Universidade do Minho
Escola de Engenharia

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Extraction of bioactive compounds from propolis for application in different matrices in food industry

outubro de 2023

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Extraction of bioactive compounds from propolis for application in different matrices in food industry

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Trabalho realizado sob a coorientação da
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outubro de 2023

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Resumo

Nos dias atuais, as indústrias tentam implementar alternativas sustentáveis para promover a economia circular. A indústria alimentar é reconhecida como uma das maiores geradoras de desperdício devido aos elevados volumes de produção. Para enfrentar esse desafio, a biotecnologia tem direcionado seu conhecimento em busca de soluções biológicas, conferindo um valor adicional a subprodutos industriais.

A própolis, um subproduto da indústria apícula, produzida pelas abelhas *Apis mellífera* a partir de exsudatos de diferentes fontes vegetais. Esta substância resinosa é conhecida pela sua composição rica em compostos bioativos, que conferem propriedades antioxidantes, antinflamatórias, antibacterianas, antitumurais e imunomoduladoras.

Neste estudo, foi realizada uma análise da composição da própolis colhida na Guarda, colhida em 2023 (G23), resultando em valores de cinzas de 0,64 ± 0,01% e de teor de humidade de 5,9 ± 0,17%. Para avaliar a eficiência de extração dos compostos bioativos presentes na própolis G23, foram utilizados os solventes EtOH 96%, H_2O e EtOH 70%, obtendo-se um rendimento de extração de 75,5%, 4,2% e 73,8%, respetivamente. Nos extratos resultantes da extração quantificou-se o conteúdo total de fenólicos (TPC), atingindo o melhor resultado referente ao solvente EtOH 96% (276,9 ± 1,0 mg GA /g). Também se calcularam os valores do conteúdo total de flavonóides (TFC), revelando o maior teor no solvente EtOH 70% (463,7 ± 0,9 mg CTE /g). Na análise da atividade antioxidante utilizaram-se três métodos diferentes, avaliando diferentes mecanismos de atuação: 2,2-diphenyl-1-picrylhydrazyl (DPPH), com valor mais elevado do solvente EtOH 96% (1,73 ± 0,14 mmol TEAC/g); ácido 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic (ABTS), alcançando o melhor valor referente ao EtOH 70% (2049,5 ± 0,2 µmol TEAC/g); e o Ferric Reducing Antioxidant Power (FRAP), com valor pertencente ao EtOH 96% de 2410,9 ± 0,1 µmol TEAC/100 g própolis. Também se estudou as correlações entre todas as análises anteriores obtendo, em todas elas, coeficientes de correlação positivos. Por último, também se realizou o ensaio de inibição da tirosinase, revelando um valor caraterístico, pertencente ao EtOH 96% (46,9 ± 0,95%). Os resultados alcançados reforçam toda a potencialidade dos extratos de própolis, podendo esta ser utilizada tanto na indústria alimentar, como ingrediente nutricional e/ou como bioconservante, e na indústria da cosmética, como ingrediente antioxidante para prevenir sinais de envelhecimento.

Palavras-Chave: Própolis, Atividade Antioxidante, Atividade Antienvelhecimento, Compostos Bioativos, Indústria Alimentar

Abstract

Nowadays, the industry is trying to introduce sustainable alternatives to promote the circular economy. The food industry is considered one of the largest generators of waste due to its high production volume. To meet this challenge, biotechnology has directed its knowledge towards finding biological solutions that add value to industrial by-products.

Propolis, a byproduct of beekeeping, is produced by *Apis mellifera* bees from the exudates of various plant sources. This resinous substance is known for its composition rich in bioactive compounds that confer antioxidant, anti-inflammatory, antibacterial, antitumor and immunomodulatory properties.

In this study, the composition of propolis harvested in Guarda in 2023 (G23) was analysed. The results were ash values of 0.64 ± 0.01% and moisture content of 5.9 ± 0.17%. To evaluate the extraction efficiency of the bioactive compounds present in the G23 propolis, the solvents EtOH 96%, H_2O and EtOH 70% were used, obtaining extraction yields of 75.5%, 4.2% and 73.8%, respectively. The total phenolic content (TPC) of the extracts obtained from the extraction was quantified, the best result being obtained with the solvent 96% EtOH (276.9 ± 1.0 mg GA / g). The values for total flavonoid content (TFC) were also calculated and showed the highest content in the solvent 70% EtOH (463.7 ± 0.9 mg CTE / g). Three different methods were used to analyse antioxidant activity, evaluating different mechanisms of action: 2,2-diphenyl-1-picrylhydrazyl (DPPH), with the highest value in solvent 96% EtOH (1.73 ± 0.14 mmol TEAC / g); 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), with the best value in solvent 70% EtOH (2049.5 ± 0.2 µmol TEAC / g); and ferric reducing antioxidant power (FRAP), with a value for 96% EtOH of 2410.9 ± 0.1 µmol TEAC / 100 g propolis. Correlations between all previous analyses were also examined, and positive correlation coefficients were obtained for all of them. Finally, the tyrosinase inhibition assay was also performed, which gave a characteristic value for 96% EtOH (46.9 ± 0.95%).

The results obtained underline the full potential of propolis extracts, which can be used both in the food industry as a food ingredient and/or as a biopreservative, and in the cosmetic industry as an antioxidant ingredient for the prevention of signs of aging.

Keywords: Propolis, Antioxidant Activity, Antiaging Activity, Bioactive Compounds, Food Industry

Table of Contents

1. Introduction 15
1.1 Waste from the Food Industry 15
1.2 Bioactive Compounds 15
1.2.1 Phenolic Compounds 16
1.2.2 Industrial Applications of Phenolic Compounds 16
1.3 Propolis 17
1.3.1 Propolis Overview 17
1.3.2 Physicochemical Composition of Propolis 18
1.3.3 Bioactive Composition of Propolis 20
1.3.4 Main Bioactivities of Propolis 22
1.3.5 Industrial Applications of Propolis 23
2. Objectives 26
3. Material and Methods 27
3.1 Sample Conditions 27
3.2 Sample Preparation 28
3.3 Bioactive Compounds Extraction 28
3.4 Propolis Samples and Extracts Characterization 29
3.4.1 Moisture 29
3.4.2 Ash 29
3.4.3 Dry Weight (DW) of Extracts Propolis 30
3.5 Total Phenolic and Flavonoid Contents of Propolis 30
3.5.1 Total Phenolic Compounds Content (TPC) 30
3.5.2 Total Flavonoids Content (TFC) 31
3.6 Antioxidant Activity of Propolis' Extracts 32
3.6.1 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) 32

3.6.2 2,2-diphenyl-1-picrylhydrazyl (DPPH) 33
3.6.3 Ferric Reducing Antioxidant Power (FRAP) 33
3.7 Anti-aging Activity of Propolis' Extracts 34
3.7.1 Elastase Inhibition Assay 34
3.7.2 Tyrosinase Inhibition Assay 35
3.8 Statistical Analysis 36
4. Results and Discussion 37
4.1 Proximate Composition Analysis 37
4.2 Efficiency of Extraction 39
4.3 Total Phenolic and Flavonoid Contents of Propolis 40
4.3.1 Total Phenolic Content (TPC) 41
4.3.2 Total Flavonoids Content (TFC) 43
4.4 Evaluation of Antioxidant Activity of Propolis 45
4.4.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) 45
4.4.2 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) 46
4.4.3 Ferric Reducing Antioxidant Power (FRAP) 48
4.5 Correlation of Antioxidant Activity with Total Phenolic and Flavonoid Content 50
4.6 Anti-aging Activity of Propolis 53
4.6.1 Tyrosinase Inhibition Assay 53
4.6.2 Elastase Inhibition Assay 55
5. Conclusions 57
6. Future perspectives 58
7. References 59
8. Appendixes 68
8.1 Appendix 1: GAE calibration curve of Total Phenolic Content (TPC) 68
8.2 Appendix 2: CTE calibration curve of Total Flavonoids Content (TFC) 68

8.3 Appendix 3: TEAC Calibration Curve of ABTS 69

8.4 Appendix 4: TEAC Calibration Curve of DPPH 69

8.5 Appendix 5: TEAC Calibration Curve of FRAP 70

List of Figures

Figure 1 – Two types of collector traps and harvested raw propolis [47]. 19

Figure 2 – Typical composition of propolis, which consists of 50% resins (including phenolic compounds), 30% beeswax, 10% essential oils, 5% pollen and 5% other inorganic substances [57]. 20

Figure 3 – An accurate representation of the propolis sample used in this study, designated G23, with a production time of about one month, with visible residues from the hives, such as bee legs and wing debris. 27

Figure 4 – Schematic overview of the extraction procedure for obtaining the bioactive compounds contained in firs propolis sample in three different solvents (EtOH 96%, H_2O and EtOH 70%) at room temperature. 28

Figure 5 – Illustration of the dry weight (DW) method for the supernatant of the extraction. 30

Figure 6 – The yield of bioactive compounds from Propolis G23 extracted at room temperature for 24 hours under stirring at 150 rpm with three different solvents, EtOH 96%, H_2O and EtOH 70%. Values are given as mean ± standard deviation (SD). Different letters (a, b) indicate significant differences ($p < 0.05$) between the extraction solvents for the same propolis sample. 39

Figure 7 – Total Phenolic Content (TPC) from G23 propolis determined by the Folin-Ciocalteu method, expressed as: **a)** milligrams GAE per gram of propolis; **b)** milligrams GAE per gram of extract. Values are expressed as mean ± SD. Different letters (a, b) indicate significant differences ($p < 0.05$) between the extraction solvents for the same propolis sample. 42

Figure 8 – Total Flavonoids Content (TFC) from G23 propolis determined by the Folin-Ciocalteu method, expressed as: **a)** milligrams CTE per gram of propolis; **b)** milligrams CTE per gram of extract. Values are expressed as mean ± SD. Different letters (a, b) indicate significant differences ($p < 0.05$) between the extraction solvents for the same propolis sample. 44

Figure 9 – Antioxidant capacity DPPH, of G23 corresponding to 96% EtOH, H2O and 70% EtOH samples extracted by the maceration method, at room temperature. Values are expressed as mean ± SD. Different letters (a, b) indicate significant differences ($p < 0.05$) between the extraction solvents for the same propolis sample..... 46

Figure 10 – Antioxidant capacity ABTS, of G23 corresponding to 96% EtOH, H2O and 70% EtOH samples extracted by the maceration method, at room temperature. Values are expressed as mean

± SD. Different letters (a, b) indicate significant differences ($p < 0.05$) between the extraction solvents for the same propolis sample..... 47

Figure 11 – Antioxidant capacity FRAP, of G23 corresponding to 96% EtOH, H_2O and 70% EtOH samples extracted by the maceration method, at room temperature. Values are expressed as mean ± SD. Different letters (a, b) indicate significant differences ($p < 0.05$) between the extraction solvents for the same propolis sample..... 49

Figure 12 – Observed correlations between phenolic content and antioxidant activity: **a)** between TPC (mg GAE/g propolis) and DPPH analysis (mmol TEAC/g propolis); **b)** between TPC (mg GAE/g propolis) and ABTS analysis (mmol TEAC/g propolis); and **c)** between TPC (mg GAE/g propolis) and FRAP assay (mmol TEAC/g propolis)..... 51

Figure 13 – Observed correlations between flavonoids content and antioxidant activity: a) between TFC (mg CTE/g propolis) and DPPH analysis (mmol TEAC/g propolis); b) between TFC (mg CTE/g propolis) and ABTS analysis (mmol TEAC/g propolis); and c) between TFC (mg CTE/g propolis) and FRAP assay (mmol TEAC/g propolis)..... 52

Figure 14 – Tyrosinase inhibitory activity of G23 corresponding to samples containing 96% EtOH, H_2O and 70% EtOH, with concentrations of 135.4 mg/mL, 5 mg/mL and 134.1 mg/mL, respectively. Values are expressed as mean ± SD. Different letters (a, b) indicate significant differences ($p < 0.05$) between the extraction solvents for the same propolis sample. 54

Figure 15 – Calibration curve of Gallic acid (0 - 150 mg/mL) as standard for Total Phenolic Content. 68

Figure 16 – Calibration curve of Catechin (0 - 200 mg/mL) as standard for Total Flavonoids Content. 68

Figure 17 – Calibration curve of Trolox-equivalent antioxidant capacity (TEAC) (0 - 1.25 mmol/L) as standard for ABTS..... 69

Figure 18 – Calibration curve of Trolox-equivalent antioxidant capacity (TEAC) (0 - 1.25 mmol/L) as standard for DPPH. 69

Figure 19 – Calibration curve of Trolox-equivalent antioxidant capacity (TEAC) (0 - 1.25 mmol/L) as standard for FRAP..... 70

List of Tables

Table 1 – Ash and moisture content (%/g DW) of G23, compared with that proposed for propolis-Guarda (G13) [124].Values are given as mean ± standard deviation (SD). 38

List of abbreviations and acronyms

ABTS: 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)

BACs: Bioactive Compounds

C: Celsius

DPPH: 2,2-diphenyl-1-picryl-hydrazyl

DW: Dry Weight

EtOH: Ethanol

FRAP: Ferric Ion Reducing Antioxidant Power

GAE: Gallic Acid Equivalent

H_2O: Distilled water

M: Molar

mmol: Millimole

nm: Nanometer

PC: Phenolic Compounds

ROS: Reactive Oxygen Species

rpm: Revolutions per minute

TE: Trolox Equivalent

TPC: Total Phenolic Content

TPTZ: 2,4,6-tripyridyl-s-triazine

UV: Ultraviolet

UV/vis: Visible ultraviolet

W: Watt

1. INTRODUCTION

1.1 Waste from the Food Industry

The industrial sector has significantly improved its production approaches over the years, but there is still great concern about the depletion of natural resources and the negative impact on the environment. In light of this, it is extremely important to explore alternatives that promote the reuse of byproducts wasted in landfills by various industries, as well as promote circular economy, reuse, and reduction [1].

The food industry is a sector essential for human survival and is therefore considered an industry with extremely high production values. This production generates significant amounts of waste, which are frequently inadequately disposed of in landfills, leading to a reduction in available land for agriculture and causing harm to both health and the environment [2]. To address this issue, the concept of a circular economy provides a range of tools to promote recycling of these waste materials and establish a system in which products are designed to minimize or eliminate waste generation. This fosters a culture in which products are created with the intention of giving them a second life with added value, while also reducing energy and raw material consumption [3].

The wastes and losses of various by-products in the food industry can serve as sources for a variety of compounds such as enzymes, nutrients, and highly potent bioactive compounds. These compounds add value to a range of industries, including food, cosmetics, and pharmaceuticals. In doing so, the industry plays a significant role in driving the shift towards sustainable production, contributing to a paradigm change [4].

