH | $6.2 \pm 0.1^{b\,A}$ | $6.3 \pm 0.007^{b\,A}$ | $6.1 \pm 0.1^{a\,A}$ | $6.25 \pm 0.07^{a\,A}$ |
| | Density | $1.03 \pm 0.01^{a\,A}$ | $0.98 \pm 0.11^{a\,A}$ | $1.08 \pm 0.01^{a\,A}$ | $0.99 \pm 0.1^{a\,A}$ |

Source: Author. Note: Different capital letters between columns indicate significant differences ($p < 0.05$); Different lowercase letters between lines indicate significant differences ($p < 0.05$); E0: Neutral sample;

E5: Sample with 5% of ethanol; G5: Sample with 5% of monopropylene glycol; PE5: Sample with 5% of ethanolic propolis extract; PG5: Sample with 5% of glycolic propolis extract.

#### 4.5.2.2 Viscosity Assays

Viscosity was proposed to ensure product quality and its proper application. This physical parameter measures a fluid's resistance to flow, that is, the higher the viscosity, the thicker and more resistant to movement the fluid will be (Oliveira, 2025). For the three proposed tests, two distinct graphs were created, grouping the ethanolic (A) and glycolic (B) formulations with each control, in addition to the neutral control. Considering the proposed application, this study established that the viscosity values need to be at least 2,500 mPa·s (millipascal-second) to be accepted for commercialization, because lower values would result in an excessively liquid formulation. Figure 13 expresses the viscosity of the formulations on day 0, 5, 10 and 15 under the light exposure conditions.

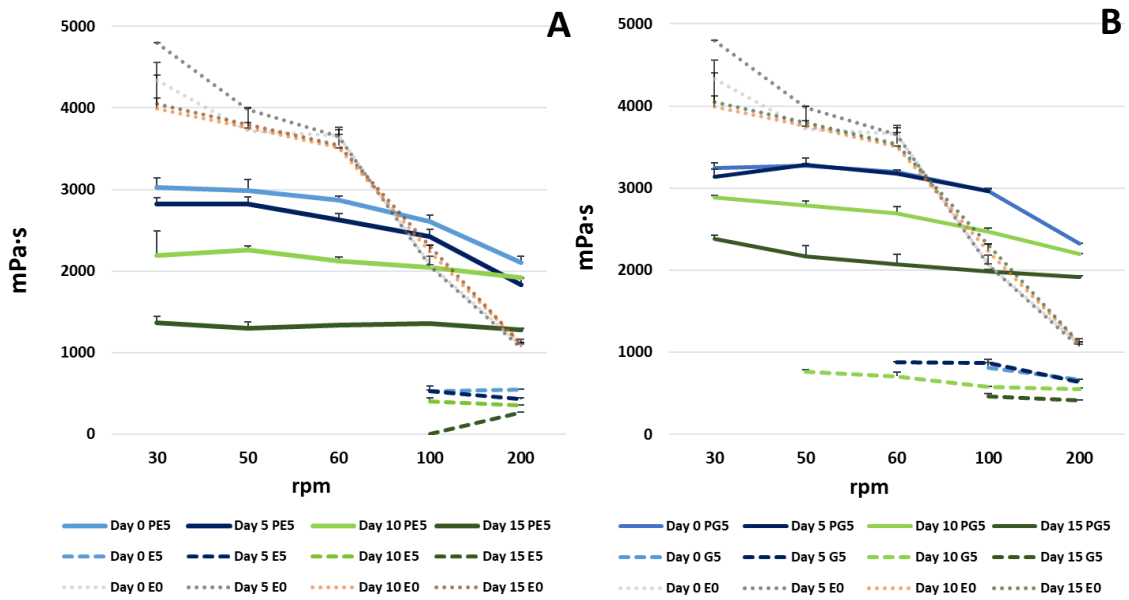



**Figure 13.** *Light test viscosity results.* Source: Author. Note: A - Ethanolic samples and controls; B - Glycolic samples and controls; E0: Neutral sample; E5: Sample with 5% of ethanol; G5: Sample with 5% of monopropylene glycol; PE5: Sample with 5% of ethanolic propolis extract; PG5: Sample with 5% of glycolic propolis extract.

