Universidade do Minho
Escola de Ciências

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e
Professora Doutora Maria de Fátima Monginho Baltazar

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STATEMENT OF INTEGRITY

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RESUMO

Propriedades antioxidantes, antimicrobianas e anticancerígenas do própolis Português do Gerês

O própolis é um produto natural produzido por abelhas, maioritariamente da espécie *Apis mellifera* L.. É uma mistura resinosa com um cheiro característico e cor variável de acordo com sua origem e idade. Ao longo dos anos, diferentes grupos de compostos foram identificados no própolis e associados a um amplo espectro de propriedades biológicas: antibacteriana, anticancerígena, antifúngica, anti-protozoária, antiviral, antioxidante, anti-inflamatória, hepatoprotetora, cardioprotetora, anti-neurodegenerativa, anti-tuberculose, anestésica, imuno-estimulante, antienvelhecimento e atividade cicatrizante.

Algumas destas propriedades biológicas foram também atribuídas ao própolis português, inclusive pelo nosso grupo de investigação, sendo a atividade antioxidante e a atividade antimicrobiana das mais bem documentadas. Assim, neste trabalho, foram avaliadas, para o própolis português do Gerês, estas duas propriedades, mas também a atividade anticancerígena, uma bioatividade menos caracterizada no própolis nacional. Primeiramente, avaliou-se a qualidade da amostra de própolis recolhida do apiário Gerês em 2021 (G21), revelando cumprir os requisitos de qualidade estabelecidos e sendo semelhantes aos estabelecidos para o própolis português tipo I, considerado análogo ao própolis do tipo Choupo, comum na Europa. A extração etanólica de G21 permitiu obter G21.EE, extrato que foi caracterizado quimicamente quanto aos teores de polifenóis totais, flavonoides totais e *orto*-difenóis, e que mostrou que o G21 é uma amostra rica em compostos fenólicos. Paralelamente, ensaios *in vitro* demonstraram que o G21.EE possui uma elevada capacidade antioxidante. Relativamente à atividade antimicrobiana, G21.EE mostrou-se bastante eficaz a inibir o crescimento microbiano, principalmente de espécies Gram-positivas do género *Bacillus*. Por fim, a atividade anticancerígena do própolis foi avaliada em melanoma, o tipo mais agressivo de cancro de pele e associado a altas taxas de resistência às terapias convencionais. G21.EE tal como G18.EE mas também a sua fração G18.EE_*n*-BuOH, mostraram-se eficazes contra células de melanoma, pelo que se usou esta fração para isolamento e fracionamento adicionais. Algumas das sub-frações provenientes deste fracionamento de G18.EE_*n*-BuOH mostraram elevada atividade anti-melanoma e os compostos nelas identificados são potenciais alvos para pesquisa de novos agentes terapêuticos.

Palavras-chave: Atividade Antimicrobiana, Atividade Antioxidante, Atividade Anticancerígena, Compostos Fenólicos, Melanoma, Própolis Português, Sub-frações.

ABSTRACT

Antioxidant, antimicrobial and anticancer properties of Portuguese propolis from Gerês

Propolis is a natural product produced by bees, mainly of the species *Apis mellifera* L.. It is a resinous mixture with a characteristic smell and colour that varies according to its origin and age. Over the years, different groups of compounds have been identified in propolis and were associated with a wide spectrum of biological properties: antibacterial, anticancer, antifungal, anti-protozoal, antiviral, antioxidant, anti-inflammatory, hepatoprotective, cardioprotective, anti-neurodegenerative, anti-tuberculosis, anaesthetic, immunostimulant, anti-ageing and healing activity.

Some of these biological properties have also been attributed to Portuguese propolis, including by our research group, with antioxidant activity and antimicrobial activity being the best documented. Thus, in this work, these two properties were evaluated for the Portuguese propolis from Gerês, but also the anti-cancer activity, a less characterized bioactivity in national propolis. Firstly, the quality of the propolis sample collected from the Gerês apiary in 2021 (G21) was evaluated, revealing to meet the established quality requirements, and proving to be similar to those established for Portuguese type I propolis, considered analogous to Poplar type, common in Europe. The ethanol extraction of G21 yielded G21.EE, an extract that was chemically characterized as to total polyphenol, total flavonoid and *ortho*-diphenol contents, and showed that G21 is a sample rich in phenolic compounds. In parallel, *in vitro* assays demonstrated that G21.EE has a high antioxidant capacity. Regarding antimicrobial activity, G21.EE proved to be very effective at inhibiting microbial growth, mainly of Gram-positive species of the genus *Bacillus*. Finally, the anticancer activity of propolis was evaluated in melanoma, the most aggressive type of skin cancer and associated with high rates of resistance to conventional therapies. G21.EE as well as G18.EE, but also the fraction G18.EE_*n*-BuOH, showed to be effective against melanoma cells, so this fraction was used for further fractionation and isolation. Some of the subfractions from this fractionation of G18.EE_*n*-BuOH showed high anti-melanoma activity, becoming potential targets for research of new therapeutic agents and the compounds identified in them are potential leads for research into new therapeutic agents.

Keywords: Antimicrobial Activity, Antioxidant Activity, Anticancer Activity, Melanoma, Phenolic Compounds, Portuguese Propolis, Subfractions.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABTS – 2,2′-azinobis(3-ethylbenzothiazolin-6-sulphonate)

Bax – Bcl-2 associated X protein

Bcl-2 – B-cell lymphoma 2

BRAF – V-RAF murine sarcoma viral oncogene homolog B

CAPE – Caffeic Acid Phenethyl Ester

CRC – Colorectal Cancer

DMEM – Dulbecco's Modified Eagle's Medium

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic acid

DPPH – α,α- diphenyl-β-picrylhydrazyl

EC_{50} – Concentration that generates half of the maximal response

EE – Ethanol Extract

ERK – Extracellular Signal-regulated Kinase

FBS – Fetal Bovine Serum

FDA – Food and Drug Administration

Folin-C – Folin-Ciocalteau reagent

FRAP – Ferric Reducing Antioxidant Power

G – Gerês propolis

G.EEs – Ethanol extracts of Gerês propolis

G11 – Gerês propolis harvested in 2011

G12 – Gerês propolis harvested in 2012

G13 – Gerês propolis harvested in 2013

G14 – Gerês propolis harvested in 2014

G15 – Gerês propolis harvested in 2015

G18 – Gerês propolis harvested in 2018

G18.EE_ *n*-BuOH – *n*-BuOH fraction resulting of G18.EE fractionation

G21 – Gerês propolis harvested in 2021

GA – Gallic Acid

GAE – Gallic Acid Equivalent

GC-MS – Gas chromatography–mass spectrometry

IC_{50} – Half-maximal inhibitory concentration

IC_{50}2021 – IC_{50} of G18.EE calculated in 2021 against A375 melanoma cell line

IC_{50}2022 – IC_{50} of G18.EE calculated in 2022 against A375 melanoma cell line

MAPK – Mitogen-activated Protein Kinase

MEK – Mitogen-activated Protein Kinase Kinase

MIC – Minimum Inhibitory Concentration

MRSA – Methicillin resistant *Staphylococcus aureus*

MSSA – Methicillin-sensitive *Staphylococcus aureus*

OD – Optical Density

OD_{600} – Optical Density at 600 nm

OD_{734} – Optical Density at 734 nm

Q – Quercetin

QE – Quercetin Equivalent

RAF – Rapidly Accelerated Fibrosarcoma

ROS – Reactive Oxygen Species

rpm – Rotations per minute

RT – Room temperature

SD – Standard deviation

SRB – Sulforhodamine B

TCA – Trichloroacetic Acid

TFC – Total Flavonoids Content

TLC – Thin Layer Chromatography

TOC – Total *Ortho*-diphenols Content

TPC – Total Polyphenols Content

TRPIQ – Technical Regulation of Propolis Identity and Quality

UV – Ultraviolet

WHO – World Health Organization

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CHAPTER I: INTRODUCTION

Since always, humanity has searched for remedies and cures for several diseases, with natural products emerging as one of the first solutions (Viegas *et al.*, 2006). Indeed, the use of natural products as remedies is recognized since ancient times (Yuan *et al.*, 2006). According to fossil records, plants have been used by humans as medicines for at least 60,000 years (Fabricant *et al.*, 2001). The advance in science allowed the discovery of the pharmacology and mechanisms of action of some bioactive compounds responsible for the therapeutic activity of some natural products, enabling their application in traditional medicine (Yuan *et al.*, 2016). Over the last years, several compounds have been isolated from natural products, and their structure determined (Viegas *et al.*, 2006). One of the most important and best-known products is morphine, which was isolated from *Papaver somniferum* bulbs and is currently used as an analgesic (Hamilton and Baskett, 2000). Other products derived from plants, microorganisms or animals are used due to their anticancer, anti-inflammatory or antidiabetic properties, for example (Harvey, 2008).

In recent years, the tendency to look to natural products as a strategy for drug discovery continues. It is estimated that about a third of the drugs approved by the FDA (Food and Drug Administration) in the last 20 years are based on natural products (Thomford *et al.*, 2018). In this context, beekeeping products have also been the subject of research due to their well-known therapeutic characteristics (Pasupuleti *et al.*, 2017). Honeybees are ancient flying insects of extreme importance to life on earth. These insects can produce specific products, like honey, propolis, royal jelly, bee venom, pollen, and beeswax; all of which have been extensively used since ancient times. Different bioactivities have already been ascribed to some of these natural products, such as anti-inflammatory, antimicrobial, and antioxidant activities (Attalla *et al.*, 2007; Freire *et al.*, 2012; Owoyele *et al.*, 2014; Junie *et al.*, 2016). Honey may be effective in cancer therapy due to the inhibition of tumour growth and metastasis and the induction of apoptosis in cancer cells (Jaganathan *et al.*, 2014; Sforcin *et al.*, 2017). Supplementation with royal jelly has been demonstrated to help control diabetes symptoms of by reducing fasting blood glucose levels and elevated insulin concentration (Pourmoradian *et al.*, 2014). Bee venom has a strong activity against bacteria, viruses, and fungi, due to a composition rich in important antimicrobial agents, like melittin and apamin (El-Seedi *et al*, 2020). Pollen has compounds such as polyphenols or flavonoids that stimulate cells playing a crucial role in inflammatory processes such as macrophages, hepatocytes, basophils, neutrophils, eosinophils (Denisow and Denisow-Pietrzyk, 2016). Beeswax is the bee product with the least attributed bioactivities and is mainly used in dermocosmetic products (Cornara *et al.*, 2017). In this dissertation, the focus will be on propolis and its characteristics and properties.