1.2 Bioactive Compounds

Bioactive compounds (BACs) are derived from plants, animals, and microorganisms, and have the ability to interact with one or more components of living tissue to produce a wide range of potential effects [5, 6].

BACs (Bioactive Compounds) exhibit a wide array of compelling biological activities, including antioxidant, anti-inflammatory, anticancer, antidiabetic, antitumor, and antiviral properties. They protect the human body from high concentrations of free radicals and reactive oxygen species

(ROS), which can react with other molecules and cause cell and tissue damage. This phenomenon is called oxidative stress and is characterized by the inability of natural antioxidants to control the formation of ROS [7-10]. Since the processes of inflammation and oxidative stress are associated with several diseases, such as cardiovascular disease, neurological disorders, hypertension, cancer, diabetes mellitus, and obesity, it is extremely important to seek alternative treatments to minimize these effects and promote human health [11]. With this in mind, research into natural products has evolved over the years as they are considered to have beneficial properties and fewer side effects compared to synthetic drugs [12, 13]. These beneficial properties are mainly attributed to its antioxidant and ROS -eliminating activities, which can slow down or inhibit the oxidation of DNA, proteins and lipids [11, 14].

A food is classified as functional when it incorporates biologically and physiologically active compounds that not only offer nutritional value but also deliver health benefits [7, 15, 16]. Through a specific mechanism, the inclusion of BACs in functional meals can help reduce or prevent the possibility of disease [17].

1.2.1 Phenolic Compounds

Currently, phenolic compounds (PC) stand as the most extensively researched class of BACs within the scientific community. This is due to their manifold health-promoting biological activities, including antioxidant, anti-inflammatory, antimicrobial, anticancer, antitumor, and antiviral effects [18, 19]. Furthermore, they serve as the primary products of plant secondary metabolism and are regarded as non-essential components in human nutrition. Phenolic compounds also play a role in pigmentation and astringency. In terms of their function, they serve as protective agents for plants, shielding them from UV rays, parasites, and insects [11].

1.2.2 Industrial Applications of Phenolic Compounds

PCs find extensive biotechnological applications across various industries. Their primary bioactivities, particularly antioxidant and antimicrobial properties, are the key factors driving their utilization in the food, packaging, cosmetics, and textile industries [20].

Within the food industry, phenolic compounds (PCs) are extensively employed as natural preservatives, effectively extending the shelf life of a variety of food products, especially when the use of artificial additives is restricted [21, 22]. In the cosmetics industry, PCs have exhibited significant potential as bioactive ingredients in products designed to offer UV protection, antioxidant and anti-aging effects, depigmentation, anti-inflammatory and antimicrobial properties, and support for the healing of skin wounds [23]. In the packaging industry, PCs have been used in formulations to produce packaging or biofilms with antioxidant and antimicrobial properties that can prevent lipid oxidation in foods [24, 25]. The textile industry is one of the most technologically advanced industries because it is one of the most polluting industries. In view of this, the use of PC has increased as natural dyes with high biodegradability, as they are less aggressive to the environment and provide UV protection when impregnated in silk and cotton [26, 27].These applications underscore the potential utility of phenolic compounds (PCs). Nevertheless, there are still certain challenges that must be addressed for the practical and feasible application of these molecules. These include the relatively low concentration of PC in plant tissues, which may not meet the industry's high-volume production requirements. which may not meet the industry's high-volume production requirements [20].

1.3 Propolis

1.3.1 Propolis Overview

Propolis is a unique natural beekeeping product, produced mainly by the honey bee species *Apis mellifera L.*, and in smaller quantities by species of stingless bees particularly the principal genus *Melipona*. It boasts valuable biological activities and health benefits [28, 29]. With advancements in analytical techniques by the International Honey Commission (IHC), a global network committed to developing novel and effective analytical methods for quality control of bee products and to establish quality standards, there has been a more comprehensive understanding of the composition of propolis and the factors influencing its production [30].

Beekeeping yields economic and social advantages and plays a crucial role in ecosystem health. Specifically, bee pollination plays a significant role in preserving biodiversity, boosting bee population, and enhancing food security [31-33]. Since honey production relies on the availability of floral resources, beekeeping can serve as a means to safeguard forest reserves and the very biodiversity where beehives are located [34, 35].

Bees collect resinous exudates with their mandibles from the shoots, leaves, flowers, fruits, twigs, and barks of various plants near their hive, to which they add wax and salivary secretions, including enzyme β – glucosidase, inside the hive to make propolis [34]. Once this sticky substance is ready, it is used to plug holes and cracks, smooth the inner walls of the hive, repair and seal the honeycomb cells, and embalm the bodies of intruders that have entered and died in the hive to eliminate the unpleasant odor and bacterial flora associated with their decay [36-39]. For this reason, propolis is crucial for the social immunity of honeybees. Due to its strong antibacterial properties, it can be considered an important component of the immune system of bees, protecting the colony from infectious and parasitic diseases [40, 41].

Propolis has a rich history that traces back to ancient Egyptian and Roman civilizations, where it found diverse applications. It was used for treating wounds, the mummification process, and as a remedy for conditions like eczema, myalgia, and rheumatism. It was even employed as a mouthwash [42]. During World War II, propolis demonstrated its efficacy as a valuable aid in wound healing. In 1969, medicine officially recognized its therapeutic potential and approved its use in an alcoholic solution (30%) [43].

1.3.2 Physicochemical Composition of Propolis

Propolis can be classified according to its physical and chemical properties such as color, texture, and chemical composition. These properties of propolis can vary greatly depending on the botanical origin, geographical location of the hives and bee population. Regarding its color, propolis can exhibit variations, typically ranging from green to brown or even red. Green propolis is widely distributed in Brazil and its main source is *Baccharis dracunculifolia* DC. Brown propolis is also found in Brazil and in Cuba, with different botanical origins such as *Luehea* sp. (Malvaceae), *Piptadenia falcate* Benth (Fabaceae), *Tabebuia* spp. (Bignoniaceae), *Tabebuia caraiba* (Mart.) Bureau (Bignoniaceae), *Vernonia* spp. (Asteraceae) and *Cecropia pachystachya* Trecul (Urticaceae). Red propolis is found in countries such as Brazil, Cuba, Mexico, China and Venezuela and is mainly grown on *Dalberguia ecastophyllum* (L.) Taub. (Fabaceae) [44, 45]. In Central Europe, including Poland, bees collect the secretion from the buds of poplars (*Populus spp.*) and alders (*Alnus spp.*). Other European countries, including England, Albania, Hungary and Bulgaria, as well as temperate countries such as the United States, western Asia, and northern Africa also use various poplar species as a source of resin. Birch (*Betula spp.*) is also a source of propolis in

northern Europe. In addition, bees also produce propolis from the shoots of willows (*Salix spp.*), oaks (*Quercus spp.*), ash trees (*Fraxinus spp.*), chestnuts (*Aesculus spp.*) and the bark of conifers such as spruce (*Picea spp.*), fir (*Abies spp.*) or pine (*Pinus spp.*) [46].

Propolis has a very distinct aroma and depending on the climate in which the hive is located, can be in a sticky, liquid or solid state, the latter being most common when propolis is collected from a hive.

The chemical composition of raw propolis exhibits significant variability, contingent upon factors such as its botanical source, environmental conditions, hive location, and the particular bee colony. Beekeepers' ability to collect high-quality and substantial quantities of propolis hinges on several aspects, including the diversity and availability of nearby plants, the time of year, and their own collection techniques and practices. It is essential for professionals to ensure that the harvested propolis is free from impurities. This collection is typically facilitated through the use of traps, which are nylon or plastic nets with small holes positioned on the roof or sides of the hive, encouraging the bees to fill these holes with propolis (**Figure 1**) [47]. The seasons of spring and autumn yield the highest propolis production, aligning with the colony's increased development and preparations for winter [48-50].



Figure 1 – Two types of collector traps and harvested raw propolis [47].

However, the typical composition is about 50% resins (including phenolic compounds), 30% beeswax, 10% essential oils, 5% pollen and 5% other inorganic substances (**Figure 2**) [46, 51]. This composition may vary as described above, but it has similar antifungal, antiviral, antiparasitic, antiproliferative, and antioxidant activities [52-55].

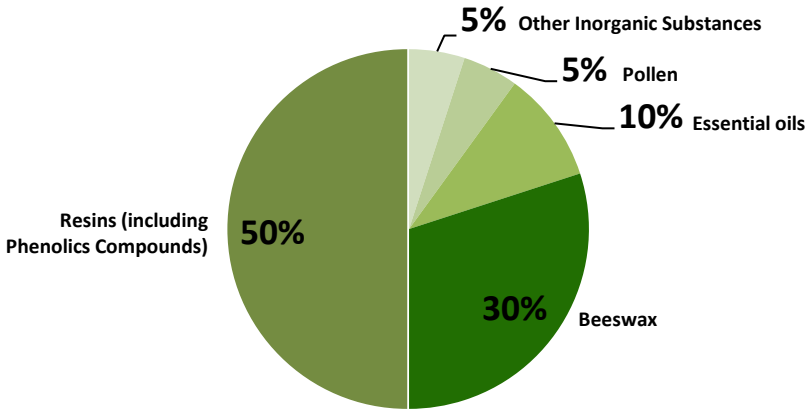


Figure 2 – Typical composition of propolis, which consists of 50% resins (including phenolic compounds), 30% beeswax, 10% essential oils, 5% pollen and 5% other inorganic substances [51].

1.3.3 Bioactive Composition of Propolis

The bioactive composition of each type of propolis and the bioactivities associated with it depend mainly on the species of bees in the colony and the macro- and microgeographic regions where the propolis is collected [48, 49]. In other words, the age of the bee and the presence of a variety of exudates from plant/tree buds, taking into account that bees have more preferences for some plant species than for others, are factors that influence the chemical content of propolis [56-59]. The most common plant/tree species are poplar (*Populus* spp.), birch (*Betula alba*), beech (*Fagus sylvatica*), horse chestnut (*Aesculus hippocastanum*), alder (*Alnus glutinosa*) and various conifers [60, 61].

Over the years, researchers have conducted several studies and analyses on various propolis extracts, not only because of their complex composition, but also because of the range of activities associated with them, and more than 300 chemical constituents have already been identified. The most important chemical classes in propolis are polyphenols, represented mainly by flavonoids such as tectochrysin, pinobanksin, pinocembrin, kaempferol, chrysin, galangin and apigenin. Other biologically active compounds in propolis are aromatic acids (benzoic acid, coumaric acid, cinnamic acid, caffeic acid, ferulic acid, gallic acid, salicylic acid, vanillic acid), aromatic esters (cinnamic and caffeic ethers), aliphatic acids (acetic acid, fumaric acid, angelic acid, crotonic acid, butyric acid, isobutyric acid, methylbutyric acid), aliphatic esters, volatile compounds (geraniol, nerol, farnesol, β-eudesmol), aromatic compounds (vanillin), hydrocarbons (eicosane, tricosane,

pentacosane), steroids (cholinasterol, fucosterol, stigmasterol), enzymes (α- and β-amylase), waxy acids (arachidic, palmitic, lauric, linoleic, oleic, stearic, myristic, cerotinic), alcohols, aldehydes, ketones, amino acids, micro- and macronutrients (Ca, K, Mg, Na, Zn, Fe, Mn, Al, Ba, Cl), vitamins (B1, B2, B6, C, E), essential oils (monoterpenes and sesquiterpenes), sugars, pollen and other organic substances. The classes of essential oils, terpenes and beeswax contained in propolis do not contribute significantly to the chemical composition and action of propolis [12, 46, 61-66].

As mentioned above, the content of phenolic compounds in propolis is reflected in its high bioactivity, the variability of which is related to its phytogeographical origin [48, 67, 68]. This variation is evident in the case of propolis collected in temperate regions, where poplar tree buds serve as the primary resin source. Such propolis tends to be abundant in phenolic acids, uncomplicated flavonoids,, and methylated and/or esterified derivatives [69, 70]. For propolis from tropical regions where the poplar plant is not abundant, bees look for alternative floral sources to produce resin [71].

For example, Brazilian propolis is very rich in prenylated derivatives of phenylpropanoic acid, such as artepilin C and caffeoylquinic acids. Brazil is a tropical country that produces a large amound of propolis, which has high bioactive activity and serves as the basis for numerous scientific studies due to this factor [61, 71-73]. However, several studies with different propolis samples have shown that the main secondary metabolites are phenolic substances, especially flavonoids, which are divided into several subclasses, such as flavanones, flavones, flavonols and dihydroflavonols, which represent the main class of phenolic compounds [61, 74].

European propolis is also the basis of study for several researchers. For example, Greek propolis has been shown to contain a wide variety of chemical compounds, including protocatechuic acid, pinocembrin, kaempferol, galangin, chlorogenic acid, ferulic acid, gallic acid, hesperetin, hydroxytyrosol, luteolin, p-coumaric acid, quercetin, caffeic acid, pinostrobin, syringic acid, resveratrol, eriodictiol, rosmarinic acid, transcinnamic acid, adipic acid, betulinic acid, isoferulic acid, naringin, vanillin, chrysoeriol, among many other compounds. Similarly, various compounds have been reported in Polish propolis, including aromatic acids, such as benzoic acid, dihydrocinnamic acid, cinnamic acid, p-hydroxybenzoic acid, vanillic acid, p-coumaric acid, p-methoxycinnamic acid, ferulic acid, dimethoxycinnamic acid, isoferulic acid, caffeic acid; fatty acids, flavonoids, chalcones and various esters [75, 76].

In Asia, propolis from various regions, especially China, has also been studied. Chinese propolis has been shown to be rich in caffeic acid, p-coumaric acid, ferulic acid, iosuofmerauric acid,

pinobanksin, naringenin, quercetin, kaempferol, apigenin, pinocembrin, chrysin, CAPE and galangin [77].

1.3.4 Main Bioactivities of Propolis

In recent years, due to its highly bioactive properties, essentially due to its phenolic composition, propolis is a natural product that has been extensively studied, not only to obtain a more accurate characterization, but also to evaluate its strong biological activities, namely antioxidant, anti-inflammatory, immunomodulatory, antiviral, antimicrobial, antibacterial, antifungal and antitumor activity [52, 68, 74].

Antioxidant activity is one of the most important and most studied activities of propolis, although there are no data yet on the safe dose for administration to humans. The chemical diversity of propolis extracts from different regions influences the antioxidant activity. The antioxidant activity is exerted by inhibiting the activity of certain enzymes (such as ascorbic acid oxygenase, cyclooxygenase, protein kinase C, cAMP), which inhibit the production of ROS species [78]. Moreira et al. (2008) and Miguel et al. (2010) indicate that Portuguese propolis is a potent antioxidant that protects human erythrocytes from free radical damage, reduces lipid peroxidation, and maintains the functional and structural integrity of cells [79, 80].