Based on the curves, it is possible to observe that the propolis samples (PE5 and PG5) decreased in viscosity over the days, although PG5 varied less than PE5, indicating that light exposure interferes more with the stability of the soap with ethanolic extract than with that with glycolic extract. The solvent-based controls (E5 and G5) followed the same pattern, but with viscosity values below 1,000 mPa·s, suggesting that the incorporation of pure solvent destabilizes the viscosity of the formulation. The neutral sample (E0) did not show changes in viscosity over the days; however, with increasing revolutions per minute, the viscosity decreased drastically. This behavior was observed only in the sample that did not contain solvent or propolis extract in its composition, suggesting that the presence of these components better stabilizes the soap formulation. Comparing graphs A and B of the viscosity test at 100 rpm, the propolis samples showed viscosities up to 2,500 mPa·s up to day 5 (PE5) and up to day 10 (PG5), unlike the control samples (E0, E5 and G5), indicating that the presence of propolis extract in the formulation helped to stabilize the viscosity for a certain period. However, from day 5 (PE5) and 10 (PG5) onwards, both formulations containing propolis reduced viscosity, thus falling outside the established parameter. Possibly, if the concentration and/or volume of propolis present were higher, the viscosity would remain constant for a longer time than observed. Values below 2,500 mPa·s would result in an excessively liquid formulation, which would not be suitable for commercialization, considering the viscosity chosen for the final soap.

To understand how the viscosity of the formulations behaves at extreme temperatures, the liquid soaps were exposed to 5℃ and 45℃ and the test was performed. Figures 14 and 15 represent the data obtained.

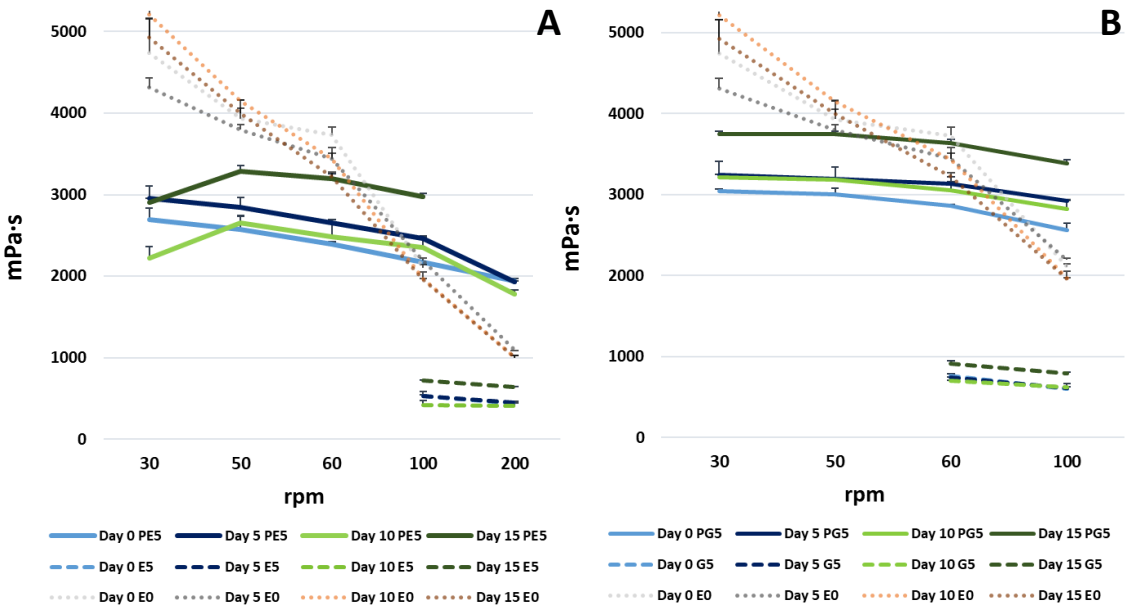



**Figure 14.** *Extreme temperature viscosity results at 5 ℃.* Source: Author. Note: A - Ethanolic samples and controls; B - Glycolic samples and controls; E0: Neutral sample; E5: Sample with 5% of ethanol; G5: Sample with 5% of monopropylene glycol; PE5: Sample with 5% of ethanolic propolis extract; PG5: Sample with 5% of glycolic propolis extract.

In general, the propolis samples showed similar behavior throughout the days, except for the samples from the 15th day. As already observed in the light test, the PG5 samples presented a wider viscosity range than the PE5 samples, suggesting that the glycolic extract contributes better to the stabilization of the formulation. At 100 rpm, the glycolic propolis samples showed viscosity greater than 2,500 mPa·s, and the controls (E5, G5, and E0) showed lower values, not meeting the acceptance criteria, as did most of the ethanolic propolis samples on most days. Therefore, at low temperatures, the incorporation of glycolic propolis extract into soaps seems to benefit the stability of the formulation.