1. Propolis and its characteristics

1.1 What is propolis?

Propolis is a natural product produced by honeybees, namely *Apis mellifera,* from resins collected from resinous sprouts and exudates of plants (Moreira *et al.*, 2008). The collected resins are mixed with salivary enzyme β-glycosidase, which partially digests the mixture, and with bee wax and other products of bees' metabolism forming the final product (Silva *et al.*, 2012). Propolis' main function is to protect the hives from predators (Silva-Carvalho *et al.*, 2015), killing them by asphyxia while covering their bodies, in this way preventing microbial putrefaction (Moreira *et al.*, 2008). It is also used to repair damages in the hive, seal the walls, and strengthen the borders of combs (Bankova, 2005a). Thus, it makes sense the Greek origin of the word meaning pro=in defence, and polis=city, that is, in defence of the city or the hive, in this case.

Propolis has a lipophilic nature, being hard and easily breakable when cold, but flexible and sticky as a glue when heated (Silva-Carvalho *et al.*, 2015). It has a characteristic smell, and its colour can vary according to its origin and age, usually raging between yellow-green, red, and dark brown **(Figure 1)** (Bankova *et al.*, 2000).

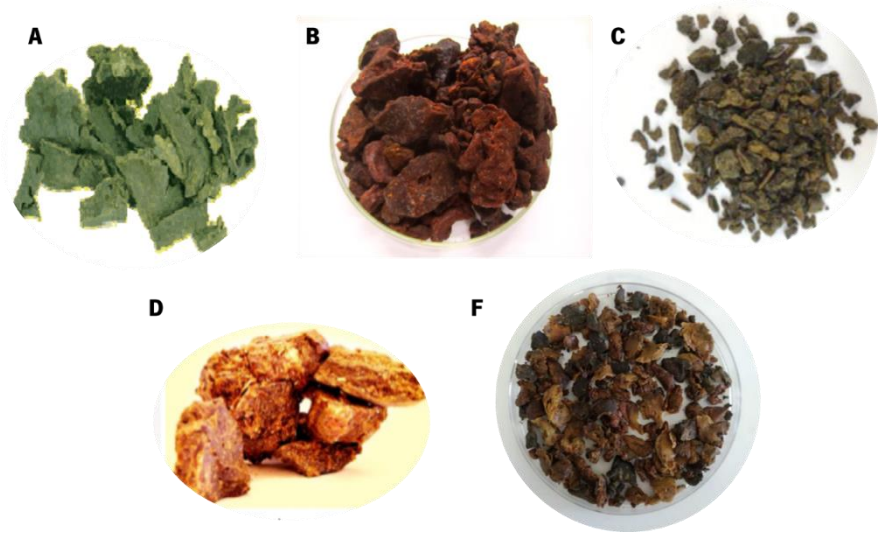

Figure 1. Different types of propolis. (A) Green propolis; **(B)** Red propolis; **(C)** Brown propolis; **(D)** Poplar propolis; **(F)** Portuguese propolis from North of Portugal (adapted from Berretta *et al.*, 2017; Santos, 2015; Bogdanov, 2016b; Peixoto, 2021).

1.2 Historical importance

Propolis is considered one of the most important "chemical weapons" of bees and has been extensively used as a remedy by humans since ancient times, at least from 300 BC (Ghisalberti, 1979)., being its use reported in numerous civilizations. The Egyptians used propolis to embalm cadavers and Jews applied it as a medicine, as mentioned in the Old Testament (Falcão, 2013). During the Roman Empire, warriors used propolis as an emergency remedy to treat wounds (Salatino *et al.*, 2005) and, in the Middle Age, Arabs used this mixture for the treatment of oral infections, dental caries, or as an antiseptic and healing agent (Castaldo and Capasso, 2002). In Europe, propolis became better known between the 17th and 20th centuries due to its antibacterial activity (Castaldo and Capasso, 2002). Its application was also documented during World War II to treat wounds. Propolis was also approved in 1969 in the former Union of Soviet Socialist Republics to treat tuberculosis (Silva-Carvalho *et al.*, 2015). Widely used over the years, propolis has been the target of several chemical and pharmacological studies in the last decades. Nowadays, propolis is used in different products such as drinks, foods, or cosmetics like antiacne creams (Moreira *et al.*, 2008), particularly in Asian countries, and the numerous properties of propolis still makes this hive resource interesting for these and other applications. It is estimated that the actual worldwide production of propolis is around 1800 to 2400 tons/year (Maximize Market Research, 2021).

The first time a propolis-based product was patented was in 1965 in Romania (Pereira *et al.*, 2002). Currently, most of the patents are Japanese, although the first patent of this country, concerning a product used to control unpleasant odours, only appeared in 1987. In Brazil, where propolis is also widely explored, the first product was patented in 1995 and is related to the prevention of tooth decay and gingivitis (Pereira *et al.*, 2002).

1.3 Geographic distribution

Propolis has a high variable chemical composition, that is related to the bee's species that produces it, the type of plants found around the hive, and the geographical and climatic conditions (Bankova *et al.*, 2000). Other factors such as the time of collection and the harvesting method can also influence the characteristics of the obtained product (Pereira *et al.*, 2002).

In Europe, North America and some regions of Asia and New Zealand, where the climate is temperate, the main plant source of resin for propolis is *Populus* spp., being propolis known as Poplar

(Bankova *et al.*, 2000). In Brazil four propolis types are known: Green, Red, Clusia and Brown and the main plant sources are *Baccharis* spp., *Dalbergia* spp., *Clusia* spp. and *Araucaria angistifolia* respectively. Red and Clusia propolis are also found in other regions of Latin America (Falcão, 2013). In Russia, bees collect the resin of birch buds, with origin in plants such as *Betula verrucose* (Bankova *et al.*, 2000). In the Mediterranean area, bees use trees from the family of *Crupessus spp. and Conifer spp.* (Silva-Carvalho, 2013) whereas in the Pacific islands a different type of propolis was found, being the resin normally collected from *Macaranga tanarius* (Falcão, 2013). In several African countries, the main resin source is *Macaranga schweinfurthii* (Silva-Carvalho *et al.*, 2015). Research in Australia has revealed that different bee species prefer different plants: for instance, *Apis mellifera* visit *Acacia paradoxa* while *Tetragonula carbonaria* bees prefer *Corymbia torelliana* trees. *Mangifera indica* is a type of plant visited by bees in countries such as Indonesia, Oman, Brazil, Myanmar and Thailand producing a distinctive propolis called "*Mangifera* propolis". **(Table 1)** (Silva-Carvalho *et al.*, 2015).

Table 1. Currently recognized types of propolis according to geographical origin, botanical source, and marker compounds (adapted from Bankova *et al.*, 2000; Fokt *et al.*, 2010; Falcão, 2013; Silva-Carvalho *et al.*, 2015; Bankova *et al.*, 2016; Kasote *et al.*, 2022).

Propolis type	Geographic area	Principal plant source	Key compounds
Poplar	Europe, North America and non-tropical regions of Asia and New Zealand	*Populus* spp	Pinocembrin, pinobanksin, chrysin, galangin, caffeic acid, ferulic acid, cinnamic acid and their cinnamic acid and its esters
Green	Brazil	*Baccharis* spp	Artepillin C, drupanin, benzoic and chlorogenic acids, prenylated p-coumaric acid, acetophenones, diterpenic acids, caffeoyl quinic acids, kaempferide isosakuranetin and kaempferol
Red	Brazil and other regions of Latin America	*Dalbergia* spp	Formononetin, isoliquiritigenin, moronic acid, mucronulatol, neovestitol liquiritigenin, hyperibone A, medicarpin and biochanin A
Clusia	Brazil and other regions of Latin America	*Clusia* spp	Polyprenylated benzophenones, nemoronose, xanthochymol and guttiferone E
Brown	Brazil	*Araucaria angistifolia* and *Eucalyptus spp.*	Communic acid, ferulic acid, caffeic acid, isopimaric acid and imbricataloic acid
Birch	Russia	*Betula spp.*	Caffeic acid, chrysin, quercetin, naringenin, CAPE, terpenes and cinnamic acid acacetin, apigenin, ermanin, rhamnocitrin, campferide, α-acetoxybetulenol

Table 1. Currently recognized types of propolis according to geographical origin, botanical source, and marker compounds (adapted from Bankova *et al.*, 2000; Fokt *et al.*, 2010; Falcão, 2013; Silva-Carvalho *et al.*, 2015; Bankova *et al.*, 2016; Kasote *et al.*, 2022) (continued).

Mediterranean	*Mediterranean area*	*Crupessus spp. and Conifer spp.*	*Flavonoids, diterpenic acids (isocuprésic, pimaric and comminic), isoagatolal, agatadiol, ferruginol and totarol*
Pacific	Pacific islands	*Macaranga tanarius*	C-prenylflavonones, alk(en)ylresorcinols, cycloartane-type triterpenes, and 27-hydroxyisomangiferolic acid
African	Nigeria, Kenya, Congo, and Cameroon	*Macaranga schweinfurthii*	Arylnaphthalene, geranylstilbenes, geranylflavone and lignans
Australian	Australia	*Acacia paradoxa* and C. *torelliana*	Cinnamic acid esters, coumaric acids, chalcones, flavonols and stilbenes
Mangifera	Indonesia, Oman, Brazil, Myanmar, and Thailand	*Mangifera indica*	Prenylflavanones, alk(en)ylresorcinols, anacardic acids and cycloartane-type triterpenes

1.4 Propolis chemical composition

Propolis is a complex mixture with 800 compounds already identified in samples from different regions, being expected that new compounds will be still identified during the chemical characterization of new samples (Kasote *et al.*, 2022). In terms of chemical composition, and despite all the different possible compounds that propolis can present, it can be inferred that it is generically constituted by 50% resin, 30% wax, 10% essential oils, 5% pollen, and 5% of other substances, which include minerals and organic compounds.