The inflammatory process is a response to frequent exposure to endogenous environmental stimuli or accidental damage. In other words, following tissue injury, the cell stimulates a complex cascade of chemical signals whose goal is to repair the tissue. Inflammation can be acute and is mediated by the activation of immune system cells that migrate to the site of damage and release growth factors, cytokines and ROS species. If this is not corrected, the result is what is known as chronic inflammation, which is fundamental to the development of various diseases such as cancer, atherosclerosis, asthma, Alzheimer's disease and Parkinson's disease [81-83].

Immunomodulatory activity refers to the ability of a particular substance to modulate or interact with the immune system in a way that affects its response. The immunomodulatory action of propolis takes place not only at the level of macrophages, which are responsible for the elimination of foreign bodies from the body. It also affects the proliferation of lymphocytes produced in response to infection [84, 85].

The bioactive compounds contained in propolis play an important role in antiviral protection. Propolis extracts have the antiviral activity to attenuate and interfere with the replication of a virus such as herpes and human immunodeficiency virus (HIV) [64, 86, 87].

The antimicrobial activity of propolis has been extensively studied and well documented as new treatments for infectious diseases are needed, especially with the increase in antibiotic-resistant pathogens [88].

Studies have shown that propolis extracts inhibit bacterial and enzymatic activity, are bacteriostatic against various bacterial genera, and can be bactericidal at high concentrations. For this reason, it has been proposed to combine propolis with certain antibiotics in order to reduce the dose of the antibiotic and thus enhance its effect [89, 90].

The antifungal activity of propolis is entirely related to the different geographical origins, as the effect against different fungi is significant, and this is of clinical interest. In one study, the antifungal activity of propolis extracts was demonstrated in the development of agrochemicals with lower economic costs [91].

Propolis is proving to be a promising natural antitumor agent because it contains a variety of phytochemical compounds that can attenuate or reduce the development of malignant properties of cancer cells. However, more thorough and detailed research on this topic is still needed [92, 93].

1.3.5 Industrial Applications of Propolis

The strong bioactivities of propolis, mainly due to the rich composition of BACs, have proven beneficial in combination with several products, and since then propolis has been the focus of many researchers. In addition, the demand for natural products with added value has increased significantly. Nevertheless, positive data have already been demonstrated in the use of propolis as a starting material for the development of various extracts used as active pharmaceutical ingredients (APIs), dietary supplements, and as an ingredient in the formulation of cosmetic products [42, 64, 94-97].

With the development and improvement of technology for the formulation of drugs through natural bioactive products, propolis represents a growing interest in the pharmaceutical and medical industry. Due to the characteristic medicinal and therapeutic properties of this biomolecule with regard to the treatment of various chronic diseases, propolis has been used in the treatment

of diabetes, burns, wounds, laryngological, neurodegenerative and dermatological diseases, gynecological problems, cardiovascular and gastrointestinal diseases, and more recently COVID - 19 [98].

Propolis is considered a bioproduct and due to its antioxidant capacity and wide antimicrobial spectrum, it is an effective agent to extend the shelf life of food or improve the quality and safety of products. There are a few challenges to overcome in the food industry, including growing consumer demand for fresh and minimally processed foods without the addition of chemical preservatives, leading to innovative approaches to food processing and the use of natural compounds as biopreservatives. In this sense, there is a growing demand for propolis in industry, as it can be added directly to certain food matrices or inserted into the formulation of packaging and edible coatings as a nutrient or preservative [99, 100].

Currently, there are many products in the cosmetic industry that contain propolis in their formulation, either in pure form or in combination with other natural products [37]. Among the many natural sources available, propolis is considered by worldwide studies to be the best herbal alternative for topical inflammation and other topical diseases [101]. The skin acts as a biological barrier protecting organs from external environmental hazards. These include biological (fungi and bacteria), physical (UV radiation) and chemical (toxic products and ozone). These risks put the skin under constant oxidative stress and can cause irreversible tissue damage. Therefore, propolis is characterized by its high antioxidant activity, which has protective and preventive properties and can therefore be included in UV protection formulations. In addition, propolis extracts have also shown significant results in anti-inflammatory, antimicrobial and healing properties and can be used in the treatment of acne [102].

In agriculture, propolis extracts have shown positive developments in the control of phytopathogenic fungi *in vivo* and *in vitro* in tomato, coffee, bean, grape, and cucumber cultures [103-106]. This can be an alternative aimed at avoiding the use of pesticides that are harmful to the environment and humans. However, the use of propolis extracts in the agricultural industry is relatively new. Nevertheless, if we consider concepts such as sustainability, natural products and organic food, this connection becomes very attractive and therefore it is necessary to invest more knowledge in this area [107].

Since propolis contains mainly photosensitive compounds, it is necessary to create a physical barrier to protect the active ingredients of these compounds from the external environment.

Microencapsulation alternatives have emerged that not only address this factor, but also provide resistance and avoid undesirable interactions between other ingredients when propolis or compounds extracted from it are added to an industrial product. Thus, to microencapsulated unstable bioactive compounds is an effective alternative to preserve and protect the functional properties of propolis when used in industry [108].

In Portugal, the beekeeping industry focuses only on the high production of honey, neglecting other products, namely propolis, which is often neglected and/or discarded, mainly due to a lack of information among producers and consumers. However, the production of propolis in Portugal is not very profitable and requires special treatments, so that the quantities required by large industrial companies are not achieved [107]. There is no information about the amount of propolis produced in Europe. In contrast, Brazil is the only tropical country that exports a significant amount of propolis [109].

2. OBJECTIVES

The primary aim of this study was to advance the concept of a circular economy within the beekeeping industry by promoting the reuse of a biological by-product, propolis, capitalizing on its biological properties. While the study primarily focused on obtaining extracts with antioxidant characteristics for potential application in the biopreservation and/or biosupplementation of food products, anti-aging activity was also evaluated.

To isolate bioactive fractions from the propolis samples, traditional solid-liquid extractions were conducted at room temperature, utilizing water and ethanol in varying proportions. The use of different solvents was aimed at optimizing the conditions for extracting bioactive compounds from propolis, specifically from the G23 sample, in order to achieve a substantial extraction yield with the highest phenolic compound content.

3. MATERIAL AND METHODS

Ethanol 96% reagent was obtained from AGA (Prior Velho, Portugal). Analytical grade. Microplate reader (Thermo Fisher Scientific, Lisboa, Portugal). Folin-Ciacalteau reagent; Catechol; Gallic Acid; Sodium Molybdate; Potassium persulfate; Methanol (Sigma Aldrich, Steinheim, Germany); Sodium Nitrite ($NaNO_3$) (Merck, Darmstadt, Germany); Aluminum chloride (Merck, Darmstadt, Germany). 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ); 2,2-diphenyl-1-picrylhidrazyl radical (DPPH); 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS); 6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid (Trolox) (Sigma Aldrich, Steinheim, Germany). Ferric chloride and the enzymes tyrosinase, elastase and reagents for all enzymatic activities were purchased from Sigma-Aldrich (Steinheim, Germany).

3.1 Sample Conditions

The propolis sample were provided by beekeeper from the interior of Portugal, namely from the Guarda district, and were collected at the end of April 2023. The propolis was transported in a thermalbox at low temperature, which was not specified.

The first sample, shown in **Figure 3**, was available in larger quantity and was labelled G23. It had a production time of about one month and it had some impurities, as it was possible to detect some characteristic wastes from the beehives, such as bee legs and wings.



Figure 3 – An accurate representation of the propolis sample used in this study, designated G23, with a production time of about one month, with visible residues from the hives, such as bee legs and wing debris.

3.2 Sample Preparation

The two propolis samples were always kept at a reduced temperature (cold room, 4 °C), in the dark and with silica to preserve all the original properties of the propolis.

Since the samples had different particle sizes, two grinding cycles of 20 seconds each at 1500 W were performed, prior to all the tests.

3.3 Bioactive Compounds Extraction

Propolis samples have some impurities that must be removed for effective characterisation. This is essential for applications in food technology, cosmetics or even pharmaceuticals, as propolis cannot be used directly due to its low solubility in water and high content of impurities [110]. Therefore, propolis needs to be purified before it can be used in these different applications.

Since the target of this work is the food sector, the extraction of bioactive compounds is usually carried out with ethanol and/or water-based solvents. Extraction with ethanol is particularly suitable for obtaining extracts rich in polyphenols, while extraction with water is ideal for extracts containing water-soluble phenolic acids [66, 111].

The bioactive compounds were extracted from the first propolis sample using the exctraction method. For this procedure, the propolis sample was added to each of the three defined solvents (EtOH 96%, H_2O and EtOH 70%) in a ratio of 1:20, obtaining a final volume of 10 mL. The solution was mixed at room temperature (20 °C) by magnetic stirring at 150 rpm for 24 hours, followed by centrifugation (Thermo Scientific™ Heraeus™ Multifuge™ X3/X3F Centrifuge Series) at 10000 g for 10 minutes (**Figure 4**). The supernatant was recovered into a 10 mL volumetric flask, which was filled with the solvent of the respective assay.

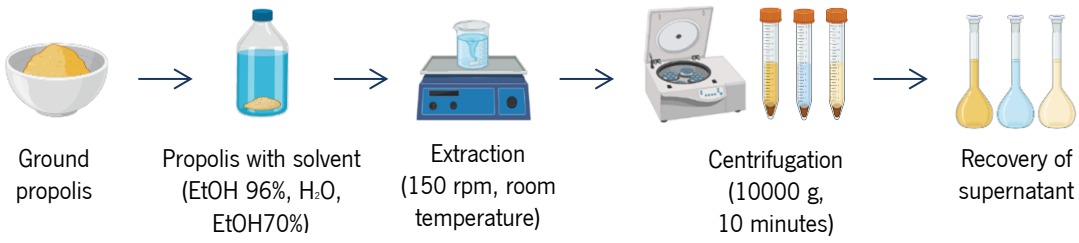


Figure 4 – Schematic overview of the extraction procedure for obtaining the bioactive compounds contained in firs propolis sample in three different solvents (EtOH 96%, H_2O and EtOH 70%) at room temperature.

3.4 Propolis Samples and Extracts Characterization

3.4.1 Moisture

The moisture content of the samples was measured by placing 0.500 g of the crushed propolis sample in duplicate in two crucibles that had previously been air-dried at 105 °C for 3 hours and weighing them. The sample was dried overnight in an air oven at 105 °C, cooled in a desiccator and weighed again.

Equation **(1)** was used to get the overall moisture content, which was then reported as a percentage per gram of dry weight (DW).

$$Moisture\ (\%/1\ g\ DW) = \frac{w_1 - w_2}{w_1} \times 100 \qquad \textbf{(1)}$$

w_1: $weight\ of\ sample$

w_2: $weight\ of\ the\ dried\ sample$

3.4.2 Ash

Following the previous procedure (moisture), the determination of the ash content was performed. 0.700 g of the propolis sample was placed in triplicate in a container and air-dried at 105 °C for 3 hours, cooled in a desiccator and weighed. The containers with the samples were then burned in a muffle furnace at 550 °C for about 14 to 16 hours, cooled in a desiccator and weighed again.

The total ash was calculated according to the following equation **(2)** and expressed as a percentage per gramme dry weight (DW).

$$Ash\ (\%/1\ g\ DW) = \frac{w_1}{w_2} \times 100 \qquad \textbf{(2)}$$

w_1: $weight\ of\ sample\ after\ 105$ °C

w_2: $weight\ of\ the\ dried\ sample$

3.4.3 Dry Weight (DW) of Extracts Propolis

To two crucibles, previously weighed and dried in an air oven at 105 °C for 3 hours, was added 1 mL of the supernatant from the extraction of the bioactive compounds. Both crucibles were placed in the oven at 105 °C, cooled in a desiccator and weighed again, determining the dry weight of this sample (**Figure 5**).

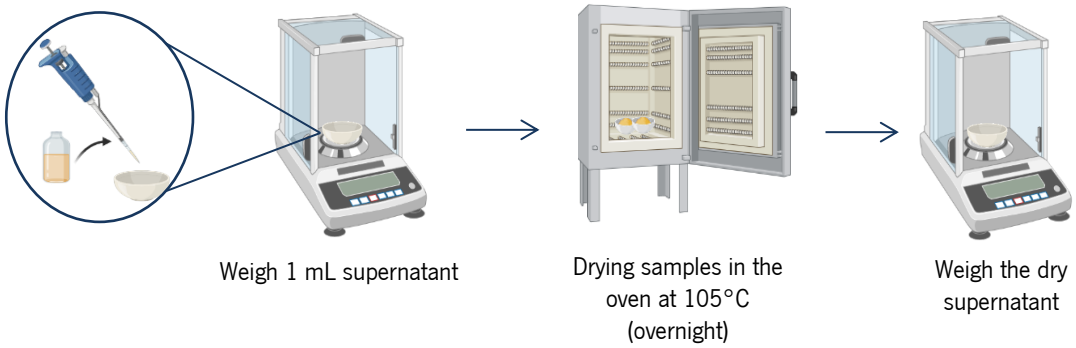


Figure 5 – Illustration of the dry weight (DW) method for the supernatant of the extraction.

3.5 Total Phenolic and Flavonoid Contents of Propolis

3.5.1 Total Phenolic Compounds Content (TPC)

The content of total phenolic compounds content in propolis extracts was determined by the Folin-Ciocalteu method, which is based on the colorimetric reduction/oxidation reaction of phenols [112]. This reagent consists of a mixture of two acids (phosphotungsten and phosphomolybdenum), which have a yellow color and are in an oxidized state. In the presence of reducing agents, such as phenolic compounds, blue colored complexes are formed [113]. Thus, it is the change in color that allows the phenolic composition of a sample to be quantified by spectrophotometry using a calibration curve of a standard phenolic compound.

The method was performed for a 96-well plate, with 20 µL of propolis extract or standard added to each well of the plate, followed by 100 µL of Folin–Ciocalteau:H_2O (1:10) and 80 µL of 7.5% sodium carbonate (Na_2CO_3). The microplate was then incubated at 42 °C for 30 minutes in the dark. The absorbance was measured at 750 nm using a UV/Vis spectrophotometer.

Gallic acid (0 - 150 mg/mL) was used as a standard to generate the calibration curve (R^2=0.999) (**Appendix 1**, **Figure 15**). The results were expressed as milligrams of gallic acid

equivalents (GAE) per gram of propolis (mg GAE/g propolis) and milligrams of gallic acid equivalents (GAE) per gram of extract (mg GAE/g extract).