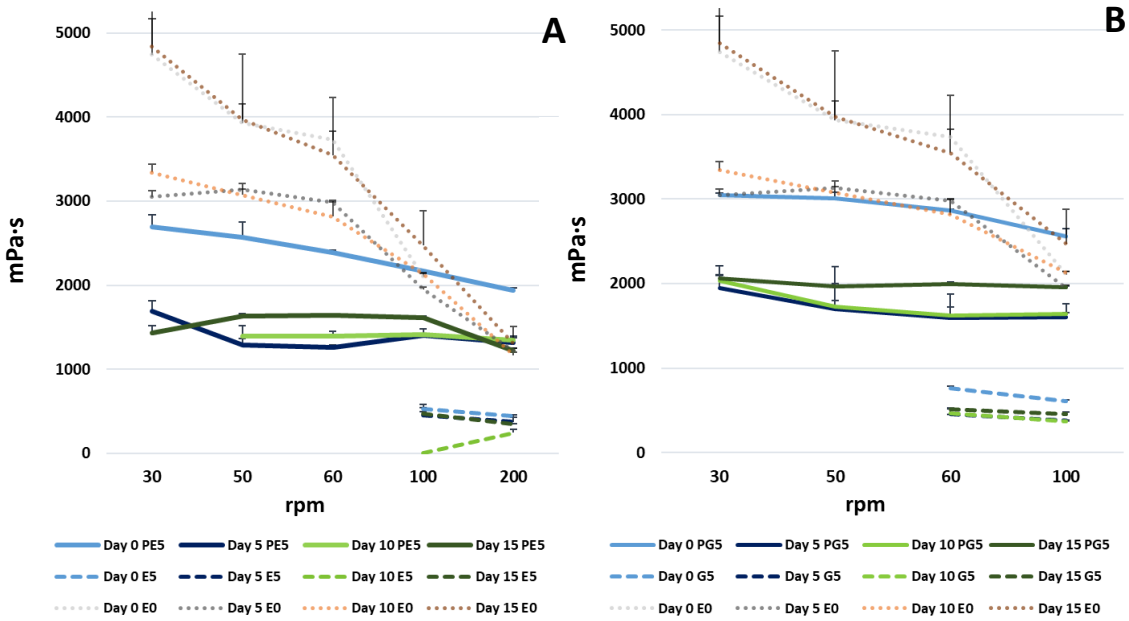



**Figure 15.** *Extreme temperature viscosity results at 45 °C.* Source: Author. Note: A - Ethanolic samples and controls; B - Glycolic samples and controls; E0: Neutral sample; E5: Sample with 5% of ethanol; G5: Sample with 5% of monopropylene glycol; PE5: Sample with 5% of ethanolic propolis extract; PG5: Sample with 5% of glycolic propolis extract.

Oliveira (2025) indicated that the changes of viscosity in different temperatures are expected, since liquids become less viscous when heated because their molecules move with more energy and become more viscous when cooled, as the energy of the molecules decreases, increasing resistance to flow. Even though the viscosity measurements were taken at 25 ± 2°C, the samples maintained the same viscosity as when exposed to 45°C. Thus, even after the removal of the high temperatures, the formulation did not return to its initial consistency, which may indicate some chemical changes in the sample. In Figure 15, the PE5 and PG5 curves on day 0, at 100 rpm, were higher than on days 5, 10, and 15, indicating higher viscosity at the beginning of the tests than at the end. Although none of samples were approved (viscosity lager than 2,500 mPa·s), PG5 results were better than PE5, suggesting again a promising incorporation of glycolic extract.

Ultimately, the viscous behavior of the samples subjected to the freeze-thaw cycle was measured and graphically represented in Figure 16. As in the other tests, all controls failed to

reach the minimum value required for acceptance at 100 rpm. For the PE5 samples, only the values from the 4th day were greater than 2,500 mPa·s. In contrast, for PG5 (Figure 16-B), all samples showed values above the minimum, which allowed their approval in the viscosity analysis.