The chemical components of resinous portion of propolis are responsible for its biological properties. The main isolated class of propolis bioactive compounds include phenolic acids and flavonoids **(Figure 2)** (Moreira *et al.*, 2008) and some compounds were already identified like phenolic acids (cinnamic and caffeic acid) and their esters, flavonoids (flavones, flavanones, flavonols, and dihydroflavonols chalcones), terpenes, aromatic aldehydes and alcohols, fatty acids, stilbenes, and β-steroids (Silva-Carvalho *et al.*, 2015).

Figure 2. Basic structure of (A) phenolic acids and (B) flavonoids (adapted from Zin *et al.*, 2020).

Essential oils found in propolis are responsible for the aroma and have also biological significance. They are mainly mono- and sesquiterpenes, although there is diversity in the compounds present

(Bankova *et al.*, 2000). In tropical regions, sesquiterpenoids are found, while samples from Brazil have prenylated acetophenones (Bankova *et al.*, 2000).

Propolis composition is different depending on the region. In temperate regions, such as Europe, the main constituents are flavonoids without B-ring substituents - such as chrysin, galangin, pinocembrin, pinobanksin and their esters - and phenylpropanoids and their esters - such as caffeic acid phenylethyl ester (CAPE) **(Figure 3)** (Huang *et al.*, 2014). CAPE is one of the main constituents of propolis from these regions and has proven to be one of the most important bioactive compounds (Bankova, 2005a; Huang *et al.*, 2014).

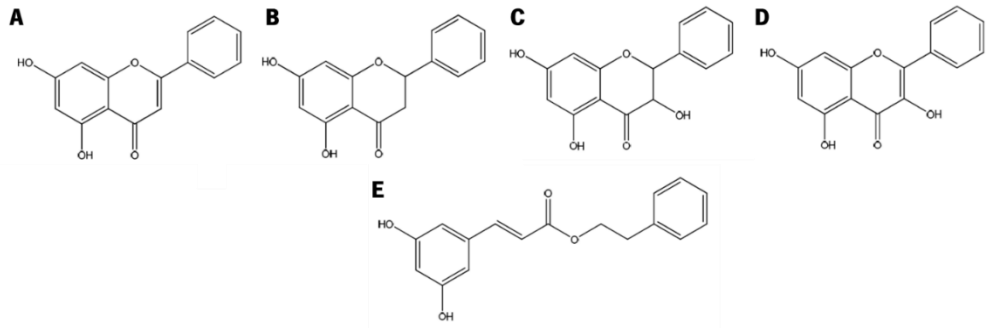

Figure 3. The basic structure of phenolic compounds found in propolis from temperate regions (A) Chrysin; (B) Pinocembrin; (C) Pinobanksin; (D) Galangin; (E) CAPE (adapted from Falcão, 2013).

In tropical regions, such as Brazil, flavonoids are also important components as in Europe, although the plant sources are different (Bankova *et al.*, 2000). Propolis includes in its composition prenylated phenylpropanoids, like artepillin C and drupanin, and caffeoylquinic acids, such as dicaffeoylquinic acid and its derivatives **(Figure 4)** (Falcão, 2013).

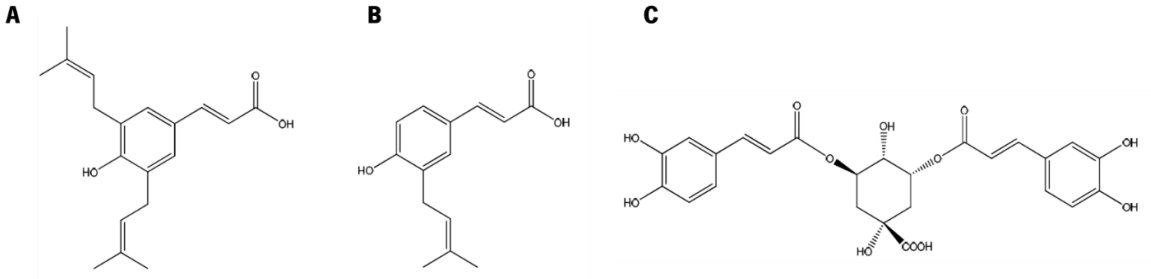

Figure 4. The basic structure of phenolic compounds found in propolis from tropical regions (A) Artepillin C; (B) Drupanin; (C) Dicaffeoylquinic acid (adapted from Falcão, 2013).

Russian propolis also contains flavones and flavonols, but they are different from those found in European propolis (Falcão, 2013). In propolis from the Mediterranean region, Pacific islands, African countries, Australia and Nepal the main chemical compounds were also described and are summarized in **Table 1**.

1.5 Quality standards

The difficulty in turning propolis into a pharmaceutical product is due to the fact that standardization of its chemical composition is difficult. Chemical standardization is mandatory to guarantee product safety and effectiveness (Bankova, 2005a). Nevertheless, due to the existence of different types of propolis with distinct and unique chemical profiles, establishing quality criteria becomes challenging (Bankova, 2005a). Currently, there is no international regulation for this natural product (Silva, 2017) and there are also no established techniques or analyses to guarantee its quality (Pereira, 2021). Thus, several methods of testing are currently used, often not comparable with each other (Pereira *et al.*, 2015; Bankova *et al.*, 2016; Lopes *et al.*, 2017). Yet, a study involving the application of propolis should ensure its quality and several parameters should be evaluated and taken into consideration (Bankova *et al.*, 2016). Criteria should be based on the concentration of compounds responsible for their biological activity, according to propolis types since each type presents a specific and typical chemical profile (Sawaya *et al.*, 2011). Propolis should also be evaluated both for its consistency, odour, taste, and colour and for its physicochemical properties such as density, melting point, and solubility in several solvents, like ethanol (Funari and Ferro, 2006). According to the International Honey Commission, a complete analysis should also evaluate parameters that can be accepted as universal, such as balsamic, ash, wax and water contents, and also the presence or absence of impurities (Lopes *et al.*, 2017). Thus, based on these parameters a good quality sample must be free of contaminants and have a high balsam content and low percentages of wax and ash. Furthermore, the active compounds should be known, and their content should be high (Falcão, 2013).

In Brazil, where the study of propolis is well established, there is the Technical Regulation of Propolis Identity and Quality (TRPIQ) of the Ministry of Agriculture, Livestock and Food Supply whose objective is to establish the characteristics and the minimum quality requirements **(Table 2)** for propolis in order to be available for national or international commerce (Brazil, 2001).

Table 2. **Standard requirements for propolis established by TRPIQ**. (Brazil, 2001).

Propolis content	TRPIQ
Ash (%)	Max. 5
Wax (%)	Max. 25
Water (%)	Max. 8
Phenolic compounds (%)	Min. 5
Flavonoids (%)	Min. 0.5
Ethanol soluble (%)	Min. 35
Oxidation activity (s)	Max. 22
Mechanical mass (%)	Max. 40

Also, for the best-known types of propolis the poplar and the green types, standard parameters have already been established (Popova *et al.*, 2007). These parameters are based on the quantification of the main compounds with biological activity, balsam, total flavone and flavonol contents, total flavanone and dihydroflavonol contents, and total phenolic contents **(Table 3)** (Bankova, 2005a; Falcão, 2013).

Table 3. **Parameters proposed for poplar and green propolis** (Popova *et al.*, 2007; Falcão, 2013).

Propolis content	Poplar (Min values, g/100g)	Green (Min values g/100g)
Ash (%)	-	Max. 5
Wax (%)	Max. 25	Max. 25
Balsam (%)	45	35
Total phenolics (%)	21	7
Flavones/flavonols (%)	5	-
Flavanones/dihydrofavonols (%)	4	-
Total flavonoids (%)	9	1
Insoluble matter (%)	Max. 5	Max. 5

In Portugal, two types of propolis have been characterized by Falcão (2013), which will be described in more detail below (see **1.7**), and some quality parameters have also been established for them **(Table 4)**. One of the characteristics of Type I propolis is its high balsamic content, which makes it a good quality propolis and gives it greater commercial value since a high balsamic content is directly related to a higher content of biologically active compounds. Type II propolis is significantly different, with a higher wax content and probably of inferior quality (Falcão 2013).

Table 4. **Parameters proposed for classification of Portuguese propolis** (Falcão, 2013).

Propolis content	Portuguese propolis type I	Portuguese propolis type II
Ash (%)	Max. 2	Max. 4
Wax (%)	Max. 25	Max. 31
Water (%)	Max. 5	Max. 5
Balsam content (%)	Min.65	Min.45
Total phenolics (%)	Min.18	Min.6
Flavones/flavonols (%)	Min. 3	Min.2
Flavanones/dihydrofavonols (%)	Min.5	Min.3
EC_{50} DPPH (mg/ml)	Max. 0.02	Max. 0.06

EC_{50} = concentration that generates half of the maximal response.

1.6 Biological and pharmacological properties

As already mentioned, propolis has been used as a therapeutic agent over the years due to its exceptional pharmacological properties. Considering the differences in propolis chemical composition, it would be expected that the biological properties would also be different, but this is not always verified (Bankova, 2005b). Kujumgiev *et al.* (1999) compared the antimicrobial activity and chemical composition of propolis from different geographical zones and proved that all propolis samples exhibit antibacterial activity regardless of location, proposing that different combinations of chemical compounds are essential for the biological activity of propolis.

The documented biological properties of propolis are the following: antibacterial, antitumour, antifungal, anti-protozoal, anti-viral, antioxidant, anti-inflammatory, hepato-protective, cardioprotective, antineurodegenerative, antituberculosis, local-anesthetic, immunostimulating, cytotoxic, genotoxic and anti-genotoxic, antiaging or cicatrizing (Ghisalberti, 1979; Falcão, 2013; Bankova 2005b; Anjum *et al.*, 2019; Kassote *et al.*, 2022). Some of the components of propolis that are responsible for such bioactivities are already known. **Table 5** outlines some of the compounds that have been associated with some propolis bioactivities.