3.5.2 Total Flavonoids Content (TFC)

The content of total flavonoids in propolis samples was determined by the aluminum chloride method [114]. This reagent is selective for flavonoids because it is able to form stable complexes between the Al3+ cation and the free hydroxyl groups in flavonoid molecules, distinguishing them from other phenolic compounds. The complexation reaction of a standard flavonoid, in this case catechin (CTE), with aluminum chloride results in a red colored complex. That is, the intensity of this coloration is proportional to the amount of complex formed and, consequently, to the content of flavonoids in the sample [115].

First, two solutions were prepared: the first was 100 g/L aluminum chloride, with 1 g aluminum chloride ($AlCl_2$) dissolved in 10 mL distilled water; the second was 50 g/L sodium nitrite ($NaNO_2$), with 0.5 g sodium nitrite dissolved in 10 mL distilled water. In addition, 1 M sodium hydroxide was prepared, adding about 2 g of sodium hydroxide (NaOH) in 30 mL of distilled water.

The method was performed for a 96-well plate, adding 25 µL of propolis extract or standard to each well of the plate, followed by 30 µL of sodium nitrite ($NaNO_2$) and incubated for 5 minutes at room temperature in the dark. Then 28 µL of aluminum chloride ($AlCl_2$) was added to the mixture. After 6 minutes in the dark and at room temperature, 125 µL of sodium hydroxide (NaOH) was added to the microtiter plate. Shake the microtiter plate in the plate reader for 30 seconds before measuring the absorbance at 510 nm using a UV/Vis spectrophotometer.

Catechin (0 - 200 mg/mL) was used as a standard to generate the calibration curve (R^2=0.998) (**Appendix 2**, **Figure 16**).

For interpolation, the flavonoid content of the tested samples is calculated according to the following equation **(7)**. Results are expressed as milligrams of catechin equivalents (CTE) per gram of propolis (mg CTE/g propolis) and milligrams of catechin equivalents (CTE) per gram of extract (mg CTE/g extract).

$$Flavonoids\ (mg\ CTE/g) = \frac{Abs\ sample - b}{a} \qquad (7)$$

Where: a is the slope of the standard curve and b is the independent term of the standard curve (y = ax + b).

3.6 Antioxidant Activity of Propolis' Extracts

3.6.1 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS)

The antioxidant capacity of the propolis extract was determined by free radical decolorization with 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) [116].

The ABTS cation radical was prepared by reacting 7 mM aqueous solution of ABTS with 148 mM potassium persulfate in distilled water. This mixture was stored in the dark at room temperature for 12 to 16 hours before use. On the day of analysis, the ABTS working solution was diluted with ethanol (1:40) until it reached an absorbance of (0.70 ± 0.02) at 734 nm.

The procedure was performed on a 96-well plate, with 10 µL of propolis extract or standard added to each well before 190 µL of the ABTS working solution (1:40) was added. The microplate was then kept at room temperature and protected from light for an additional 30 minutes. A UV/Vis spectrophotometer was then used to determine the absorbance at 734 nm.

Trolox-equivalent antioxidant capacity (TEAC) (0 - 1.25 mg/L) was used as a standard to generate the calibration curve (R^2=0.973) and was used to plot the results (**Appendix 3: Figure 17**). The following equation **(3)** was used to determine the percentage of inhibition effect.

$$\%\ inhibition_{sample} = \frac{Abs\ (734)_{\ ABTS\ Blank} - Abs\ (734)_{\ sample}}{Abs\ (734)_{ABTS\ Blank}} \times 100 \qquad \textbf{(3)}$$

Where: $Abs\ (734)_{\ ABTS\ Blank}$: $absorbance\ the\ ABTS$;

$Abs\ (734)_{\ sample}$: $absorbance\ of\ each\ sample$.

The TEAC value of the samples was calculated according to equation **(4)** as follows. Results are expressed as millimole of Trolox equivalent antioxidant capacity (TEAC) per gram of propolis (mmol TEAC/g propolis).

$$TEAC_{sample} = \frac{\%\ inhibition}{slope\ of\ standard\ curve} \qquad \textbf{(4)}$$

3.6.2 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The antioxidant capacity of the propolis extract was determined by using the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH).

The 8.87 mM DPPH stock solution was prepared by dissolving 35 mg DPPH in 10 mL ethanol and stored in the refrigerator for two days. The 5 mM DPPH working solution was obtained by diluting the DPPH stock solution with methanol to give an absorbance of 1.0 ± 0.02 at 520 nm using a microplate reader.

The procedure was performed in a 96-well plate. 10 µL of the propolis extract or standard was added to each well before 190 µL of the DPPH working solution was added to each well, so that the total reaction volume was 200 µL. The microplate was then stored at room temperature for an additional 30 minutes and protected from light. A UV/Vis spectrophotometer was then used to determine the absorbance at 520 nm.

Trolox equivalent antioxidant capacity (TEAC) (0 - 2.5 mg/L) was used as a standard to construct the calibration curve (R^2=0.998) and plot the results (**Appendix 4, Figure 18**). The following equation **(5)** was used to determine the percentage of inhibition:

$$\%\ inhibition_{sample} = \frac{Abs\ (520)_{\ Blank} - Abs\ (520)_{\ sample}}{Abs\ (520)_{Blank}} \times 100 \qquad (5)$$

$Abs\ (520)_{\ Blank}$: *absorbance the blank*

The TEAC value of the samples was calculated according to equation **(6)** as follows. Results are expressed as millimole of Trolox equivalent antioxidant capacity (TEAC) per gram of propolis (mmol TEAC/g propolis).

$$TEAC_{sample} = \frac{\%\ inhibition}{slope\ of\ standard\ curve} \qquad (6)$$

3.6.3 Ferric Reducing Antioxidant Power (FRAP)

The Ferric Reducing Antioxidant Power (FRAP) assay was used to evaluate the antioxidant activity of the propolis extract [117].

The reagent used was 300 mM acetate buffer (adjusted to pH 3.6 by addition of acetic acid) with 40 mM hydrochloric acid (HCl), dissolved in distilled water, and 10 mM TPTZ (0.03 g TPTZ in

30 mL HCl). The working solution FRAP was freshly prepared by mixing acetate buffer, TPTZ solution and 20 mM ferric chloride ($FeCl_3.6H_2O$) solution.

The procedure was performed on a 96-well plate, with 20 µL of the propolis extract or standard added to each well before 280 µL of the ABTS working solution was added. The microplate was then stored at 37 °C for an additional 30 minutes and protected from light. Absorbance was measured at 593 nm using a UV/Vis spectrophotometer.

Trolox equivalent antioxidant capacity (TEAC) (0 - 1.25 mg/L) was used as a standard to generate the calibration curve (R^2=0.999) (**Appendix 5, Figure 19**). The results were expressed as milligrams of TEAC per gram propolis (mmol TEAC/propolis).

3.7 Anti-aging Activity of Propolis' Extracts

3.7.1 Elastase Inhibition Assay

Elastase is an enzyme involved in the degradation of elastin. This protein gives elasticity to tissues, especially connective tissue, blood vessels and lungs.

The elastin inhibition test is characterized by the inhibition of the enzyme elastase, i.e., the higher the percentage of inhibition, the more the enzyme is inhibited and consequently the greater the anti-aging activity of the by-product.

A buffer solution of 0.2 mM Tris-HCl was prepared and adjusted to pH 8 by adding distilled water. The substrate N-succinyl-Ala-Ala-p-nitroanilide (0.8 mM) was prepared by adding about 11 mL of the Tris-HCl buffer. The enzyme (1 U/mL) was prepared by dissolving 42 µL of the elastase enzyme in approximately 1.9 mL of the buffer solution.

The procedure was performed in a 96-well plate. To each well, 50 µL of the propolis extract or standard was added, then 160 µL of the Tris-HCl buffer solution and 20 µL of the prepared substrate. The microplate was incubated at room temperature and protected from light for 10 minutes. Finally, 20 µL of the elastase enzyme was added. The absorbance was measured at 410 nm using a UV/Vis spectrophotometer.

The following equation **(8)** was used to calculate the percentage of inhibition:

$$Inhibition\ (\%) = \frac{Abs_{control} - Abs_{sample}}{Abs\ control} \times 100 \qquad (8)$$

3.7.2 Tyrosinase Inhibition Assay

Tyrosinase is an enzyme involved in the production of melanin, a pigment responsible for the pigmentation of skin, eyes, and hair, and for the darkening of plants after harvest. Inhibition of its activity may have various industrial interests, such as extending the shelf life of food in the food sector, for the cosmetics industry and dermatology in the treatment of hyperpigmentation disorders. In addition to these applications, tyrosinase is also involved in various studies aimed at the production of antioxidants and drugs, the formation of biopolymers and the production of biosensors [118-122].

The tyrosinase inhibition assay is used to measure the ability of a particular compound to inhibit tyrosinase activity. In other words, the higher the percentage of inhibition, the more the enzyme is inhibited and consequently the greater the anti-aging activity.

A 50 mM phosphate buffer solution was prepared and adjusted to a pH of 6.5 with distilled water. The tyrosinase enzyme (1 U/mL) was prepared by dissolving about 100 µL of the enzyme in about 1600 µL of the phosphate buffer solution.

Before proceeding to the actual procedure, two controls were prepared: the positive control was prepared with Kogic acid (1 mg/mL), and the negative control was prepared with distilled water and 10% DMSO. In addition, the propolis extracts were dissolved with 10% DMSO.

The procedure for determination of tyrosinase inhibition was performed in a 96-well plate, with 10 µL of propolis extract or control added to each well, then 20 µL of tyrosinase enzyme and 170 µL of mixed solution (10:10:9) prepared previously with L-tyrosinase: phosphate buffer: distilled water. The microplate was incubated at 37 °C for 10 minutes. The absorbance was measured at 490 nm using a UV/Vis spectrophotometer.

For interpolation, the tyrosinase inhibition of the tested samples is calculated according to the following equation **(9)**:

$$Inhibition\ (\%) = \frac{Abs_{control} - Abs_{sample}}{Abs\ control} \times 100 \qquad (9)$$

3.8 Statistical Analysis

Results are expressed as mean ± standard deviation (SD). For the estimations of propolis TPC, TFC, antioxidant activity and antiaging activity mean values, 3 independent experiments, each with 3 replicates, were done. For the other parameters, regarding propolis characterization (ash and moisture) 3 independent replicates were used. Statistical analysis was performed using the Excel and significant differences between the antioxidant, phenolic, and flavonoid activities of the different propolis samples were determined by ANOVA using GraphPad Prism software (version 8.0.1; GraphPad Software).

4. Results and Discussion

To evaluate the potential of bioactive compounds in propolis samples from the interior of Portugal (Guarda), we first performed a light characterization of composition of these samples. To obtain the bioactive compounds extracted from the propolis extracts, extractions were performed with three different solvents (water and 70% and 90% ethanol) and the extraction parameters were adjusted to obtain extracts rich in phenolic compounds with high bioactive potential. To evaluate this bioactivity, the hidro-ethanolic and aqueous extracts resulting from the extractions were first subjected to colorimetric methods to quantify the total content of polyphenols and flavonoids. Antioxidant tests were also performed, such as DPPH and ABTS, which are radical scavenging tests, and a test that evaluates the reducing power of iron ions, FRAP. Anti-aging, tyrosinase and elastase tests were also performed.

To continue this project, an extraction of the bioactive compounds of propolis in sunflower oil (100%) was also performed.

4.1 Proximate Composition Analysis

The ash content and moisture content of the propolis sample G23 were determined. The ash content is a test that quantifies the inorganic composition of the samples after the combustion of an organic substance and reveals the total composition of the mineral elements. The moisture content refers to the amount of water present in the sample and is very important to obtain a real total weight.

The immediate ash composition and moisture content of G23 are summarized in **Table 1**.

Over the centuries and in the face of climate change, bees have selected substances from various plant sources to satisfy their needs, especially for the production of propolis to defend their own hive. The diversity of flora surrounding the hives in different geographical locations is also an important factor in the properties and chemical composition of propolis [48, 94, 123, 124].

Falcão et al. (2013), recognizes two groups of Portuguese propolis, type I and type II. These groups are distinguished by their wax, ash and balsam values, as well as by their composition and phenolic content. This author suggests that propolis samples from the Azores and the north and central coast of Portugal fall into the type I propolis group, while samples from the central and

southern interior of Portugal and the island of Madeira belong to the II type. For instance, a concrete propolis sample from the Guarda district showed ash values of 1.9% and moisture content of 3.6% [124].

A look at **Table 1** shows that the ash content of G23 is slightly lower (0.64 ± 0.01%) than the values reported in the literature for propolis samples from Guarda (G13) (1.90 ± 0.02%) and from the entire central interior of Portugal (1.3% - 4.2%) [124]. However, the values are consistent with propolis samples from northwestern Spain, which show ash content between 0.31% - 0.81% [125]. Since the presence of ash in propolis is due to the accumulation of other non-resinous materials, i.e., geographical locations where resin is scarce, it is assumed that the plant species available for the production of G23 propolis can fully supply the resins required for healthy production.

In contrast to the ash content, the moisture content of the obtained G23 propolis (5.9 ± 0.17%) was higher than the values reported in the study of Falcão et al. (2013), who analysed samples from different regions of the central interior of Portugal, including the Guarda district, reaching a moisture content between 3.6% - 4.5% [124]. Dias et al. (2012) also studied the characteristics of different propolis samples from the Trás-os-Montes region and obtained moisture values between 3.4 ± 0.2% to 5.4 ± 0.4%. This range is close to the moisture value of G23 propolis [126].

The difference in values compared to G23 propolis is quite small, and this can be explained by factors related to the harvesting of propolis itself. The fact that it was harvested on a wetter day may have contributed to the higher moisture content.

Table 1 – Ash and moisture content (%/g DW) of G23, compared with that proposed for propolis-Guarda (G13) [124].Values are given as mean ± standard deviation (SD).

	Propolis - Guarda (G23)	Propolis - Guarda (G13) [124]
Ash (%)	0.64 ± 0.01	1.9 ± 0.02
Moisture (%)	5.9 ± 0.17	3.6 ± 0.3

4.2 Efficiency of Extraction

The extraction of bioactive compounds contained in propolis is a fundamental step in this study, since all subsequent work depends on the efficiency of this step. Therefore, to use propolis properly, it must be purified by solvent extraction, removing waxes and organic residues to preserve its phenolic content and bioactivity [127].

In order to extract the bioactive ingredients from G23 propolis without impairing its bioactivity, the cold solid-liquid extraction was used. In this method, the parameters of temperature, stirring, solid-liquid ratio and extraction time were kept constant in all experiments, the only changing parameter being the solvent used.

To better analyse the efficiency of the three extractions performed, the yield values for each solvent (EtOH 96%, H_2O and EtOH 70%) are presented in the form of a bar graph (**Figure 6**).