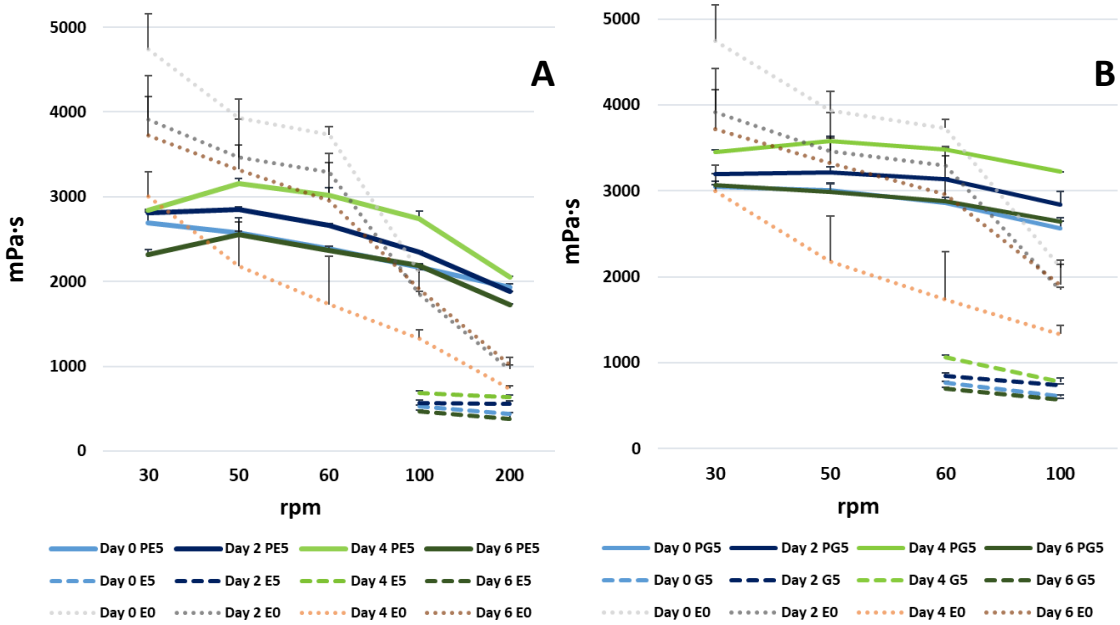



**Figure 16.** *Freeze-thaw cycle viscosity results.* Source: Author. Note: A - Ethanolic samples and controls; B - Glycolic samples and controls; E0: Neutral sample; E5: Sample with 5% of ethanol; G5: Sample with 5% of monopropylene glycol; PE5: Sample with 5% of ethanolic propolis extract; PG5: Sample with 5% of glycolic propolis extract.

Analyzing all graphics by their curve, almost every sample had behavior of Non-Newtonian Fluids, it means that viscosities changed in response to external forces, therefore, the higher the rotations per minute, the lower the values in mPa·s. This behavior may indicate a plastic or pseudoplastic characteristic of the liquid soap. Nonetheless, freeze-thaw and 5°C exposure graphs (B) showed that, at low temperatures, glycolic propolis formulations showed a tendency to behave like a Newtonian fluid, in other words, the viscosity remains constant regardless of the force applied (Oliveira, 2025). Therefore, based on the overall assessment of the viscous performance of the samples, the incorporation of glycolic extracts of propolis may contribute better to the stability of the liquid soap.

#### 4.5.2.3 Colorimetric analysis

In general, polyphenols are classified as non-flavonoids, colorless, and flavonoids (excluding anthocyanins, flavanols and proanthocyanidins) which are yellow or brown, depending upon their structures. Briefly, flavones and flavonols tend to be yellow, thus, the richness in apigenin, chrysin, quercetin and galangin of the samples, provides a natural yellow liquid soap (Shoji, T., 2007).

Considering the possible oxidation processes, the samples were evaluated by color when subjected to different conditions. Figure 17 show the color changes of the formulas after exposure to light for 15 days.

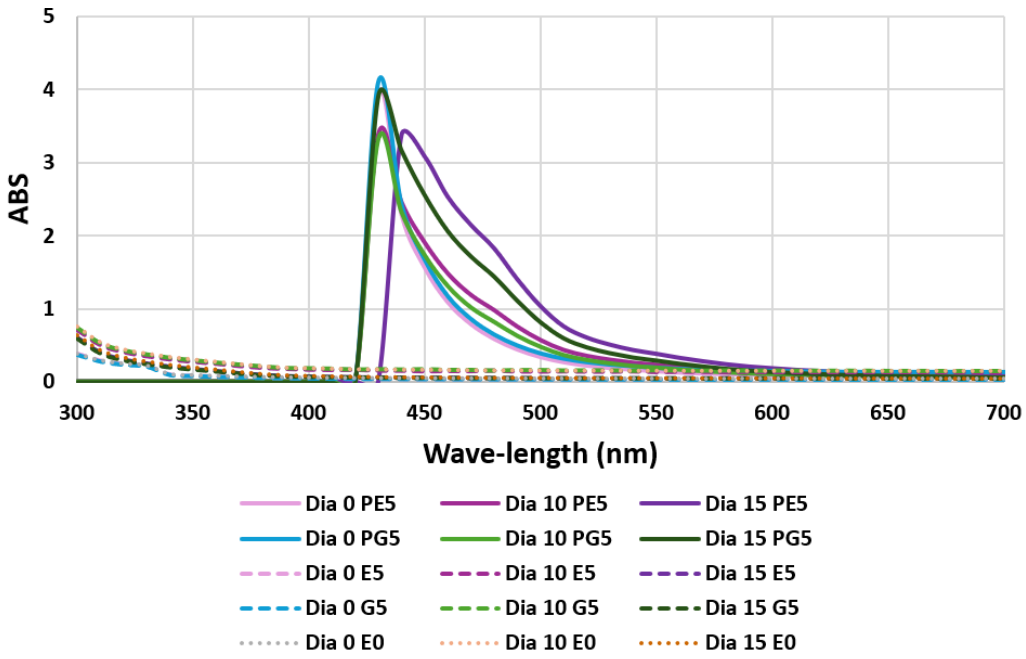



**Figure 17.** *Light test colorimetric results of ethanolic and glycolic samples*. Source: Author.

Note: E0: Neutral sample; E5: Sample with 5% of ethanol; PE5: Sample with 5% of ethanolic propolis extract; G5: Sample with 5% of monopropylene glycol; PG5: Sample with 5% of glycolic propolis extract.