Table 5. Principal compounds responsible for the biological properties attributed to propolis (adapted from Bankova, 2005b; Silva-Carvalho *et al.*, 2015).

Biological properties	Propolis compounds	References
Antibacterial	CAPE, caffeic acid, cinnamic acid, artepillin C, pinocembrin, galangin, and pinobanksin	Bankova *et al.*, 2000; Bankova, 2005b; Anjum *et al.*, 2018
Antitumour	CAPE and artepillin C	Castaldo and Capasso, 2002; Slavov *et al.*, 2013
Antifungal	Pinocembrin, p-coumaric acid and caffeic acid	Farnesi *et al.*, 2009; Anjum *et al.*, 2018
Anti-protozoal	Caffeic acid, chrysin, moronic acid, protocatechuic acid, p-coumaric acid and apigenin	Anjum *et al.*, 2018
Anti-viral	Kaempferol, acacetin, quercetin, galangin, isorhamnetin and chrysine	Anjum *et al.*, 2018; Silva *et al.*, 2019
Antioxidant	Galangin and pinocembrin	Farooqui and Farooqui, 2012; Anjum *et al.*, 2018
Anti-inflammatory	CAPE and galangin,	Anjum *et al.*, 2018
Hepato-protective	Dicaffeoylquinic acid	Anjum *et al.*, 2018
Cytotoxic	Prenylated p-coumaric acids and diterpenic acids	Bankova *et al.*, 2000
Cicatrizing	Bioflavonoids, arginine, vitamin C and provitamin A, and B complex	Anjum *et al.*, 2018

Due to all these properties and the fact that there is currently a particular interest in natural products, propolis continues arousing the interest of the pharmaceutical, cosmetic, and food industries. This introduction will focus on propolis antimicrobial, antioxidant, and anticancer properties, as they will be the target of research in this dissertation.

1.6.1 Antimicrobial properties

Antimicrobial properties of propolis have been extensively investigated and documented over the past few years (Mirzoeva *et al.*, 1997; Ota *et al.*, 2001; Gekker *et al.*, 2005; Scazzocchio *et al.*, 2006; De Castro *et al.*, 2010; Gutiérrez, 2012; Cui *et al.*, 2013; Bonvehí and Szweda *et al.*, 2015; Silva *et al.*, 2019; Agbor *et al.*, 2020; Berretta *et al.*, 2020) probably due to the need for alternative treatments against infectious diseases since, as it is well known, the resistance of pathogens to antimicrobial drugs has increased (WHO, 2019). Propolis activity against several types of microorganisms such as bacteria, yeast, viruses, or parasites is well studied (Machado *et al.*, 2007; Fokt *et al.*, 2010; Sforcin and Bankova, 2011; Yildirim *et al.*, 2016; Przybyłek and Karpinski, 2019 Salatino, 2022), being flavanones, flavones, and CAPE, the main bioactive components identified as responsible for antimicrobial activity in the European propolis type (Bankova, 2005b). It has already been demonstrated *in vitro* that propolis acts

directly on microorganisms and that *in vivo* it stimulates the mechanisms of the immune system that cause the death of microorganisms (Sforcin and Bankova, 2011). In this dissertation, the activity of Gerês propolis on bacteria and unicellular fungi will be tested and therefore its activity on these types of microorganisms will be explored in more detail.

1.6.1.1 Antibacterial properties

Studies have focused on assessing the efficacy of propolis against Gram-positive and Gram-negative bacteria, being shown that Gram-positive are generally more susceptible than Gram-negative bacteria (Bankova *et al.*, 2000; Silva-Carvalho *et al.*, 2015). Although not yet clear, it is thought that this difference in susceptibility is due to the presence of a more complex cell wall in Gram-negative bacteria and a higher lipid content, which is directly related to the presence of an external membrane that can prevent propolis action (Pinto *et al.*, 2011).

According to the literature, the most commonly used bacteria in trials to evaluate the antibacterial activity of propolis are *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) (Salatino, 2022). However, this propolis activity has been widely evaluated against several bacteria, in particular: *Aeromonas hydrophila*, *Bacillus* spp, *Branhamella catarrhalis*, *Brucella abortus*, *Corynebacterium* spp., *Diplococcus pneumonae*, *Enterococcus* spp., *Helicobacter pylori*, *Micrococcus luteus*, *Mycobacteria* sp., *Mycobacterium tuberculosis*, *Staphylococcus* spp., *Streptococcus* spp., *Salmonella sp*, *Nocardia asteroids*, *Rhodococcus equi*, *Klebsiella* spp., *Pseudomonas aeruginosa*, *Proteus* spp., *Shigella dysenteriae Actinomyces naeslundii, Lactobacillus acidophilus, Peptostreptococcus micros, Actinobacillus actinomycetemcomitans, Capnocytophaga gingivalis, Porphyromonas* spp., *Prevotella* spp., *Fusobacterium nucleatum,* and *Veillonella parvula* (Fokt *et al.*, 2010; Bogdanov, 2016a).

In *Bacillus subtilis*, *Escherichia coli* and *Rhodobacter sphaeroides* propolis cinnamic and flavonoid compounds can change the ionic permeability of the membrane and consequently decrease bacterial motility due to the change the membrane potential (Mirzoeva *et al.*, 1997). Studies using *Helicobacter pylori* as a bacterial model have shown that CAPE is a competitive inhibitor of the peptide deformylase (Cui *et al.*, 2013), an essential enzyme for bacterial survival, and propolis can thus be considered a potential therapeutic agent for gastrointestinal diseases caused by this bacterium. Artepillin C is a common compound in propolis from Brazil. Using artepillin C isolated from a propolis extract, Veiga *et al.*, (2017) demonstrated a high activity of this compound against *Staphylococcus aureus* (Minimum

inhibitory concentration (MIC) = 0.53 μg/ml). This compound was also effective against *Porphyromonas gingivalis* (Gram-negative) by increasing cell membrane permeability (Yoshimasu *et al.*, 2018). The polyphenols (pinocembrin, apigenin, quercetin, and CAPE) present in Chilean propolis exhibited antibacterial activity against *Streptococcus mutans* and decreased biofilm proliferation (Veloz *et al.*, 2019). A more recent study proved that propolis slows the growth of Gram-positive bacteria cultures, like *Streptococcus mutans* and *Lactobacillus* spp, which are responsible for tooth decay (Agbor *et al.*, 2020). To summarize, the analysis of the mechanisms of propolis antibacterial activity showed that propolis compounds can increase cell membrane permeability, reduce ATP production, decrease bacterial mobility, change membrane potential, and stimulate the body's immune system (Przybyłek and Karpinski, 2019).

Additionally, the combination of propolis with antibiotics has been shown to decrease the required dose of antibiotics. This synergism between propolis and several classes of antibiotics enhances the antibacterial effect against both Gram-positive and Gram-negative bacteria and has been studied with several antibiotics, namely ampicillin, cefoxitin, cefixime, chloramphenicol, clindamycin, cotrimoxazol, erythromycin, gentamicin, imipenem, linezolid, mupirocin, netilmicin, penicillin, tetracycline and tobramycin (Hossain *et al.*, 2022). *Escherichia coli* is a pathogenic bacterium that causes intestinal and urinary tract infections and is highly resistant to treatment. The combined effect of propolis and antibiotics (ofloxacin, ceftriaxone and ertapenem) revealed that propolis potentiates the treatment effect (Lavigne *et al.*, 2020). The synergetic activity of Portuguese propolis combined with gentamicin was also evaluated, enabling a decrease in the therapeutic dosage of this antibiotic (Freitas *et al.*, 2022b).

1.6.1.2 Antifungal properties

Currently, there is a demand for new antifungal drugs and, consequently, the antifungal properties of propolis should be understood (De Castro *et al.*, 2010). Since fungal infections are extremely difficult to treat and long-term use of commercial antifungal medicines has side effects, it is important to prevent negative effects. As for antibacterial activity, the antifungal properties of propolis are thought to be related to its flavonoids and other phenolic components (Farnesi *et al.*, 2009).

The antifungal activity of propolis was vastly investigated on yeasts mainly on *Candida* spp. (*C. albicans, C. dubliniensis, C. glabrata, C. krusei, C. parapsisolis* and *C. tropicalis*) and *Saccharomyces cerevisiae*, but also on filamentous fungi such as *Alternaria solani*, *Alternaria alternata*, *Aspergillus* spp., *Botrytis cinerea*, *Cladosporium* spp., *Cryptococcus sp.*, *Fusarium* spp., *Histoplasma encapsulatum*,

Madurella mycetomi, *Microsporum* spp., *Mucor mucedo*, *Penicillium* spp., *Piedra hortae*, *Phialophora jeanselmei*, *Rhizopus stolonifera, Rhodotorula mucilaginosa, Trichophyton* spp. and *Trichosporon cutaneum* (Fokt *et al.*, 2010; Bogdanov, 2016a)

Using *Saccharomyces cerevisiae* as a model, De Castro *et al.* (2010) found that cytochrome c and the metacaspase *YCA1* gene are involved in a Brazilian propolis-caused yeast cell death. The authors also concluded that mitochondrial function, vacuole acidification and autophagy are important factors in cell death. Bonvehí and Gutiérrez (2012) explored Spanish propolis extracts and demonstrated that *S. cerevisiae* was also susceptible to these extracts, with a MIC values varying between 0.7 - 1.5 mg/ml. Another report studied Brazilian propolis action against several *Candida* strains isolated from saliva, a fungus associated with several diseases (Ota *et al.*, 2001). Susceptibility to propolis was detected *in vivo* by a decrease in yeast numbers in patients who used propolis as a mouthwash (Ota *et al.*, 2001). Later, Sariguzel *et al.* (2016) analysed the *in vitro* antifungal activity of propolis from Turkey against some *Candida* strains (*C. albicans*, *C. glabrata*, *C. tropicalis* and *C. parapsilosis*) and obtained satisfactory results for all with a MIC range of 0.185 to 3 µg/ml. According to Stahli *et al.* (2021) propolis caused *C. albicans* to lose the integrity of its cell walls and exhibit lower metabolic activity. Various ethanol extracts of Polish propolis showed activity against several clinical isolates of *C. albicans*, and some also inhibited biofilm formation by *C. glabrata* and *C. krusei* (Gucwa *et al.*, 2018).