The results show that water was the solvent with the lowest efficiency in the extraction of bioactive compounds, with a yield of only 4.2%. On the other hand, ethanol confirmed its high efficiency in extraction, with 96% ethanol showing an extraction yield of 75.5%, similar to the extracts in 70% ethanol with a yield of 73.8%. Some authors report that increasing the ethanol content leads to a higher extraction yield [100, 128]. However, statistical analysis of the results obtained shows that the difference in ethanol content does not represent a significant ($p > 0.05$) extraction difference.

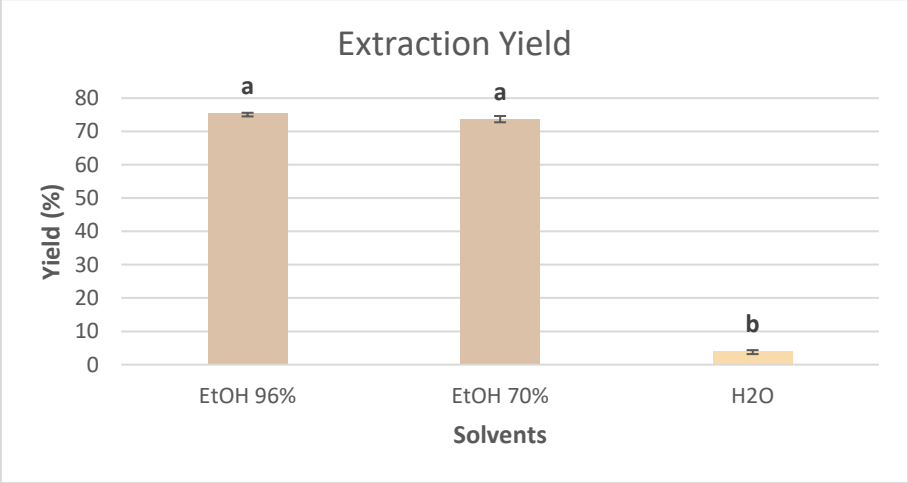


Figure 6 – The yield of bioactive compounds from Propolis G23 extracted at room temperature for 24 hours under stirring at 150 rpm with three different solvents, EtOH 96%, EtOH 70% and H_2O. Values are given as mean ± standard deviation (SD). Different letters (a, b) indicate significant differences ($p < 0.05$) between the extraction solvents for the same propolis sample.

The chemical composition of propolis depends on various factors, such as the season in which this bioproduct is harvested or the geographical location of the apiary. Nevertheless, even for the same propolis sample, the choice of solvent for the extraction affects both the yield and the composition of the extracts. In general, ethanol is the first choice for the extraction of the biologically active components of propolis, as it results in extracts richer in phenolic compounds, thus emphasizing the biological value of this natural product [128]. Water is considered a biocompatible solvent and allows multiple applications for aqueous extracts. However, aqueous extracts of propolis usually have a much lower content of bioactive compounds compared to hydroethanolic extracts [129, 130]. In addition, many of the constituents of propolis are lipophilic, which complicates extraction and compromises yield, which can be more efficient when organic solvents are used [107]. Thus, the results obtained in the extraction of the bioactive compounds contained in Propolis G23 are consistent with those published in the literature.

The ideal temperature is one of the most important parameters in the process of extracting bioactive compounds from propolis to achieve optimal yield. According to the literature, increasing the temperature not only shortens the extraction time, but also increases the efficiency of the process, as the intermolecular forces of the solvent decrease, allowing it to better penetrate into the particles [131]. However, there is scientific evidence that an increase in temperature may affect the stability of antioxidant components [132]. In view of this, the influence of temperature was not evaluated in the present study because the priority was to keep the antioxidant compounds of interest stable for a food matrix.

4.3 Total Phenolic and Flavonoid Contents of Propolis

The phenolic and flavonoid composition in propolis extracts are important parameters for evaluating the quality and biological potential of this bioproduct [79]. In particular, flavonoids are associated with the antioxidant properties of various plant products, such as propolis. In addition, some phenolic acids and their esters also have an interesting antioxidant effect [133, 134].

The complexity of the phenolic content of propolis results from the total plant resin and the variety of plant combinations produced by the bee itself, especially in places with phytogeographic diversity. However, some of these compounds are detected only in trace amounts, which may

mean that the bee has no preference for certain plants or trees, or that the plant or tree is rare in the vicinity of the hive [48, 135].

Therefore, we quantified the total phenolic and flavonoid content to understand the compositional potential of G23 propolis from inland Portugal.

4.3.1 Total Phenolic Content (TPC)

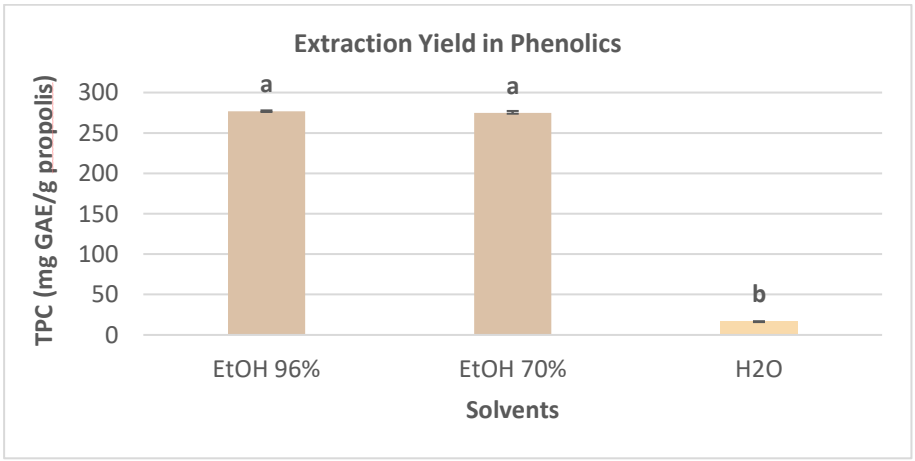
Total phenolic content was quantified using the Folin-Cicocalteu reagent, which is characterized by an oxidation-reduction reaction.

The results of this analysis were expressed in gallic acid equivalents (GA) per gram of propolis and per gram of extract are presented in a bar chart in **Figure 7: a), b)**.

G23 propolis showed significantly higher ($p < 0.05$) values for phenolic content with the solvent ethanol, EtOH 96% (276.9 ± 0.99 mg GAE / g propolis) and EtOH 70% (274.8 ± 2.41 mg GAE / g propolis), than compared to water, H_2O (19.6 ± 0.10 mg GAE / g propolis) (**Figure 7: a)**). In **Figure 7: b)**, the results represent the total extracted content of propolis, including phenolic compounds, with 96% EtOH extraction (366.7 ± 1.14 mg GAE /g extract), 70% EtOH (372.6 ± 2.81 mg GAE /g extract) and H_2O (406.1 ± 0.49 mg GAE /g extract).

Considering the results of the three extractions performed (**Figure 6**), the values obtained for phenolic content in each test are consistent with each other, because it was expected that the extracts with the highest extraction yield would contain a greater amount of phenols. The results show that there is no significant ($p > 0.05$) difference in the phenolic content of propolis extracts treated with 96% EtOH and 70% EtOH. Therefore, 70% EtOH is an effective solvent for the extraction of all phenolic compounds from propolis.

a)



b)

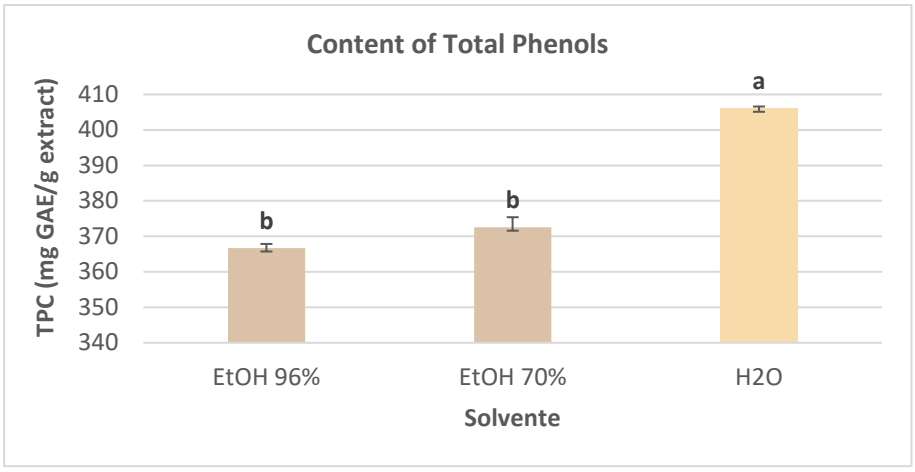


Figure 7 – Total Phenolic Content (TPC) from G23 propolis determined by the Folin-Ciocalteu method, expressed as: **a)** milligrams GAE per gram of propolis; **b)** milligrams GAE per gram of extract. Values are expressed as mean ± SD. Different letters (a, b) indicate significant differences ($p < 0.05$) between the extraction solvents for the same propolis sample.

Falcão et al. (2013) conducted an intensive and in-depth study of numerous propolis samples from all regions of Portugal, including the archipelagos. After examining the total phenolic composition, they obtained TPC values between 200 - 400 mg phenols /g extract. In the Guarda district (G13), a value of 291 ± 0.1 mg GAE /g propolis was obtained in 80% ethanolic extracts [124]. The results obtained for the TPC of the ethanolic extracts of G23 propolis are in the range of the values reported in this study. The slight difference could be due to the climatic characteristics of the site, the seasonal timing of the propolis harvest and the bees' preference for certain plant species, or/and even from the difference in the composition of the solvent [61, 135].

In northwestern Spain, total phenolic content of 200 - 340 mg GAE / g was reported in 70% ethanolic extracts of 19 propolis samples [125]. Other studies with different propolis species from South America and Brazil have shown similar phenolic contents (200 - 300 mg / g) [136].

Park et al. (1998) demonstrated that extraction with aqueous solvent gave relatively low values for total phenolic content and described that this solvent had lower absorption in aqueous extracts of propolis compared with ethanolic extracts. The TPC results for propolis G23 extracted with water also showed the lowest value of phenols. This is consistent with what is reported in the literature [137].

In this way, propolis G23 is not only within the ranges of values for total phenolic content described in the previously reported studies, but also within different ranges of values determined for different samples of European propolis extracts [138].

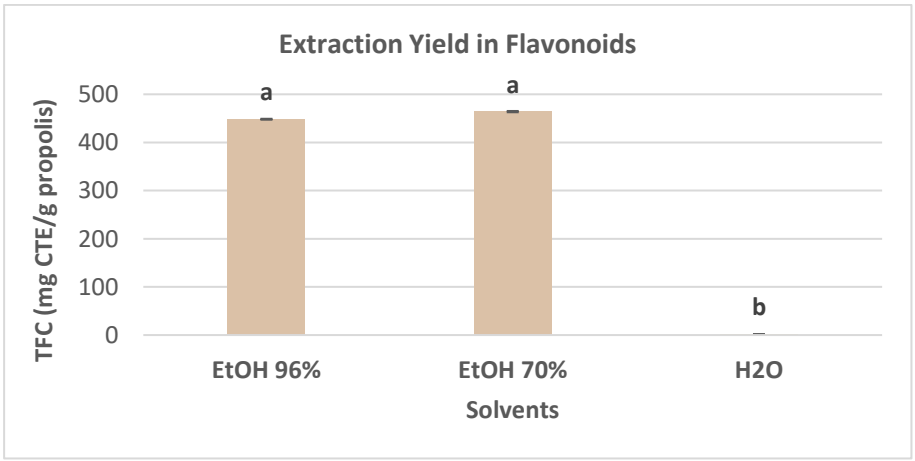
4.3.2 Total Flavonoids Content (TFC)

The flavonoid content in propolis was quantified by a colorimetric aluminum chloride ($AlCl_3$) method.

The values obtained for the total flavonoid content in the G23 propolis samples were plotted in a bar graph (**Figure 8; a), b)**).

After a brief analysis of **Figure 8: a), b)**, it is immediately apparent that the solvent ethanol had predominantly much higher TFC values than the TFC for the aqueous extracts. In contrast to the analysis of total phenolic content, in **Figure 8: a)**, 70% EtOH proved to be slightly (3%) more efficient in extracting flavonoid content (463.7 ± 0.93 mg CTE / g) than 96% EtOH, which had a value of 448.3 ± 1.61 mg GA / g propolis. H_2O, on the other hand, as expected and given by its low extraction yield, proved to be insufficient in terms of the TFC value obtained 1.6 ± 0.07 mg CTE / g propolis. In **Figure 8: b)**, the results represent the total extracted content of propolis, including phenolic compounds, with 96% EtOH extraction (593.6 ± 1.07 mg CTE / g extract), 70% EtOH (628.7 ± 1.87 mg CTE / g extract) and H_2O (38.0 ± 0.33 mg CTE / g extract).

a)



b)

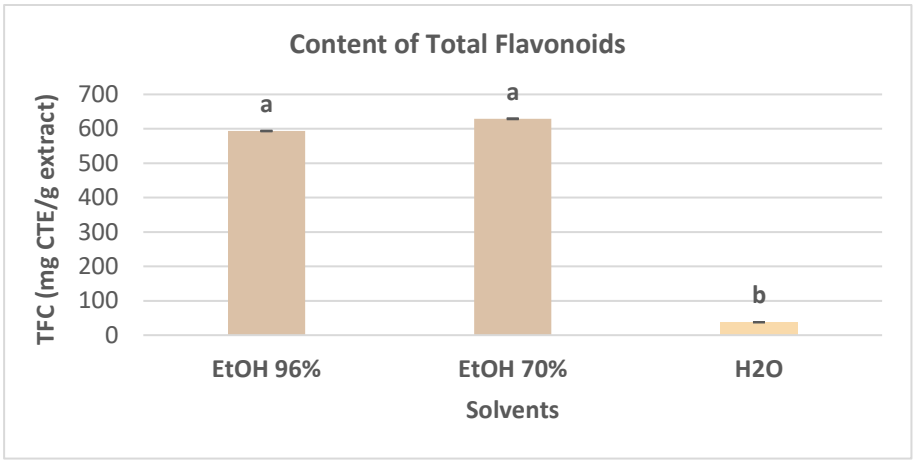


Figure 8 – Total Flavonoids Content (TFC) from G23 propolis determined by the Folin-Ciocalteu method, expressed as: **a)** milligrams CTE per gram of propolis; **b)** milligrams CTE per gram of extract. Values are expressed as mean ± SD. Different letters (a, b) indicate significant differences ($p < 0.05$) between the extraction solvents for the same propolis sample.

Zin et al. (2018) investigated the influence of two solvents (70% EtOH and 95% EtOH) on different extractions and flavonoid content in propolis extracts. In this study, the maceration extraction method with 70% EtOH resulted in higher flavonoid content compared to 95% EtOH [128]. This was also confirmed in the study on G23 propolis. The TFC values for 70% EtOH are six times higher than in the above study under the same conditions (70% EtOH with 24-hour extraction).