The word color referring to a wavelength of light is related to the (non-physical, but conventional) division of the visible spectrum into colors. According to the authors, yellow color could be detected in wavelengths between 560 and 580 nm, but the addition of red and green frequencies results also in yellow, but in a different wavelength (Scarinci, A. L., &

Marineli, F., 2014). Therefore, after scanning the propolis formulas (yellowish) in a spectrophotometer, in the visible region (300 nm - 700 nm), the absorbance peak was around 430 nm for samples in day zero of assay.

In the ethanolic sample, the absorbance peaks remained between 430 and 450 nm, even after 15 days, while the glycolic sample remained stable around 430 nm. Although Figure 16 did not show any change in the peak profile, the soap appeared to acquire a slightly more orange color after 15 days (Figure 22), suggesting slight oxidation.

Similar to the previous test, the soaps stored at 5 ℃ maintained peaks at 430 nm throughout the days (Figure 18), and visually, no change in the yellowish coloration was perceived (Figure 19). Therefore, as expected, the colors of the formulas remained stable at low temperatures. The same behavior was observed in freeze-thaw cycle colorimetric results (Figure 20). In contrast, samples at 45℃ passed to 430 nm on day zero to around 515 nm (Figure 21), consequently, underwent a high change of color change, which signaled degradation of phenolic by oxidation.

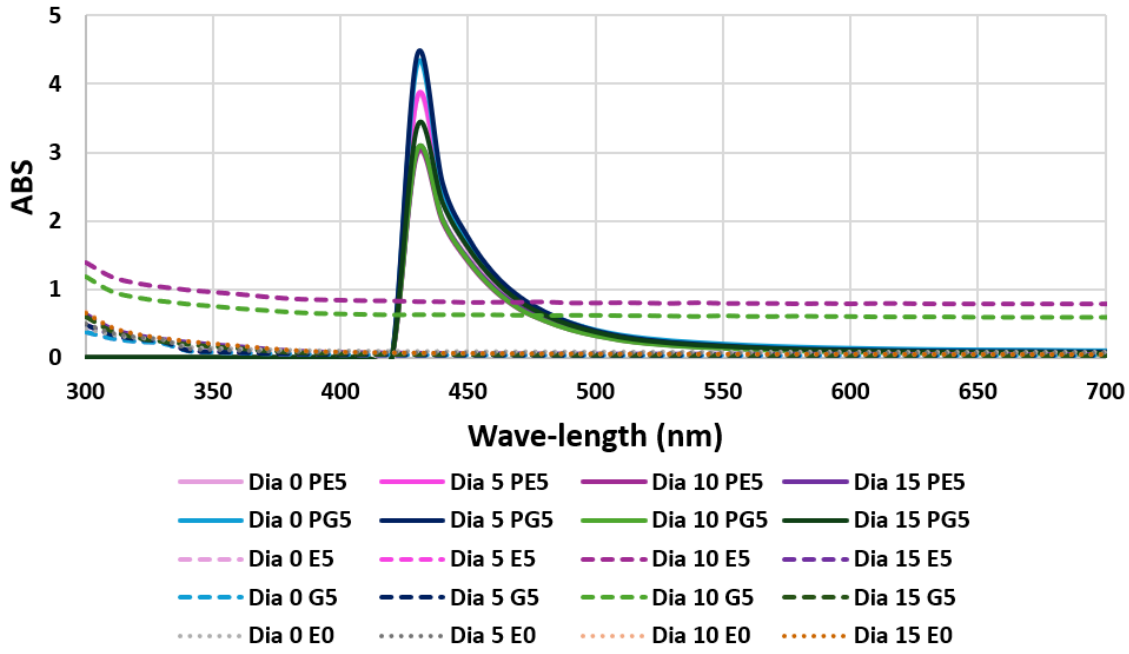



**Figure 18.** *Extreme temperature colorimetric results of ethanolic and glycolic samples at 5 ℃.* Source: Author. Note: E0: Neutral sample; E5: Sample with 5% of ethanol; PE5: Sample with 5% of ethanolic propolis extract; G5: Sample with 5% of monopropylene glycol; PG5: Sample with 5% of glycolic propolis extract.