Propolis activity against filamentous fungi has been studied and observed too, namely against dermatophytes like *Trichophyton mentagrophytes* and *Trichophyton tonsurans* (Al-Daamy *et al.*, 2015). Brazilian green and red propolis also showed activity against fungi that cause dermatophytosis. For all three of the tested species (*Trichophyton rubrum*, *Trichophyton tonsurans* and *Trichophyton mentagrohytes*), the red propolis was more effective than the green propolis and the *T. rubrum* was the most susceptible to propolis antifungal effects (Siqueira *et al.*, 2009). Polish propolis with a chemical composition rich in pinocembrin, chrysin, pinobanksin, apigenin, kaempferol, p-coumaric acid, ferulic acid, and caffeic acid showed antifungal activity against *Aspergillus niger*, *Aspergillus ochraceus, Colletotrichum gloeosporoides, Alternaria solani, Fusarium solani, Rhizopus stolonifera, Botrytis cinerea, Cladosporium cladosporoides, Mucor mucedo, Penicillium expansum, Penicillium chrysogenum* (Pobiega *et al.*, 2019). Embaby *et al.* (2019) also evaluated the antifungal activity of propolis against phytopathogenic fungi, propolis being able to inhibit *Aspergillus alternata*, *Aspergillus niger*, *Fusarium sp.* and *Penicillium expansum* in a concentration-dependent manner.

1.6.2 Antioxidant properties

Damage caused by free radicals and reactive oxygen species (ROS) is associated with the appearance and progression of several diseases such as cardiovascular disease, heart disease, anaemia, cancer, or inflammation and is also directly related to ageing (Moskovitz *et al.*, 2002; Vaibhav *et al.*, 2011). Therefore, there is a need to find compounds with antioxidant properties that protect organisms from the effects caused by oxidative stress (Zehiroglu and Sarikaya, 2019). Propolis, like other bee products, is a supplier of potential natural antioxidant compounds that can neutralise the effects of oxidative stress this way being a potential therapeutic or adjuvant agent in the treatment of numerous diseases caused by oxidative stress (Kocot *et al.*, 2018).

The antioxidant properties of propolis depend on its composition (Zabaiou *et al.*, 2017). According to several researches, phenolic compounds such as phenolic acids and flavonoids display high antioxidant capacity (Bors *et al.*, 1990; Heim *et al.*, 2002; Russo *et al.*, 2002; Gregoris and Stevanato, 2010; Lagouri *et al.*, 2014; Anjum *et al.*, 2018; Wieczorek *et al.*, 2022). Phenolic compounds are hydrogen ion donors, being the hydroxyl group from their phenolic ring able to transfer its hydrogen atom to a free radical, resulting in electron stabilisation and protecting cells from the damaging effects of free radicals **(Figure 5)** (Kurek-Górecka *et al.*, 2013; Anjum *et al.*, 2018; Rungratanawanich *et al.,* 2018).

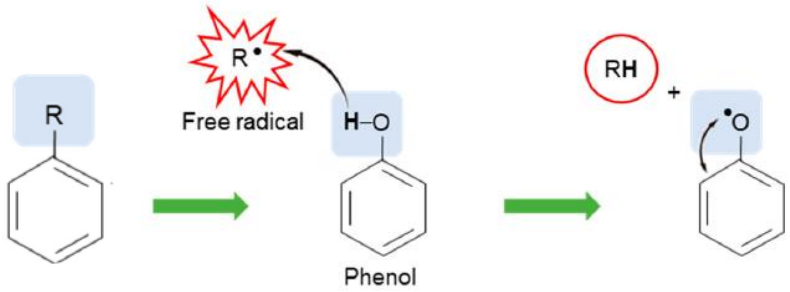

Figure 5. Mechanism by which phenolic compounds neutralize free radicals (adapted from Rungratanawanich *et al.*, 2018).

The evaluation of the antioxidant capacity of propolis extracts can be done using several methods such as DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) and FRAP (ferric reducing antioxidant power assay) (Wieczorek *et al.*, 2022), among others. These chemical methods are an easy way to assess antioxidant capacity and to screen several samples to find the most promising to advance to studies in the biological environment (Banskota *et al.*, 2000; De-Melo *et al.*, 2014).

Antioxidant activity of propolis from different regions of the world has been tested. Banskota *et al.* (2000) compared propolis samples from Brazil, Peru, the Netherlands and China and prepared

extracts using water and methanol as solvents. Brazilian and Chinese propolis showed the best antioxidant capacity when extracted with water while in the case of the Netherlands and Peruvian propolis the methanol extracts were the most effective in DPPH free radical scavenging. An extract of propolis from Chile, containing galangin, caffeic acid, *p*-coumaric acid, ferulic acid and CAPE, showed an antioxidant activity similar to superoxide dismutase, a well-known antioxidant enzyme (Russo *et al.*, 2004). Different types of extracts (methanol, methanol 80% (w/v), and water) were prepared with propolis from two locations in Greece and the best antioxidant activity was observed for methanol extracts. Moreover, the most active sample was the one with the highest amount of total phenolic compounds (Lagouri *et al.*, 2014). More recently, Nichitoi *et al.* (2021) investigated the antioxidant properties of two hydroalcoholic extracts (70% and 50% ethanol) prepared from Romanian propolis samples being the 50% (w/v) hydroalcoholic extract the best one. Different solvents allow solubilising and therefore extracting different types of compounds and therefore different solvents and/or solvent mixtures are tested to prepare propolis extracts (Watanabe *et al.*, 2011). Commonly used solvents are polar solvents such as ethanol, ethanol and water, water, methanol, and chloroform (Fokt *et al.*, 2010; Watanabe *et al.*, 2011; Kubiliene *et al.*, 2015). Ethanol and ethanol/water mixtures are the most widely used because allow obtaining extracts with higher phenolic compounds and lower wax contents (Watanabe *et al.*, 2011; Dönmez *et al.*, 2020; Kara *et al.*, 2022). However, solvent effectiveness always depends on raw propolis composition.

1.6.3 Anticancer properties

According to the World Health Organization (WHO, 2021), cancer can start in any organ or tissue of the body and is characterised by uncontrollable cell growth. This can lead the cells to invade adjacent parts of the body, possibly spreading to other organs. The invasion of other organs is the most advanced stage of the disease, known as metastasis, and is the main cause of cancer death. According to this same institution, cancer is the second leading cause of death globally, being in 2020 responsible for nearly 10 million deaths (WHO, 2021).

Currently, cancer treatment involves surgical removal, radiotherapy, and chemotherapy according to the type and grade of cancer (Elumalai *et al.*, 2022). However, these traditional therapeutic methodologies carry adverse reactions that impair the quality of patients' lives (Rayan *et al.*, 2017). Understanding the molecular mechanisms underlying cancer has led to the development of a vast number of anticancer drugs, however, some of these have an associated rate of therapeutic resistance. Thus, the discovery and development of new drugs based on natural products have been subject of research. It is

estimated that about 60% of anticancer drugs currently used in clinical context are derived from natural sources like plants, marine organisms, and microorganisms (Cragg and Newman, 2005).

Natural products like propolis have been investigated to identify new drugs for treatment of various types of cancer (Silva-Carvalho *et al.*, 2015). Propolis has demonstrated an antitumoral activity against cancers such as head and neck, brain and spinal cord, blood, skin, breast, pancreas, liver, colon, prostate, kidney and bladder (He *et al.*, 2006; Cogulu *et al.*, 2009; Missima *et al.*, 2010; Wu *et al.*, 2011; Markiewicz-Żukowska *et al.*, 2013; Silva-Carvalho *et al.*, 2014; Valença *et al.*, 2013; Patel, 2015; Frión-Herrera *et al.*, 2020; Rodrigues *et al.*, 2021; Freitas *et al.*, 2022a; Oliveira *et al.*, 2022). The antitumoral action of propolis and some of propolis-isolated components (CAPE, drupanin, baccharin, artepillin C, quercetin, kaempferol, and *p*-coumaric acid) was also evaluated *in vivo* as well as *in vitro* (Silva-Carvalho *et al.*, 2015).

Antitumour action has been mainly associated with CAPE and artepillin C present in propolis (Castaldo and Capasso, 2002; Slavov *et al.*, 2013). Lee *et al.* (2005) proved that CAPE interrupts the cell cycle in the G2/M phase and could induce cell apoptosis. Several studies have shown that CAPE is an inhibitor of NF-κB (Slavov *et al.*, 2013; Silva-Carvalho *et al.*, 2015), has a cytotoxic effect on oral cancer (Lee *et al.*, 2000) and inhibits proliferation of the colorectal cell line SW480 (He *et al.*, 2006). CAPE also demonstrated to decrease melanoma tumour growth by inhibiting PI3K/AKT/XIAP pathway (Pramanik *et al.*, 2013). In breast cancer MCF-7 and MDA-231 cell lines, CAPE inhibited *in vivo* tumour growth, decreased cell proliferation *in vitro* and reduced the formation of vascular endothelial growth factor in MDA-231 cells, preventing angiogenesis (Wu *et al.*, 2011).

Telomerase reverse transcriptase activity is directly related to the immortality of cancer cells. The role of a Turkish propolis sample in the activity of this catalytic subunit of telomerase was studied in human leukemic cells. The results demonstrate a considerable decrease in transcriptase expression in the bone marrow cell cultures of propolis-treated patients (Cogulu *et al.*, 2009). Another research project tried to encapsulate propolis in nanoparticles – propolis nanofoods – which were more effective in a human pancreatic cancer cell line than non-encapsulated propolis (Kim *et al.*, 2008). Frión-Herrera *et al.* (2020) tested the ability of Cuban propolis to prevent metastasis in two cell lines of colorectal cancer (CRC) (HT-29 and LoVo) and observed a decrease in cell viability, inhibition of the clonogenic capacity of CRC cells and interruption of the cell cycle inducing apoptosis proving that, besides the antiproliferative action on CRC cells, propolis decreases its metastatic potential too.