In the study by Falcão et al. (2013), the TFC of propolis from Guarda district (G13) was 59.5 ± 0.1 mg / g extract, which is the highest result for flavonoid content in inland Portugal, and total flavonoids range from 50 to 232 mg/g. Again, the TFC of G23 propolis was significantly higher ($p < 0.05$) than the total flavonoid content of all regions of Portugal (26.4 ± 0.2 mg galagin / g extract - 114.1 ± 0.1 mg / g extract) [124].

These differences in the amount of flavonoids can be explained by the fact that flavonoids are characteristic of certain areas, as well as by the years in which the propolis was harvested, because if the storage conditions are not suitable, the flavonoid content may lose its bioactivity. In addition, the composition of propolis depends strongly on the natural environment, plants and climate in which it occurs [124].

4.4 Evaluation of Antioxidant Activity of Propolis

Quantification of antioxidant activity was analysed by three methods that have similar principles of action based in antiradicalar activity (DPPH and ABTS) and one method with a different mechanism of action based in the reduction of iron III (FRAP). Values for the ability of G23 propolis to eliminate DPPH and ABTS radicals were expressed in Trolox equivalents per gram of extract (mmol TEAC/g) and (µmol TEAC/g), respectively, which was used as a standard for establishing the calibration line (**Appendix 3, Figure 17**; **Appendix 4, Figure 18**; **Appendix 5, Figure 19**).

4.4.1 2,2-diphenyl-1-picrylhydrazyl (DPPH)

DPPH analysis is based on the quantification of the antioxidant ability to reduce the DPPH radical to hydrazine, and the results are usually expressed in the effective concentration 50% (EC_{50}). In other words, the EC_{50} expresses the minimum concentration of antioxidant required to reduce 50% of the initial DPPH concentration. This molecule is characterized by being a stable violet radical that turns yellow when reduced [138].

The values obtained for the evaluation of the antioxidant capacity of the G23 propolis extracts to reduce DPPH radical are shown in a bar chart in **Figure 9**.

After analysing the results for DPPH, the solvent EtOH 96% showed the highest antioxidant capacity (1.73 ± 0.14 mmol TEAC/g). In contrast, H_2O had a much lower antioxidant capacity (0.12 ± 0.01 µmol TEAC/g). Although 70% EtOH did not have the highest antioxidant capacity, the value 1.49 ± 0.05 µmol TEAC/g was very close to that of 96% EtOH.

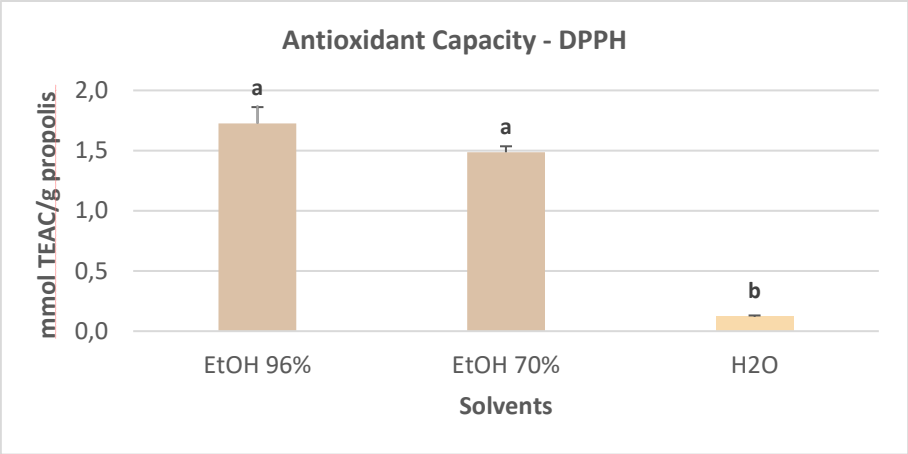


Figure 9 – Antioxidant capacity DPPH, of G23 corresponding to 96% EtOH, and 70% EtOH and H_2O samples extracted by the maceration method, at room temperature. Values are expressed as mean ± SD. Different letters (a, b) indicate significant differences ($p < 0.05$) between the extraction solvents for the same propolis sample.

Mihai et al. (2011) conducted a study relating the polyphenolic composition and antioxidant activity of propolis extracts from Transylvania. The extraction conditions were identical to those of G23 propolis, but only one solvent (70% EtOH) was used in this study. These ethanolic extracts were subjected to an evaluation of antioxidant activity, namely the DPPH test, expressed in TEAC equivalents per gram extract of propolis, with a range of values between (0.29 ± 0.10 mmol TEAC/g propolis - 1.24 ± 0.01 mmol TEAC/g propolis) [139]. Based on the results of the DPPH test for propolis G23, it can be concluded that the value for the extract treated with 70% EtOH is slightly higher than that reported in the literature. This can also be explained by the different composition of propolis, which is influenced by the geographical location, the time of harvest of this bioproduct and the plant species characteristic of the different regions [94, 123].

4.4.2 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS)

The ABTS method is also based on the quantification of the antioxidant capacity of the reduction of the ABTS radical. However, in this analysis, ABTS is generally stabilized by potassium persulfate (K_2SO_5), which has a dark green color. When this radical is exposed to an antioxidant, the solution is dechlorinated and becomes a light green color [116].

The values obtained for the evaluation of the antioxidant capacity of the G23 propolis extracts to reduce the ABTS radical are presented in a bar chart in **Figure 10**.

A glance at **Figure 10** shows that the ethanolic extracts of G23 had higher values for the total antioxidant capacity of the samples, with 70% EtOH showing the highest value 2049.5 ± 0.15 µmol TEAC/g, followed by 96% EtOH, which obtained 1862.2 ± 0.04 µmol TEAC/g. The aqueous extracts, on the other hand, showed difference significantly ($p < 0.05$) antioxidant capacity values 149.2 ± 0.05 µmol TEAC/g.

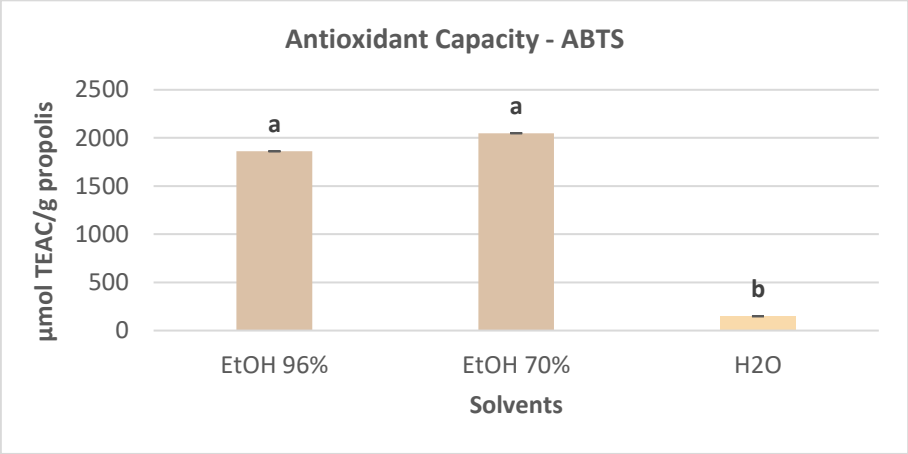


Figure 10 – Antioxidant capacity ABTS, of G23 corresponding to 96% EtOH, H2O and 70% EtOH samples extracted by the maceration method, at room temperature. Values are expressed as mean ± SD. Different letters (a, b) indicate significant differences ($p < 0.05$) between the extraction solvents for the same propolis sample.

Bonvehí et al. (2011), conducted an intensive study with propolis samples from northeastern Spain and performed various tests to obtain a complete composition of these extracts, including antioxidant activity (ABTS). The extracts treated with 70% EtOH showed a high antioxidant capacity and obtained values between 560 - 1430 µmol TEAC/g propolis, corresponding to ABTS inhibition values of 23.7% - 54.7% [125]. Based on the values obtained, it can be concluded that the range of values reported in the described study is lower than the result for the G23 propolis extract treated with 70% EtOH. Thus, it can be seen that the G23 propolis has a greater activity in inhibiting the ABTS radical, and thus has a greater antioxidant effect than the propolis from the northeast of Spain. However, the difference is in the range of the usual variability due to environmental differences between both samples. In addition, it should be noted that the propolis extracts

extracted with 70% EtOH reach values above 2000 µmol TEAC/g, which underlines the performance of this bioproduct.

According to the results obtained, the antioxidant activity was higher in the ABTS assay than in the DPPH assay. This difference in the results of these two radical scavenging tests can be explained by the fact that the DPPH radical reacts mainly with lipophilic antioxidants, while the ABTS radical reacts with hydrophilic and lipophilic antioxidants [140]. This explain also the fact that the difference is greater for the 70 % EtOH extract than for the 96% EtOH extract, and even greater for the aqueous extract.

4.4.3 Ferric Reducing Antioxidant Power (FRAP)

FRAP is a method based on evaluating the ability of antioxidant compounds to reduce the 2,4,6-tripyridyl-1,3,5-triazine (TPTZ) complex, resulting in a purple complex with Fe (II). Briefly, what happens in this assay is the reduction of Fe (III) to Fe (II) at low pH, resulting in the formation of a colored Fe (II) tripyridyl-triazine complex [141].

The values obtained for the evaluation of the antioxidant capacity of the G23 propolis extracts using the ABTS assay are shown in a bar graph in **Figure 11**.

The G23 propolis samples showed the highest activity with 96% ethanol (2410.9 ± 0.03 µmol TEAC / g propolis), followed by 70% ethanol (2252.9 ± 0.09 µmol TEAC / g propolis) and finally, with a significantly lower ($p < 0.05$) value, H_2O 12.1 ± 0.01 µmol TEAC / g propolis.

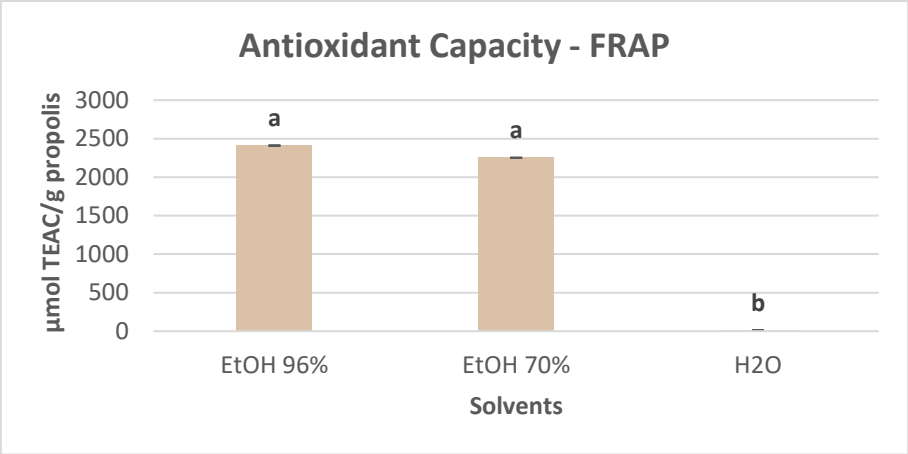


Figure 11 – Antioxidant capacity FRAP, of G23 corresponding to 96% EtOH, H_2O and 70% EtOH samples extracted by the maceration method, at room temperature. Values are expressed as mean ± SD. Different letters (a, b) indicate significant differences ($p < 0.05$) between the extraction solvents for the same propolis sample.

Mohatar et al (2020) conducted a study on different propolis extracts from different countries to evaluate their antioxidant activity using specific methods, namely FRAP. He found values in the range of 22 ± 1 - 304 ± 1 µmol ascorbic acid/g, with extracts from Brazil having the highest FRAP values and extracts from Argentina having the lowest. Also in this study, the author points out that even samples with reduced activity have considerable antioxidant activity, which can be combined with alternatives to eliminate oxidative stress responsible for a number of diseases [142].

In a recent study, Can et al. (2022) investigated various properties of propolis from the Marmara region in Turkey. They obtained a range of values between 104.1 - 534.4 µmol FeSo4.7H_2O/g for the FRAP test. He also found that the extracts with the highest antioxidant activity also had the highest levels of flavonoids and total phenols [143].

The results obtained with the G23 propolis extracts were expressed in TEAC equivalents and the above studies were performed with different compounds. Therefore, it is not possible to compare the values with the studies presented above, but it can be concluded that the results show that the extract has antioxidant activity in the removal of Fe (III).

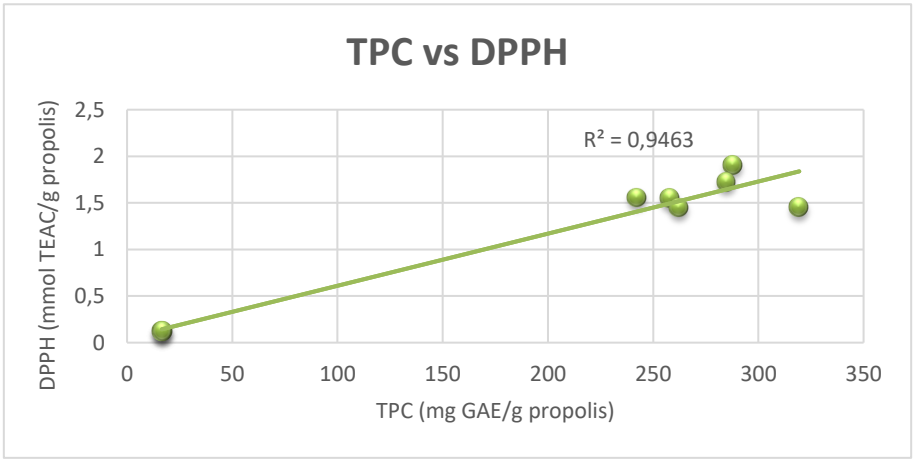
A few years ago, Zehra et al (2015) studied the antioxidant capacity of Azerbaijani propolis extracts treated with 95% ethanol. They found FRAP values in the range of 170 - 438 µmol TEAC/g propolis [144]. The FRAP values of propolis G23 are again higher than those reported in the literature.

This variation may be due to the different geographical origin, composition and resulting bioactivities of propolis.