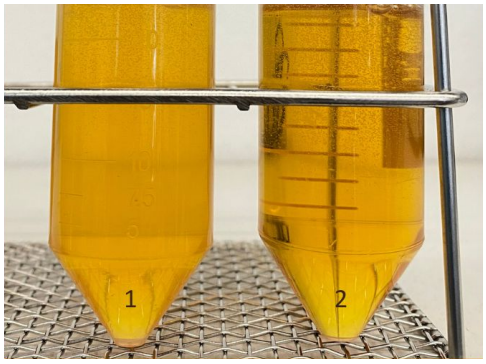



**Figure 19.** *Extreme temperature of ethanolic samples at 5 ℃ comparison results.* Source: Author. Note: 1: Day 0 of ethanolic samples at 5℃; 2: Day 15 of ethanolic samples at 5℃.

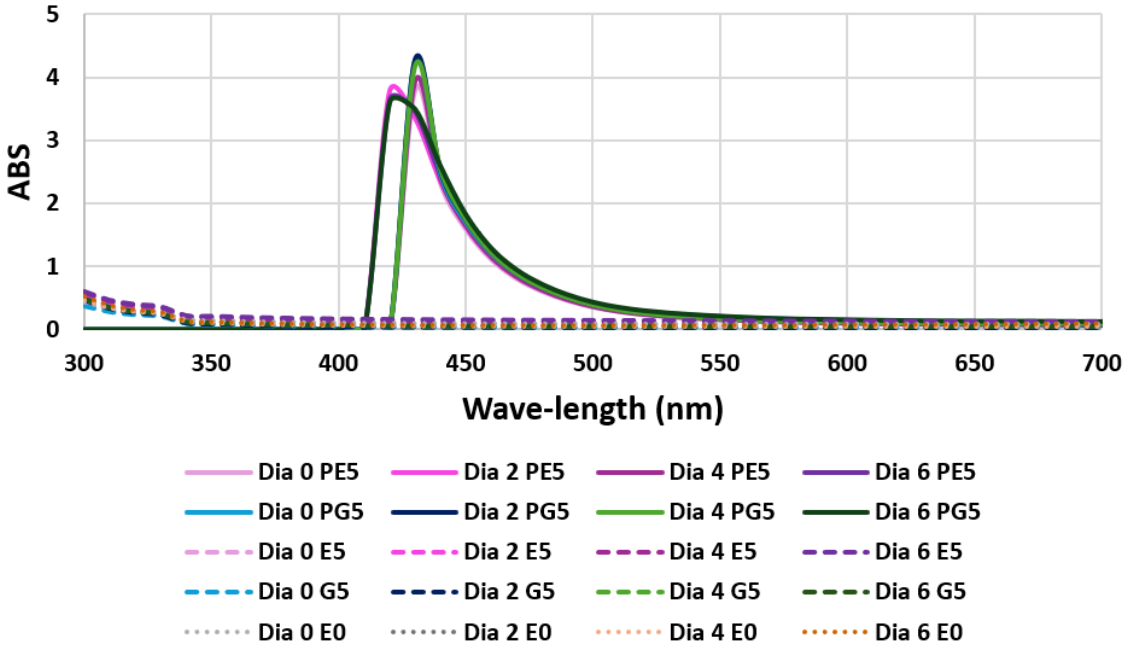



**Figure 20.** *Freeze-thaw cycle colorimetric results of ethanolic and glycolic samples.* Source: Author. Note: E0: Neutral sample; E5: Sample with 5% of ethanol; PE5: Sample with 5% of ethanolic propolis extract; G5: Sample with 5% of monopropylene glycol; PG5: Sample with 5% of glycolic propolis extract.