Besides propolis role as a cancer progression inhibitor, its antitumour properties can be enhanced when it is combined with other compounds (Hermansyah *et al.*, 2022). For example, Alsherbiny *et al.* (2021) evaluated the effect of propolis associated with doxorubicin, a drug commonly used for the treatment of breast cancer and observed a synergistic activity, showing that propolis, in a dose-dependent manner, significantly enhanced the effect of doxorubicin in MCF-7 cells. Immunotherapy is another way of treatment that is effective in some tumour types and propolis has proven to be a successful adjuvant here too. One of its compounds, artepillin C, has been shown to increase the ratio of CD4/CD8 T cells and total T helper cells, thus activating the immune system (Kimoto *et al.*, 1998). A human clinical trial showed that supplementing breast cancer patients with propolis reduces some of the main consequences associated with radiotherapy due to the DNA-protecting effect of propolis against damage induced by ionizing radiation (Ebeid *et al.*, 2016).

In summary, propolis anticancer mechanism is related to its ability to induce apoptosis, block oncogene-specific signalling pathways and consequently decrease cell proliferation and growth, prevent angiogenesis and metastasis and, in some cases, stimulate macrophage activity and production of antibodies to eliminate the tumour (Slavov *et al.*, 2013; Patel, 2015; Silva-Carvalho *et al.*, 2015; Frión-Herrera *et al.*, 2020). Moreover, this bee product also shows very promising results when associated with conventional treatments for several types of cancer (Kimoto *et al.*, 1998; Ebeid *et al.*, 2016; Alsherbiny *et al.*, 2021; Elumalai *et al.*, 2022; Hermansyah *et al.*, 2022).

1.7 Propolis research in Portugal

Propolis and its characteristics have also been explored in Portugal, with researchers focused on the chemical characterization and the evaluation of the biological properties of propolis from different regions of the country. One of the first biological activities studied in Portuguese propolis was the antioxidant action. Cruz *et al.* (2008) studied samples from three different areas of Algarve (mountain-like, transition-like and maqui-like) to evaluate the antioxidant activity of propolis methanol extracts. The maqui-like samples exhibited the highest levels of phenolic compounds, the greatest ability to eliminate free radicals and promote the reduction of Fe (III) to Fe (II) more easily, being the sample with the greatest antioxidant action. Later, using other samples of propolis from different areas of the Algarve collected at two different seasons (winter and spring), Miguel *et al.* (2010) evaluated the content of total phenols and flavonoids and the antioxidant properties and studied the effect of the extraction solvent and the harvest time. The mixture of water/ethanol showed to be the best solvent and higher contents of phenols were

found in hydroalcoholic extracts of propolis harvested in spring than in winter. One of the samples had the highest levels of polyphenols and the best antioxidant activity regardless of the harvest season. Moreira *et al.* (2008) also found high antioxidant activity as well as high levels of phenolic components in propolis samples collected in Bornes, in the Northeast, and Fundão in the Centre of Portugal. Both extracts showed to protect human erythrocytes from free radical damage by decreasing lipid peroxidation (Valente *et al.*, 2011). Later, Moreira *et al.* (2011) used samples from the same locations to understand the action of Portuguese propolis in hereditary spherocytosis, a type of anaemia, showing that propolis reduces the fragility of the erythrocyte membrane. Using flow cytometry, propolis from Côa region (Beira Alta, Portugal) showed to increase intracellular antioxidant activity *in vivo* (Cruz *et al.*, 2016). More recently, our research group has proved that propolis from Alves (Alentejo) (Cruz, 2021; Passão, 2021), Caramulo (Pereira, 2021), Gerês (Freitas *et al.*, 2019; Peixoto *et al.*, 2021) and Pereiro (Guarda) (Peixoto *et al.*, 2022) have high antioxidant potential as well.

Neuroprotective effects of Northeast Portuguese propolis were evaluated and propolis showed to moderately protect cortical neurons subject to stress stimuli through the inhibition of caspase-3 activation (Cardoso *et al.*, 2011).

Antimicrobial properties of Portuguese propolis were also the subject of some studies. Silva *et al.* (2012) evaluated the phenolic profile, antimicrobial activity, and role in inflammation of propolis from different regions of Portugal (Bragança, Coimbra and Beja). Antimicrobial action was tested in both Gram-positive and Gram-negative bacteria as well as in yeast and filamentous fungi (Silva *et al.*, 2012; Falcão *et al.*, 2014). The effect of Portuguese propolis extracts against pathogenic protozoa namely *Plasmodium falciparum*, *Leishmania infantum*, *Trypanosoma cruzi* and *Trypanosoma brucei* was also analysed and *in vitro* tests revealed higher effectiveness against *Trypanosoma brucei* (Falcão *et al.*, 2014). Propolis micro-extracts from several Alentejo regions have been found to be particularly effective against *Bacillus subtilis* e *Pseudomonas savastanoi* (MIC varying from 100 to 500 μg/ml) and further inhibited the mycelial growth of several species of filamentous fungi (Passão, 2021). Other phytopathogenic fungi such as *Botrytis cinerea* and *Penicillium expansum* are susceptible to propolis, which inhibited the growth of the mycelium in vitro and reduced the lesion size caused by *Penicillium expansum* on apples (Pereira, 2021; Pereira *et al.*, 2022).

Some researchers were also interested in the potential antitumour capacity of Portuguese propolis. Extracts of propolis from Bornes and Fundão exhibited selective toxicity against human renal carcinoma cells and inhibited the growth of renal carcinoma cells in a concentration-dependent manner

(Valente *et al.*, 2011). Valença *et al.* (2013) explored propolis action on cell viability, proliferation, metabolism, and death of a human colon carcinoma cell line (HCT-15) and observed a cytotoxic effect on these cancer cells. Various types of fractions (hexane, chloroform, and residual ethanol) of an ethanol extract of propolis were used in this study, with the chloroform one being the most effective in reducing cell viability. Cytotoxicity showed to be related to the effect on glycolytic metabolism of tumour cells since there was a decrease in glucose consumption and lactate production. Silva-Carvalho *et al.* (2014) used an ethanol extract of propolis collected from Pereiro focusing on its effects on cell proliferation, cell cycle and death, migration, metabolism and angiogenesis. The viability of different tumour cells was affected, being human breast (MDA-MB-231) and prostate (DU145) cancer cell lines more susceptible as cell proliferation and migration decreased, cell cycle was altered, and cell death increased.

Regarding the chemical composition of Portuguese propolis, Falcão *et al.* (2010) characterized the phenolic composition of Northeast Portuguese propolis and identified 37 phenolic compounds, such as phenolic acids and flavonoids characteristic of temperate zone propolis, but also several new compounds never documented before like methylated and esterified phenolic compounds, or flavonoid hydroxylates and pinocembrin, pinobanksin and *p*-coumaric derivatives. Later, in her PhD Dissertation, Falcão (2013) characterized propolis from six different Portuguese regions and, as already mentioned, defined two different types of propolis in Portugal, according to their geographic origin and characteristics. One is similar to propolis from temperate zones, has poplar as plant source, contains phenolic compounds typical of these zones such as flavonoids and their methylated or esterified forms, phenylpropanoid acids and their esters, and includes samples from the north, central coast and archipelago of the Azores, showing appreciable bioactivities due to the high phenolic content. Type II propolis was considered atypical, exhibiting a different colour and an unusual composition in quercetin and kaempferol glycosides, some of which were never identified. This propolis type has more wax but is less active and includes samples from the central interior, south and Madeira Island. Differences between two propolis types were ascribed to different botanical species as the resin sources (Falcão *et al.*, 2012). Thus, although Portugal is in an area that includes the European propolis type, Portuguese propolis can also be produced from other botanical sources, such as *Cistus ladanifer*, if species of the genus *Populus* are absent (Falcão, 2013).

Propolis from Gerês is probably the most studied Portuguese propolis. It was studied for the first time by Freitas (2015) during her Master thesis, where it was characterized the chemical composition and biological activities of samples collected over four consecutive years. All the samples exhibited a very

similar behaviour regarding both the bioactivities and phenolic profiles (Freitas *et al.*, 2019). These propolis samples from Gerês were effective in inhibiting the growth of several Gram-positive bacteria, mainly species of the genus *Bacillus*. This type of antimicrobial activity remains constant in propolis samples from the following years. Propolis from Gerês has a promising antioxidant potential as well, with samples harvested from 2011 to 2021 displaying EC_{50} values varying from 25.2 ± 2.5 to 10.90 ± 0.34 µg/ml (Freitas, 2015; Peixoto, 2018; Oliveira, 2022) Gerês propolis shows synergism with gentamicin, allowing a reduction of the therapeutic dose of this drug. Honey combined with propolis showed stronger antibacterial activity than the exhibited by each sample individually (Freitas *et al.*, 2022b). The hydroethanolic extract (70%) of G15 was shown to be a potential product for oral application (Gonçalves, 2017). G17.EE is especially effective against *Microsporum audourii* (Gomes, 2019) revealing antifungal potential. G19.EE was used to textile functionalisation, which showed significant antioxidant and antibacterial activity levels (Cardoso, 2021).

Given the constancy of Gerês propolis over the years, Freitas *et al.* (2022a) later prepared an ethanol extract of propolis from Gerês collected in 2018 (G18.EE), further fractionated this extract into four fractions - ethyl acetate (EtOAc), *n*-hexane, *n*-butanol (*n*-BuOH), and water. The cytotoxicity of the extract and its fractions was evaluated in renal cancer cell lines (A498, 786-O and Caki-2) and non-neoplastic renal cells (HK2). EtOAc was the most effective fraction as presenting considerable cytotoxic activity and high selectivity index. Three subfractions were obtained through the partition of EtOAc but their effect was lower than that shown by the upstream EtOAc fraction, suggesting that the combined effect of other constituents gives this fraction its greater antitumor activity (Freitas et al., 2022a). The anti-melanoma activity of G18.EE and its fractions were also evaluated later by Oliveira (2022), being the most cytotoxic the *n*-BuOH fraction. These results highlight the potential of Gerês propolis and its fractions as a promising research source for the identification of compounds with antitumour activity.