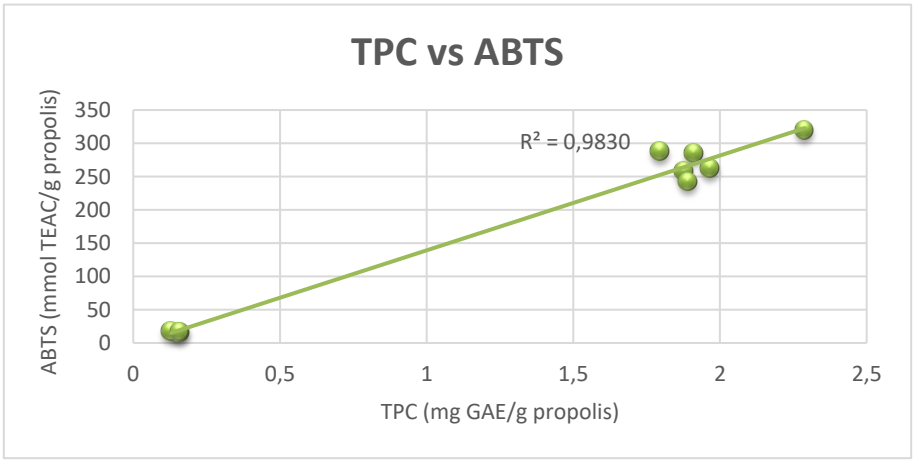
4.5 Correlation of Antioxidant Activity with Total Phenolic and Flavonoid Content

Since three different methods were used to quantify the antioxidant activity of the G23 propolis extracts, they appear to be correlated with each other, notwithstanding the small difference observed in the ethanolic extracts, which showed an insignificant inverse relationship. In addition, the relationship between the total phenolic activity and the free radical scavenging activity of the propolis samples of DPPH, ABTS and FRAP was studied, and a positive correlation was found in these cases (**Figure 12: a), b), c)**).

a)



b)



c)

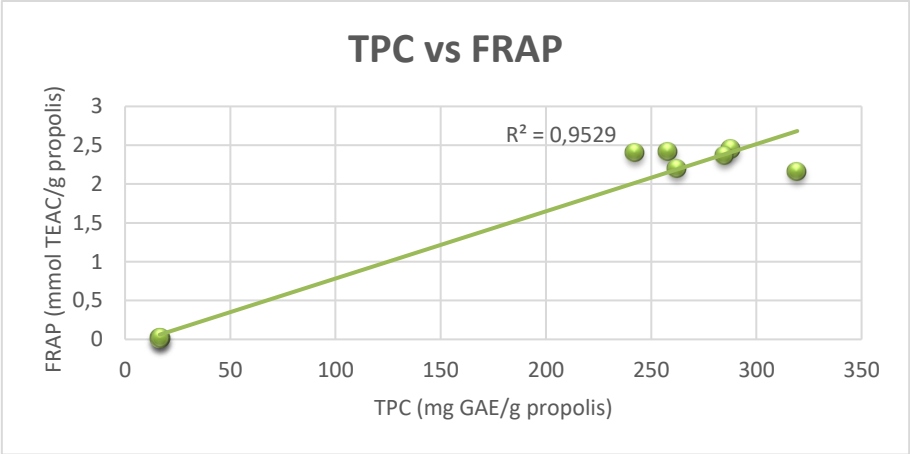
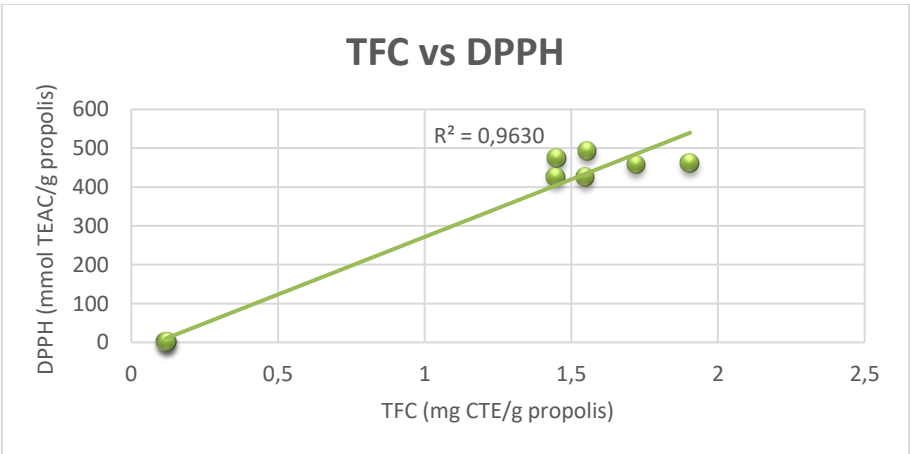


Figure 12 – Observed correlations between phenolic content and antioxidant activity: **a)** between TPC (mg GAE/g propolis) and DPPH analysis (mmol TEAC/g propolis); **b)** between TPC (mg GAE/g propolis) and ABTS analysis (mmol TEAC/g propolis); and **c)** between TPC (mg GAE/g propolis) and FRAP assay (mmol TEAC/g propolis).

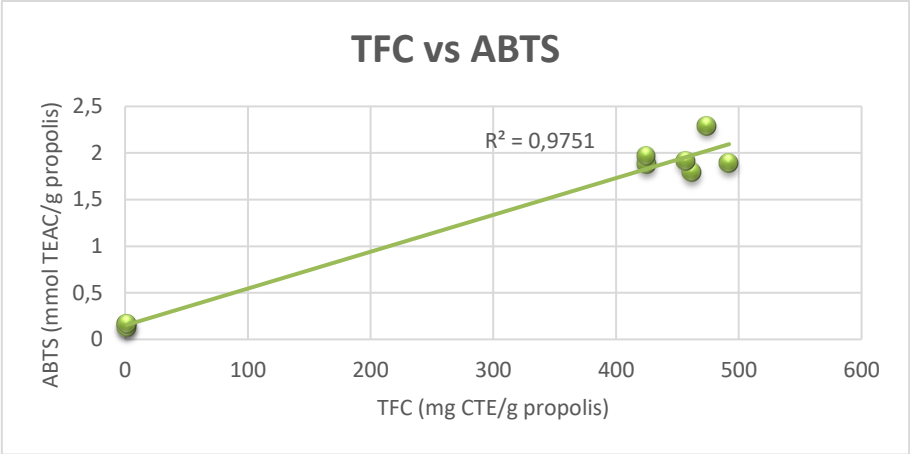
Total phenolic content and antioxidant activity were compared and it was found that there was a relationship between the two, as the sample with the higher phenolic content also had the higher antioxidant capacity.

According to **Figure 13: a), b), c)**, the correlation between antioxidant capacity and total flavonoid content shows a positive correlation with correlation coefficients of 0.96, 0.98 and 0.95 for DPPH, ABTS and FRAP tests, respectively.

a)



b



c)

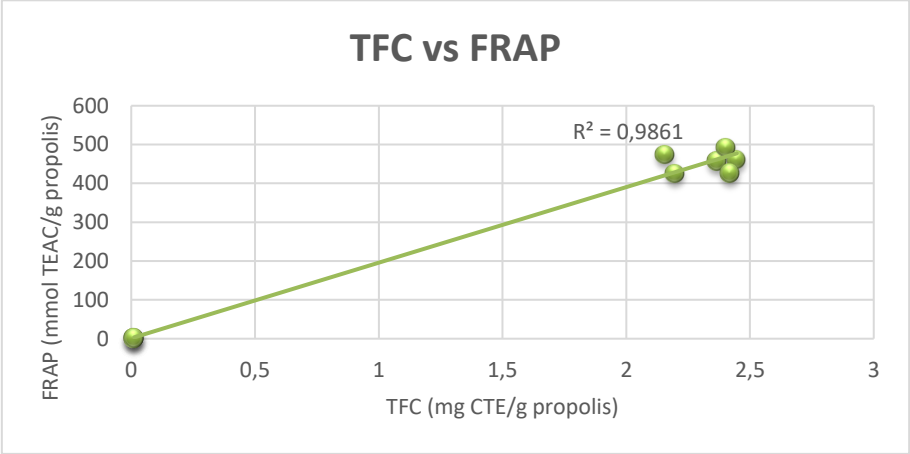


Figure 13 – Observed correlations between flavonoids content and antioxidant activity: a) between TFC (mg CTE/g propolis) and DPPH analysis (mmol TEAC/g propolis); b) between TFC (mg CTE/g propolis) and ABTS analysis (mmol TEAC/g propolis); and c) between TFC (mg CTE/g propolis) and FRAP assay (mmol TEAC/g propolis).

Flavonoids are considered one of the most important classes of phenolic compounds and are also extensively studied as they are the most abundant and effective antioxidant in propolis [145]. With this background, several authors have studied the antioxidant activity of Argentine propolis and concluded that the correlation between flavonoid content and antioxidant activity is quite significant [146, 147].

Yamauchi et al (1992) also conducted a study with Chinese propolis extracts from which they isolated benzyl caffeate to investigate its antioxidant activity. This compound showed significant antioxidant activity, leading to the conclusion that there are other components of propolis besides flavonoids that also confer antioxidant properties to this bioproduct [148].

The correlation between the content of total flavonoids and the antioxidant activity of G23-propolis is quite consistent with the literature, since the G23-propolis extracts had high flavonoid contents and consequently high antioxidant activity. This fact is reflected in the results obtained, since the values for both flavonoid content and antioxidant activity are significantly higher ($p > 0.05$) than in all the literature consulted.

When analysing the antioxidant activity of these three tests, we concluded that all propolis extracts exhibited antioxidant activity, with the ethanolic extracts performing significantly ($p < 0.05$) better than the aqueous extracts. In fact, all values for the tests with the aqueous extracts were very low, indicating little or almost no antioxidant activity.

4.6 Anti-aging Activity of Propolis

4.6.1 Tyrosinase Inhibition Assay

Tyrosinase inhibitory activity quantifies the ability of certain compounds to reduce or inhibit the action of the enzyme tyrosinase. This enzyme is involved in the processes responsible for catalyzing the first two stages of melanin synthesis. Apart from pigmentation, the main function of this compound is to protect the skin from harmful factors such as ultraviolet radiation by absorbing UV rays and eliminating ROS. Reducing melanin synthesis is important for the treatment of hyperpigmented diseases such as melasma, spots, and scarring of skin lesions [149, 150]. This enzyme is also involved in the process of enzymatic browning of foods, which is considered to be detrimental to the quality of plant-based foods and beverages. Therefore, the control of tyrosinase activity at the processing stage can make an important contribution, e.g., in the preservation of foods [151, 152].

The ability of G23 propolis extracts to inhibit the enzyme tyrosinase was evaluated and the results are shown in **Figure 14**.

After analysing **Figure 14**, the first thing to note is that the solvent 96% EtOH showed a much higher percentage of tyrosinase inhibition compared to the other solvents (46.9 ± 0.9%). Interestingly, the extracts treated with 70% EtOH showed non relevant activity (20.9 ± 1.6%), with a similar extract concentration. This may mean that more lipophilic compounds from propolis may be responsible for the greater tyrosinase inhibition activity. Since the H_2O solvent had a very low

extraction yield, low tyrosinase inhibition values were expected, and only 15.4 ± 1.19% were obtained.

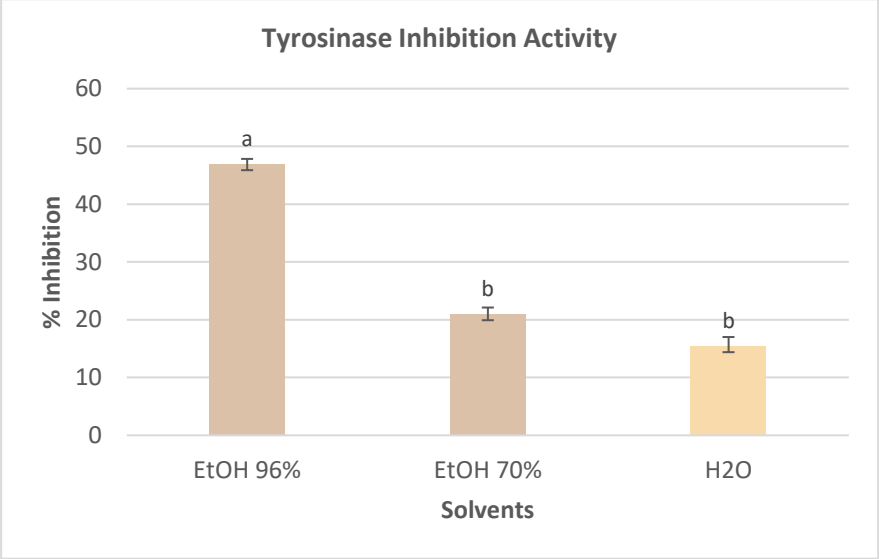


Figure 14 – Tyrosinase inhibitory activity of G23 corresponding to samples containing 96% EtOH, 70% EtOH and H_2O, with concentrations of 135.4 mg/mL, 134.1 mg/mL and 5 mg/mL, respectively. Values are expressed as mean ± SD. Different letters (a, b) indicate significant differences ($p < 0.05$) between the extraction solvents for the same propolis sample.

To date, there is little literature on the tyrosinase inhibition capacity of propolis extracts. However, Stavropoulou et al. (2021) described the tyrosinase inhibition potential of 20 samples from different regions of Greece. The highest tyrosinase inhibitory potential was characteristic of the terpenoid-rich samples (68.46 - 85.69%), where the propolis concentration was 200 µg/mL. However, the samples with high content of flavonoids showed inhibition range between 40.14% - 56.66% [153].

In another study, the tyrosinase inhibitory capacity of 21 propolis samples from different locations in Morocco was evaluated, and two of these samples showed very high inhibitory capacity, with IC_{50} values of 0.050 mg/mL and 0.037 mg/mL. However, a negative correlation was observed between the IC_{50} and the concentration of polyphenolic compounds. According to the author of this study, this can be explained by the chelating mechanism of the copper ion (Cu^{2+}) located in the active site of tyrosinase between the vicinal 30,40-dihydroxyl group of catechol [154].

Deniz et al. (2021) also investigated the tyrosinase inhibitory effect of 92 herbal extracts and propolis extracts. For the propolis extracts, they achieved IC_{50} tyrosinase inhibition values of 169.70 ± 2.96 µg/mL, corresponding to an inhibitory effect of more than 50% (64.38 ± 2.89%) [155].

Since flavonoids represent a very important class of tyrosinase inhibitors, as the hydroxyl groups on the A and B rings characteristic of these compounds cause the formation of Cu^{2+} chelators in tyrosinase, a stronger inhibitory effect on this enzyme can be expected in samples with a high content of flavonoids [156].

Since the quantification of tyrosinase inhibitory activity of the G23 propolis extracts was only informative and it was necessary to repeat this assay with equal concentrations of the extracts, it is not possible to compare these results with the limited literature found.

4.6.2 Elastase Inhibition Assay

This test is based on evaluating the effect of a substance that inhibits the enzyme elastase. This enzyme is responsible for the degradation of elastin as well as collagen and fibronectin, which are essential for the elastic properties of the extracellular matrix. Elastase is responsible for the first two steps of the melanin biosynthesis pathway and thus for eye, skin and hair coloration in humans. Its unregulated activity causes pigmentary disorders of the skin known as urticaria pigmentosa and age-related hyperpigmentation of the skin [157, 158].