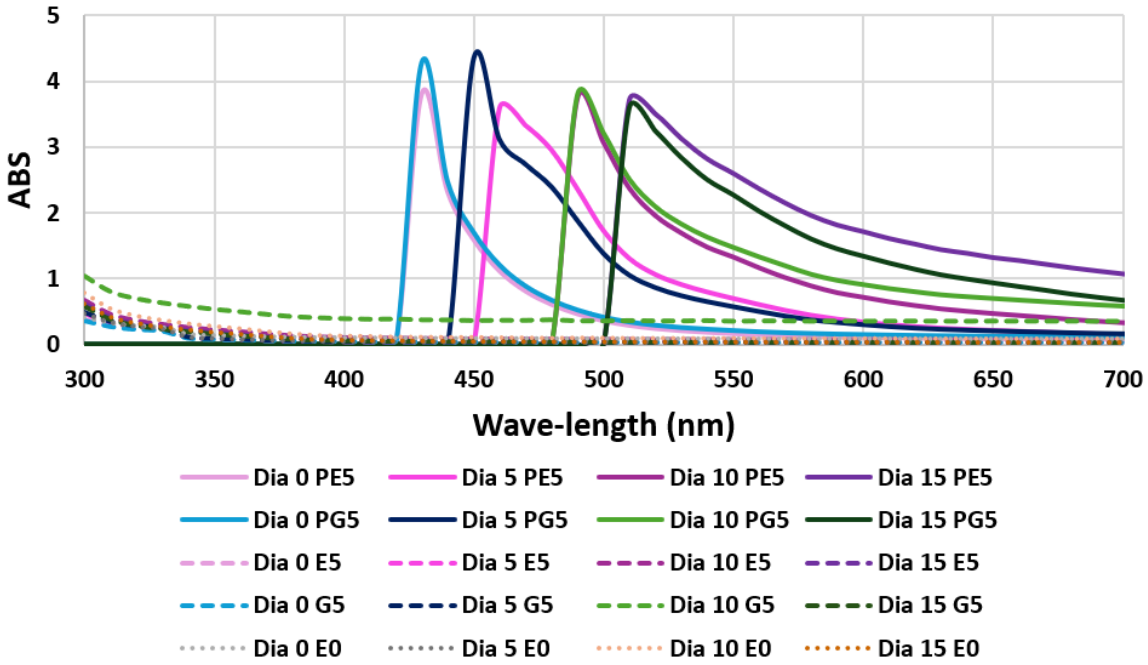



**Figure 21.** *Extreme temperature colorimetric results of ethanolic and glycolic samples at 45℃.* Source: Author. Note: E0: Neutral sample; E5: Sample with 5% of ethanol; PE5: Sample with 5% of ethanolic propolis extract; G5: Sample with 5% of monopropylene glycol; PG5: Sample with 5% of glycolic propolis extract.

Figure 22 shows the light test on the 15th day and temperature color comparison results. Over 15 days, the color of liquid soap at 5℃ continues a translucent yellow, while light exposure turns the sample orange and at 45℃, the formula darkened to a dull brown. In Figure 23, the day 6 of freeze-thaw cycle color was compared with the other conditions results on day 5.

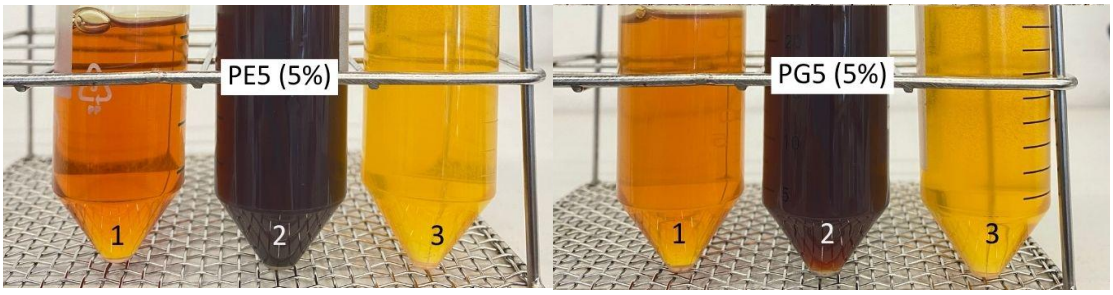



**Figure 22.** *Day 15 of light and temperature color comparison results.* Source: Author. Source: Author. Note: 1: Day 15 color of Light test; 2: Day 15 color of Extreme temperature test (45°C); 3: Day 15 color of Extreme temperature test (5°C).

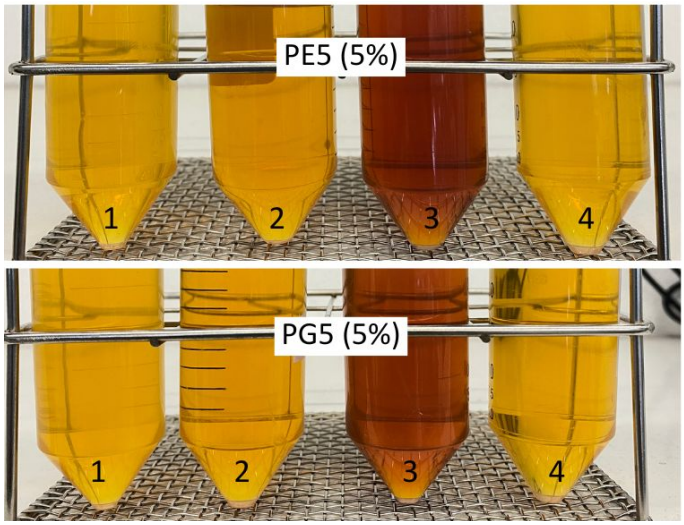



**Figure 23.** *Day 6 of freeze-thaw cycle color comparison results.* Source: Author. Note: 1: Day 6 color of Freeze-thaw cycle; 2: Day 5 color of Light test; 3: Day 5 color of Extreme temperature test (45°C); 4: Day 5 color of Extreme temperature test (5°C).