2. Melanoma and propolis: what we know so far

2.1 Melanoma: a public health problem

Skin cancer incidence has increased the most in recent years (Ashraf *et al.*, 2020). The main factor responsible for all skin cancer types is exposure to UV (Ultraviolet) radiation, meaning that the decrease in cases depends largely on behavioural change in the population (Arnold *et al.*, 2018). Skin cancer can have its origin in pigment-producing cells, the melanocytes, in which case it is called melanoma, or it can have its origin in keratinocytes, which can lead to basal cell carcinoma or squamous cell carcinoma (Leiter *et al.*, 2020). In this Master thesis, the antitumour capacity of propolis in melanoma will be evaluated and therefore this type of skin cancer will be discussed in more detail.

Melanoma is most common in western countries where most of the new cases appear (Schadendorf *et al.*, 2015). In Europe, melanoma is responsible for about 20,000 deaths per year (Forsea, 2020). In Portugal, 1,071 new cases and 289 deaths due to this skin disease were reported in 2020 (Global Cancer Observatory, 2021). Melanoma appears when melanocytes start to grow in an uncontrolled way, forming a malignant tumour (Dildar *et al.*, 2021). It can appear in any part of the body where pigment cells are present, though more commonly in the skin, especially in areas more exposed to the sun, it can also appear in extracutaneous regions (eyes, intestines, esophagus, meninges, oral and anogenital mucosa) (Mihajlovic *et al.*, 2012; Shain and Bastian, 2016). The mechanism leading to the transformation of normal melanocytes into melanoma cells is not yet fully clarified, but it is known that it is mainly a consequence of mutations in genes encoding proteins that regulate proliferation and growth. For example, activating mutations of proto-oncogenes and in constituents of the signal transduction pathway (Ras-Raf-Mek-Erk) (Mandalà and Voit, 2013). Resistance to apoptosis is also a characteristic of melanoma cells, as low expression of pro-apoptotic proteins has been detected in such cells (Broussard *et al.*, 2018).

Although melanoma is the rarest type of skin cancer, it is associated with the highest mortality rate (Forsea, 2020). If diagnosed at an early stage it can be removed by surgery, increasing the patient survival rate. However, late diagnosis leads to high mortality rates due to its high capacity to metastasise, spreading rapidly throughout the body and making treatment much more challenging (White *et al.*, 2002). In addition, patients with this type of disease develop resistance to available conventional therapies. Considering these factors, it is clear that melanoma is the most worrying skin cancer type (Sousa *et al.*, 2010).

2.2 Treatment options

There are a few therapeutic options for melanoma, such as surgical removal, chemotherapy, immunotherapy, or targeted therapy, depending on the characteristics of the tumour (location, stage, and genetic profile) (Domingues *et al.*, 2018). As already mentioned, in the early stages, tumour removal by surgery is a very effective option (White *et al.*, 2002).

Chemotherapy was the first type of therapy to emerge to combat the advanced stages of this disease. (Domingues *et al.*, 2018; Bomar *et al.*, 2019). For example, dacarbazine, a standard chemotherapeutic agent, was the first drug approved by FDA for metastatic melanoma and demonstrated an effective response in less than 5% of the patients (Heo *et al.*, 2016). The failure of this type of therapy is related to the development of therapeutic resistance (Soengas and Lowe, 2003) and therefore, currently, chemotherapy is not a frontline therapy for melanoma (Wilson and Schuchter, 2016). The combination of several chemotherapeutic drugs has also been tested, as well as the combination of chemotherapy with other types of therapy, such as immunotherapy, but none improved the patient overall survival (Luke and Schwartz, 2013; Wilson and Schuchter, 2016).

The increasing knowledge of the relationship between cancer and the immune system allowed the emergence of immunotherapy, which is a treatment approach for advanced melanoma (McDermott *et al.*, 2014). One of the drugs used is ipilimumab, an antibody that targets the CTLA-4 (Cytotoxic T-lymphocyte antigen 4) protein that maintains immune homeostasis by negatively regulating the immune responses. Ipilimumab increases the body's immune response against melanoma cells by blocking the action of CTLA-4 (cytotoxic T-lymphocyte–associated antigen 4) (Heo *et al.*, 2016). This therapeutic agent revealed an enhanced overall survival in melanoma (Hodi *et al.*, 2010). Later, another drug appeared, Nivolumab, which is an antibody that blocks the action of PD-1 (programmed cell death protein 1) allowing the action of T cells against cancer cells. (Gellrich *et al.*, 2020). However, this kind of therapy has some side effects, such as the toxicity to normal tissues and the development of autoimmune diseases due to the immune system modulation (Caspi, 2008). Immunotherapy is a common approach especially in patients without *BRAF* mutation (Robert *et al.*, 2015).

Along with the advance in the understanding of the mechanism associated with melanoma appearance, targeted therapy has emerged (Dhomen and Marais, 2009). This treatment involves small molecule inhibitors or antibodies that inhibit crucial targets for disease progression (Domingues *et al.*, 2018). In the case of melanoma, it is very common to find mutations that result in constitutive activation of proteins that regulate proliferation and growth. Thus, these proteins become an interesting therapeutic

target (Dhomen and Marais, 2009). The most common mutation is in the *BRAF* gene (V-RAF murine sarcoma viral oncogene homolog B), a part of the mitogen-activated protein kinase (MAPK) signaling pathway (Sosman *et al.*,2012). Briefly, the normal function of this signalling pathway relies on binding of a growth factor to its receptor, which activates RAS (rat sarcoma virus) that consequently activates RAF (rapidly accelerated fibrosarcoma) kinases, one of which is BRAF. This activation causes the activation of MEK (mitogen-activated protein kinase kinase) which causes the activation of ERK (extracellular signal-regulated kinase), resulting in cell proliferation. When a mutation occurs in *BRAF* this pathway becomes constitutively active leading to tumour development **(Figure 6B)** (Wong and Ribas, 2016). Approximately 66% of melanoma patients have activating *BRAF* mutations. (Davies *et al.*, 2002) and the most common mutation is found at amino acid 600, in which valine is substituted by glutamic acid ($BRAF^{V600E}$) (Ascierto *et al.*, 2012).

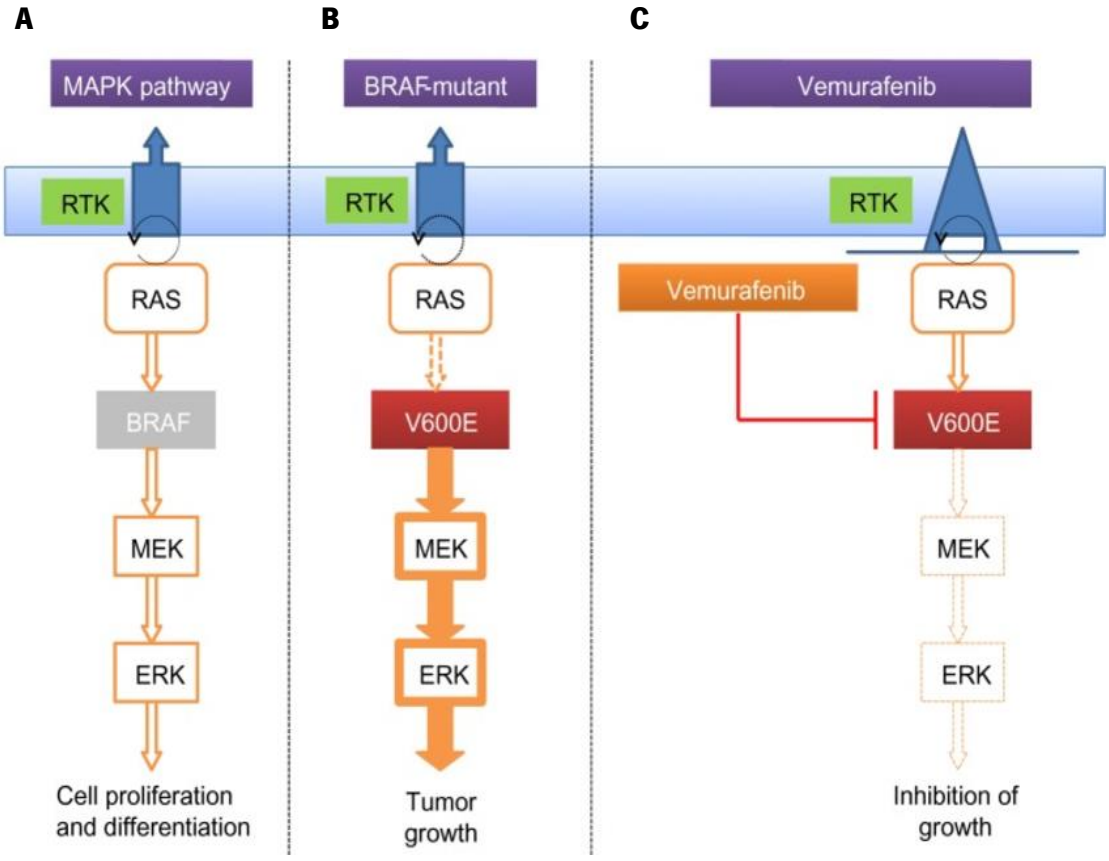

Figure 6. **Effects of *BRAF* mutation on MAKP signaling pathway. (A)** MAKP signaling pathway; **(B)** MAKP signaling pathway in *BRAF*-mutated condition; **(C)** MAPK signaling with the inhibition of BRAF by vemurafenib, a BRAF inhibitor used in melanoma treatment (adapted from Swaika *et al.*, 2014).