As with the tyrosinase assay, the analysis of the behavior of propolis extracts in inhibiting the enzyme elastase is still very recent, and there are few studies in the literature. However, Deniz et al. (2021), in addition to evaluating the tyrosinase inhibition activity of propolis extracts, also performed the elastase inhibition assay and obtained a value of 45.43 ± 5.22% [155].

The evaluation of the inhibitory effect of elastase enzyme was performed with the G23 propolis extracts. However, after several repetitions of this procedure, the values always remained above the control of this assay (Tris-HCl buffer). In these three replicates, some dilutions of the G23 extract were made to make this analysis valid, but the values always remained above the control. Furthermore, the control would have to be repeated, which led me to the conclusion that this test should be repeated to quantify the potential of G23 propolis extracts to inhibit elastase.

An analysis of the anti-aging activity of the G23 propolis samples shows the potential of these extracts.

As for the tyrosinase test, considerably good values have been obtained when ethanolic extracts (96%) are used, which may represent alternatives of biological origin for the cosmetic industry as an additional ingredient in a product formulation or for the food industry as a biopreservative to extend the shelf life of a food product [149, 151, 152]. Regarding the elastase inhibitory effect, the potential of the ethanolic extracts of propolis G23 can be seen, and there is a need to repeat this test to confirm what has been described above.

It should also be noted that the geographical origin, climate and time of harvest are extremely important factors that affect the bioactivity of propolis in some way. Thus, considering that the G23 propolis was taken after one month of production and always stored at low temperatures, the values obtained in the anti-aging analyses were favorable.

5. Conclusions

The propolis from Guarda (G23) (in inland Portugal) proved to be very soluble in ethanol and sparingly soluble in water, which resulted in quite different extraction yields with respect to these two solvents. Thus, the extraction yield of G23 propolis reached a maximum value when treated with 96% ethanol or 70% ethanol.

Propolis is a bioproduct characterized by its chemical composition rich in bioactive compounds. Since the main objective of this work was based on the evaluation of antioxidant and anti-aging activity, different methods were used to analyse the potential of G23 propolis.

The composition of total phenolic compounds and flavonoids was an important step to understand the amount of bioactive compounds extracted from propolis in this study. In both tests, the levels in the ethanolic extracts were considerably high. In both tests, the values were significantly higher in the ethanolic extracts ($p < 0.05$). Since the aqueous extracts had poor solubility, as reflected in the extraction yield, the amount of total phenols and flavonoids decreased significantly ($p > 0.05$).

Regarding the antioxidant activity evaluated by different methods (ABTS, DPPH, and FRAP), it was concluded that the ethanolic extracts showed much greater antioxidant activity compared with the aqueous extracts. In addition, the correlations between the antioxidant activity and the content of phenols and flavonoids were studied, and it was found that a higher content of total phenols and extracted flavonoids led to a greater antioxidant activity.

In the anti-aging tests (inhibition of tyrosinase and elastase), the potential of ethanolic extracts of propolis G23 was also verified. As mentioned above, the chemical composition and bioactivity of propolis can vary significantly depending on the geographical location, plant species and climate to which it is exposed. In this case, EtOH 96% extracts showed better performance.

It can be concluded that propolis extracts are potential natural ingredients that can be used in various products in different industries, namely food and cosmetics. It also proves to be a viable alternative that could lead to a significant reduction in food waste in the long term.

6. FUTURE PERSPECTIVES

In a possible continuation of this work, it would be interesting to perform an in-depth analysis of the composition of G23 propolis and identify any phenolic compounds that may be present through an HPLC-MS analysis. In addition, it would also be useful to evaluate the phenolic profile with different concentrations of ethanol at room temperature to obtain more comparative conditions with the present study. This would provide more informative details to choose the most efficient extraction that extracts the maximum amounts of bioactive compounds and maintains their bioactivity throughout the process.

For future work on this study, it would be interesting to analyse the behavior of propolis in natural lipophilic solvents, such as 100% sunflower oil. It would also be useful to quantify the content of total phenols and flavonoids and analyse the antioxidant activity of the extracts.

As for the anti-aging tests performed, they serve as a starting point for possible application in the field of cosmetics, as a promising source of natural bioactive compounds that can be used in formulations of products of natural origin, and as a means of inhibiting or eliminating ROS and preventing skin diseases.

It was also planned to perform both anti-inflammatory and antibacterial tests with the G23 propolis extracts, but due to the limited time to perform this study, this is left as a suggestion for further work. Since propolis is known for its antioxidant power and is often associated with anti-inflammatory activity, it is expected to be able to eliminate ROS, a very important property in the cosmetic industry. In addition, the study of antibacterial activity will aim to provide promising results in terms of extending the shelf life of a food product and ensuring food safety.

In order to expand the possible applications of propolis extracts for other purposes, namely in the therapeutic field, it would also be interesting to evaluate the biological properties of propolis in different cell lines, such as colon adenocarcinoma cells (Caco-2). Therefore, the aim would be to investigate the ability of propolis extracts to inhibit tumor cells as well as their ability to eliminate oxidative stress.

The detailed study of the phenolic profile can not only help to establish a value for propolis extracts, but also reveal the natural source of antioxidant and anti-aging compounds that can be combined or provide sustainable alternatives for large-scale industry in the food and cosmetic sectors.

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8. APPENDIXES

8.1 Appendix 1: GAE calibration curve of Total Phenolic Content (TPC)

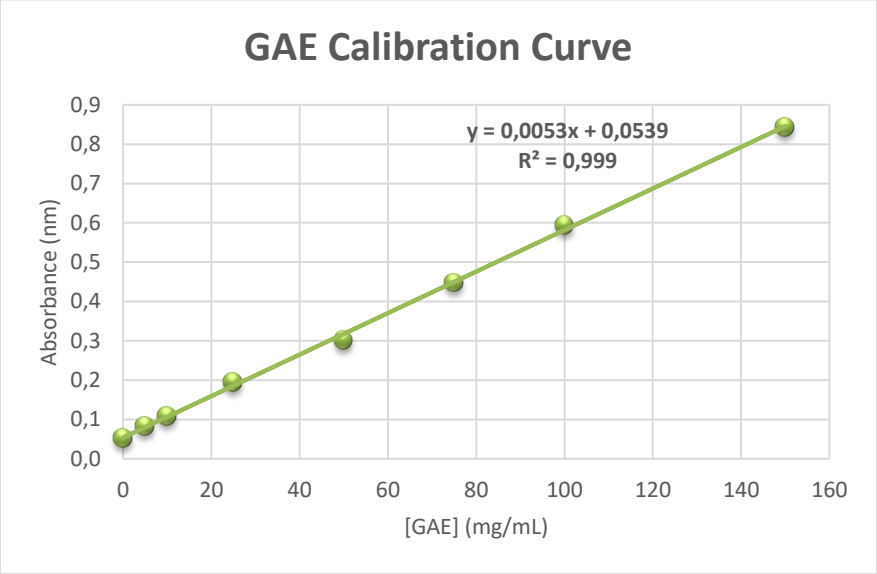


Figure 15 – Calibration curve of Gallic Acid (0 - 150 mg/mL) as standard for Total Phenolic Content.

8.2 Appendix 2: CTE calibration curve of Total Flavonoids Content (TFC)

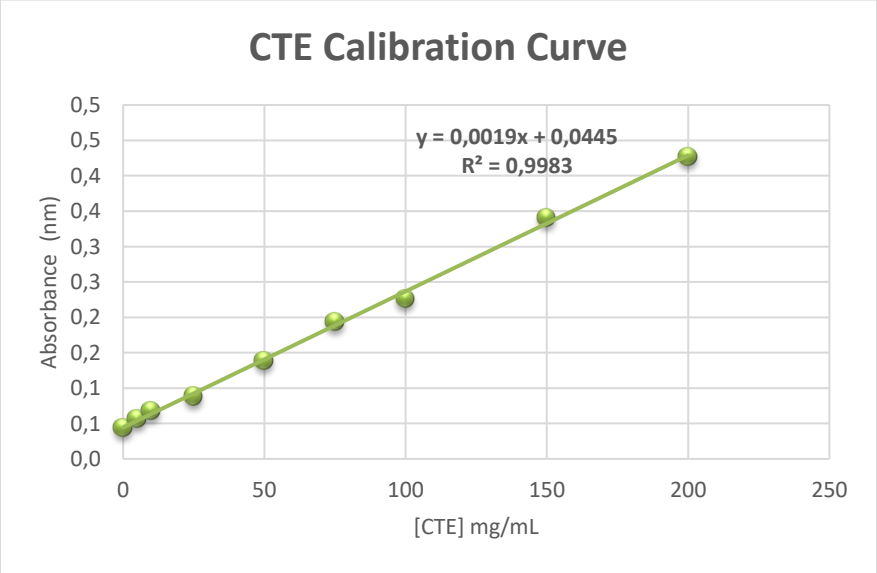


Figure 16 – Calibration curve of Catechin (0 - 200 mg/mL) as standard for Total Flavonoids Content.

8.3 Appendix 3: TEAC Calibration Curve of ABTS

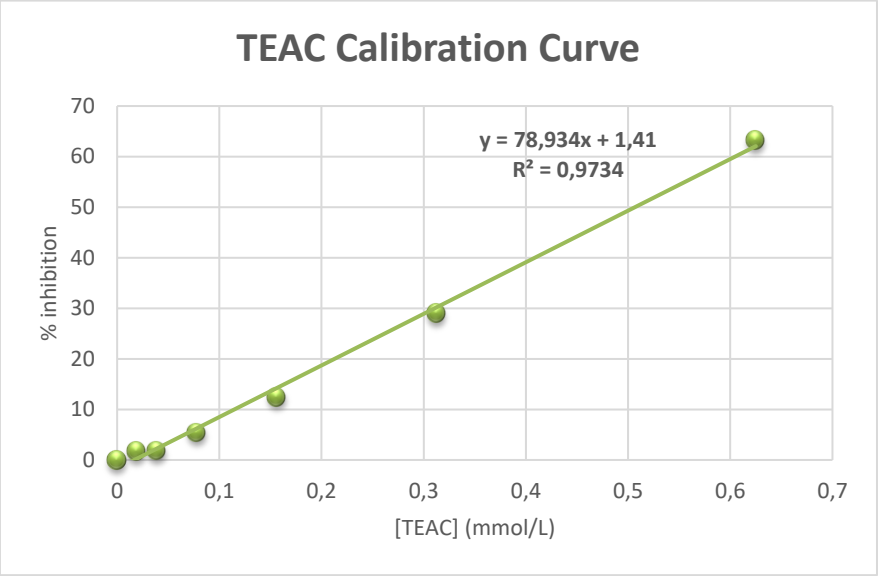


Figure 17 – Calibration curve of Trolox-Equivalent Antioxidant Capacity (TEAC) (0 - 1.25 mmol/L) as standard for ABTS.

8.4 Appendix 4: TEAC Calibration Curve of DPPH

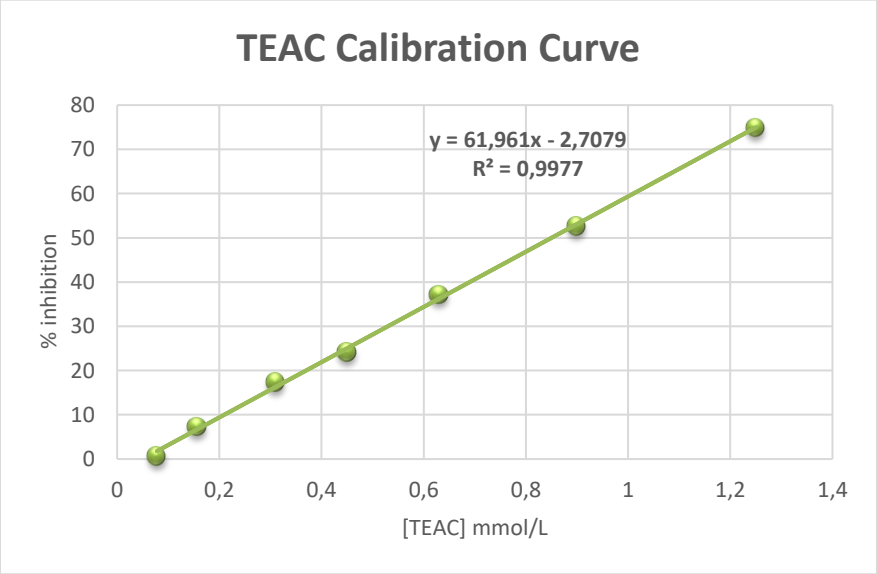


Figure 18 – Calibration curve of Trolox-Equivalent Antioxidant Capacity (TEAC) (0 - 1.25 mmol/L) as standard for DPPH.

8.5 Appendix 5: TEAC Calibration Curve of FRAP

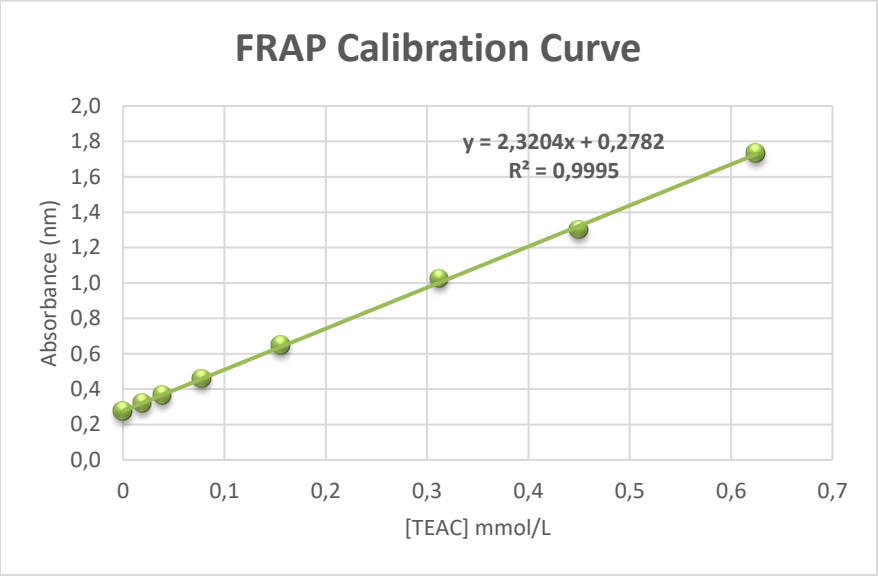


Figure 19 – Calibration curve of Trolox-Equivalent Antioxidant Capacity (TEAC) (0 - 1.25 mmol/L) as standard for FRAP.