Considering the direct relationship between phenolic content and antimicrobial activity, the TPC of the formulation was evaluated at the beginning of the stability tests and at the end (on the 15th day) of the sample exposed to 45 °C. The clear color change in the propolis formulations at high temperatures was the main criterion for selecting this sample for evaluation, as it was the darkest. The results were organized in Table 18.

Table 18 - TPC in liquid soap formulation.

| Samples | Day 0<br>TPC (mg GAE/mL) | Day 15<br>TPC (mg GAE/mL) |
|---|---|---|
| PE5 | $2.078 \pm 0.008^{a}$ | $1.306 \pm 0.003^{b}$ |
| PG5 | $1.968 \pm 0.004^{ab}$ | $1.705 \pm 0.006^{ab}$ |

Source: Author. Note Different lowercase letters indicate significant differences ($p < 0.05$); PE5: Sample with 5% of ethanolic propolis extract; PG5: Sample with 5% of glycolic propolis extract.

Temperature, chemical interactions, and enzymatic reactions contribute to the decrease in phenolic components (Akhan *et al.*, 2025). As expected, the assay indicated significant variation in phenolic compounds over the days, which is associated with the darkening of the samples, suggesting that the liquid soap underwent oxidation. To avoid this, two approaches could be adopted in future formulations: increasing the concentration of extracted propolis and/or increasing the cosmetic incorporation of the extract, since propolis is abundant in antioxidant activity. Alternatively, the addition of a second antioxidant agent could be tested, such as vitamin E, for example. However, knowing that propolis can act as an oxidation inhibitor, the effort to make it act in this way would be a differentiating factor for the final product, targeting the natural cosmetics market.

## 5.CONCLUSIONS AND FUTURE PERSPECTIVES

The results presented in this study highlight the potential of propolis from the Montesinho Natural Park as a valuable bee product. Propolis is a highly bioactive natural product due to its rich phenolic profile. To extract the phenolic compounds, maceration and ultrasound extraction were made with several solvents. Spectrophotometric analysis revealed that propolis maceration with monopropylene glycol (MMPG) was more efficient than maceration with ethanol 70% (ME70), yielding higher total phenolic content (65.9 vs. 63.1 mg GAE/mL), total flavonoids (23.5 vs. 18.4 mg QE/mL), and antioxidant activity (DPPH: 88.4 vs. 78.1 mg AAE/mL and ABTS: 86.1 vs. 75.4 mg AAE/mL), respectively. The UHPLC-DAD-ESI/MS qualitative analysis showed similar chemical profiles between both solvents, composed of 44 compounds, predominantly flavonoids (chrysin, pinocembrin, galangin, pinobanksin) and phenolic acids (caffeic acid, *p*-coumaric acid, ferulic acid) and their methyl esterified derivatives.

The phenolic profile of propolis gives it an antimicrobial activity, which was evaluated against Gram-positive, Gram-negative, yeast and fungus by disk diffusion, MIC and MBC. Antimicrobial assays revealed high inhibitory, bactericidal and antifungal potential of both propolis extracts. ME70 was slightly stronger against *Staphylococcus aureus*, *Salmonella enterica* and *Candida albicans*, while MMPG stood out for its potent bactericidal action against *Pseudomonas aeruginosa*, *S.aureus* and *Cutibacterium acnes*.

Considering the growing demand for natural matrices, propolis shows promising potential as an ingredient in the cosmetics industry. The incorporation of 5% of the extracts into the liquid soap seems to corroborate with the stability of the pH (6.0) and viscosity, especially at 5 ℃. Although high temperatures (45 ℃) triggered slight oxidation, the final product showed promising bactericidal and antifungal potential against *Escherichia coli*, *S. enterica*, *S. aureus*, *Cutibacterium acnes* and *C. albicans*. Overall, both extracts had promising perspectives as an antiseptic agent in cosmetics, even though the MMPG performed better in stability assays.

In addition, the present work unlocked some possibilities for propolis extract incorporation in different cosmetic products, namely face and intimate soap, and also to

cleaning and antiseptic products, due the chemical characteristic and the response against various microorganisms.

Although this study was just the beginning to better understand the future of propolis extract applications potential in cosmetics, mainly the propolis from Montesinho Natural Park which is rich in botanical and natural properties. Therefore, to continue this line of work, other analyzes could be done for the extract and cosmetic formulation. For propolis extract, a new study should be done to examine different solvents and its concentrations and to ascertain if the amplitude of ultrasonic extraction interferes in phenolic extraction results. Also, for the liquid soap a new phase 2 needs to be made, to correct some observed problems. After, a thirst phase of stability assay needs to be conducted, namely accelerated stability, challenge test, shelf-life analyzes and different in vivo tests to analyze the interaction skin-product, which as patch test, hydrations and elasticity of the skin and self-assessment studies for consumer perception. It would also be interesting to evaluate the propolis extracts and products against bacterial biofilms, resistant microorganism strains and its application in treatment wounds.

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