BRAF inhibitors have been developed for the treatment of patients carrying this mutation (Sun *et al.*, 2020). Vemurafenib was the first to be approved by the FDA and has revealed to be more effective than chemotherapy, being able to reduce tumour progression and increase patient overall survival

(Figure 6C) (Chapman *et al.*, 2011, 2017). Dabrafenib and encorafenib, appeared latter and proved to improve progression-free survival (Hauschild *et al.*, 2012; Koelblinger *et al.*, 2018). However, the major problem with these targeted therapies is the development of multiple resistance mechanisms after 5-7 months (Rizos *et al.*, 2019; Sun *et al.*, 2020; Bomar *et al.*, 2019). In addition, 90% of patients experience side effects such as arthralgia, fatigue, nausea, diarrhoea, and headache (Livingstone *et al.*, 2014). The combination of BRAF and MEK inhibitors is the most recent type of treatment approved by the FDA, nonetheless associated resistance is also a therapeutic concern (Kakadia *et al.*, 2018).

2.3 Propolis: a potential source of therapeutic agents

The resistance of melanoma to conventional therapies demands new treatments and sources of therapeutic agents (Yi *et al.*, 2020). Studies of propolis effects on melanoma are still limited but the activity of propolis from different regions on this type of cancer has been reported (Patel 2015; Cisilotto *et al.* 2018; Pereira *et al.*, 2021; Popova *et al.*, 2021). In this work, the cytotoxic properties of Gerês propolis will be evaluated on human melanoma cell lines hence research studies using propolis and melanoma will be described in more detail.

Vongsak *et al.* (2016) evaluated the anticancer capacity of propolis produced by three distinct species of stingless bees - *Tetragonula pagdeni, Lepidotrigona ventralis*, and *Lepidotrigona terminata* - in human melanoma (SK-MEL-28) and normal human (Hs68) cell lines. Propolis from *T. pagdeni* was the most effective in inhibiting cancer cell growth (Half-maximal inhibitory concentration (IC_{50}) = 33.38 μg/ml) and the less toxic to normal cells (IC_{50} = 228.75 μg/ml). Cisilotto *et al.* (2018) evaluated the cytotoxic mechanisms of two stingless bees (*Scaptotrigona bipunctata* and *Melipona quadrifasciata anthidioides*) propolis extracts from Brazil in the same melanoma cell line and in human melanocyte (NGM) cell line. Both extracts inhibited the growth of melanoma cell lines more effectively than normal cell lines, with a selectivity index close to 2. The combined action of these extracts with vemurafenib increased the cytotoxic effect, which may be related to their ability to increase ROS accumulation, causing DNA damage and apoptosis. The ethanol extract of a Chinese propolis increased the apoptotic rate and decreased cell viability in melanoma tumour cells A375. This extract promotes mitochondria-mediated intrinsic apoptosis and changes in Bax and Bcl-2 protein expression (IC_{50} = 112 μg/ml) (Zheng *et al.*, 2018b).

Zheng *et al.* (2018a) also showed that pinocembrin, a compound present in propolis, induces apoptosis, caused endoplasmic reticulum stress, and suppressed autophagy in A375, all of which are pro-death mechanisms. Chen *et al.* (2014b) isolated propolin C from Taiwanese propolis and tested its

cytotoxic activity in human melanoma cells A2058. The compound was able to induce apoptosis by activating caspase-8 and Bid, and by provoking cytochrome c release, leading to DNA fragmentation and consequently apoptosis (IC_{50} =8.5 μM). Later, Chen *et al.* (2014a) used propolis extracts from different regions of Taiwan and the extract with the highest capacity to induce apoptosis (IC_{50} = 2.3 μg/ml) was characterized by the highest levels of phenolic content and propolin C and D. Propolin activity was evaluated in an isolated form, but none exhibited better results than the extracts, suggesting that it is the combined action of propolins that confers cytotoxic capacity. CAPE is one of the compounds most associated with propolis antitumour ability. CAPE suppressed melanoma tumour (SK-MEL-28) growth in a dose-dependent manner by blocking the PI3K/AKT/XIAP signalling pathway, indicating that AKT/XIAP is the target of CAPE (Pramanik *et al.*, 2013). Pichichero *et al.* (2011) showed that chrysin, a flavonoid found in various propolis types, may induce apoptosis in a dose-dependent manner in melanoma cell line A375, with downregulation of ERK 1/2 and activation of p38.

Anti-melanoma activity was recently demonstrated for Portuguese propolis. The effect of an ethanol extract of propolis from Gerês collected in 2018 (G18.EE) and its three fractions (EtOAc, *n*-hexane, *n*-BuOh) was described in two *BRAF*-mutated melanoma cell lines (A375 and WM9) (Oliveira *et al.*, 2022). The highest cytotoxic activity was displayed by the *n*-BuOh fraction against both melanoma cell lines (IC_{50} = 8.14 μg/ml for A375 and IC_{50} = 11.22 μg/ml for WM9). This fraction has the highest proportion of CAPE, suggesting that this compound may be responsible for the melanoma cytotoxic activity of propolis from Gerês. The effect of the extract and its fractions on ROS production was also investigated, and an accumulation of ROS and increased levels of pro-apoptotic proteins was observed. Thus, G18.EE and its fractions seem to promote ROS-mediated apoptosis in melanoma (Oliveira *et al.*, 2022). Altogether, these results prove that propolis and its constituents are a promising target to find new therapeutic agents for melanoma.

3. Contextualisation and aims of this work

Propolis, like other natural products, is an interesting object of pharmacological and chemical studies due to its multiple bioactivities, including antioxidant, antimicrobial and antitumour activities (Fokt *et al.*, 2010; Silva-Carvalho *et al.*, 2015; Bankova, 2005b). In the latest years, there has been a high interest in the potential of this natural product for the development of new drugs or its use as an adjunctive therapeutic agent (Silva-Carvalho *et al.*, 2015; Parolia *et al.*, 2022). However, propolis is a variable mixture of natural compounds and therefore the major barrier to its use and acceptance by the medical community is its difficult standardization (Bankova, 2005a; Bankova, 2005b; Silva-Carvalho *et al.*, 2015). As a result, it is still necessary to establish standardised working methods to obtain comparable results. In addition, chemical characterisation should be linked to the association of a certain type of propolis compounds with a specific type of biological activity (Bankova, 2005a).

In Portugal, the professionalisation rate of beekeepers is low, with beekeeping being mainly a complement to the main professional activity. Bees produce several products of commercial interest, however most beekeepers only market honey, due to a lack of information on the commercial value of other products such as propolis. The use of specific grids for propolis harvesting allows a production increase without compromising other processes in the hive (Federação Nacional dos Apicultores de Portugal, 2016) and our group recently showed that an increase in pore grid size also contributes to a higher propolis production (Freitas *et al.*, submitted). Nevertheless, research on the biological properties and chemical composition of Portuguese propolis has evolved favourably in recent years, showing that Portuguese propolis is a valuable product with potential therapeutic applications (Cruz *et al.*, 2008; Miguel *et al.*, 2010; Cardoso *et al.*, 2011; Moreira *et al.*, 2011; Valente *et al.*, 2011; Silva *et al.*, 2012; Falcão, 2013, Falcão *et al.*, 2014; Silva-Carvalho *et al.*, 2014; Freitas *et al.*, 2019, 2022a, 2022b; Peixoto *et al.*, 2021; Oliveira *et al.*, 2022; Pereira *et al.*, 2022). Thus, the general purpose of this work is to further understand the potential of Gerês propolis as a therapeutic element through the assessment of its biological activity and chemical composition. This work focuses on propolis harvested in 2021 (G21) which was extracted with ethanol to obtain the respective ethanol extract G21.EE.

The first objective of this work is to assess the quality of G21, to see if its characteristics are in agreement with those defined in the literature. Moreover, being phenolic compounds identified as one of the main responsible for the propolis bioactivities, the phenolic composition of G21.EE was also determined. Antioxidant activity is well documented in propolis and worth of research too, given the continuous search for new antioxidant compounds that combat the harmful effects of free radicals.

G21.EE antioxidant activity was assessed and compared with results obtained previously for samples from the same apiaries (Freitas *et al.*, 2019; Peixoto *et al.*, 2021), which in the case of Gerês propolis has been shown the particularity to remain constant over the past years (Freitas *et al.*, 2019). Antimicrobial properties are widespread in propolis and samples from Gerês have been shown to possess this activity too, especially against Gram-positive bacteria (Freitas *et al.*, 2019, 2022b), making this activity worth of searching for G21.EE, especially having in mind the search for new therapies for infectious diseases.

Another propolis activity is its ability to combat cancer cells. Considering the constancy of bioactivities that propolis do Gerês has already demonstrated for antibacterial and antioxidant activities (Freitas *et al.*, 2019), it was decided to verify if this constancy was also true for anticancer properties, namely in melanoma. Oliveira *et al.* (2022) reported that G18.EE and one of its fractions (G18.EE_*n*-BuOH) have high cytotoxic effect on two *BRAF*-mutated melanoma cell lines (A375 and WM9). Melanoma is the rarest type of skin cancer but is associated with a high mortality rate (Forsea, 2020) and existing therapies are not considered sufficiently effective (Sousa *et al.*, 2010). Thus, investigating propolis activity in this type of cancer is important due to the need for new treatments and sources of therapeutic agents (Yi *et al.*, 2020). Besides the cell line with *BRAF* mutation (A375), a wild-type melanoma cell line (SK-MEL-23) was used to assess possible susceptibility differences to propolis. Treatment options for patients with non-*BRAF* melanoma mutations are limited and there is insufficient development of new therapeutic strategies (Fedorenko *et al.*, 2015; Rane and Minden, 2019). There is also a lack of research on the effect of propolis in these cases. Therefore, besides *BRAF*-mutated melanomas being more common and aggressive, effective therapies should also be developed for non-*BRAF*-mutated cases.

CHAPTER II: MATERIALS & METHODS

1. Propolis sample: origin, characteristics and quality analysis

A propolis sample harvested from Gerês in September 2021 (G21) was kindly supplied by the beekeeper Amadeu Fortunas and used in this work. G21 was collected in an apiary located near the Cávado river, between the villages of Paradela and Sirvozelo, in Montalegre, Gerês, Portugal (41º45'41.62'' N; 7º58'03.34'' W). The sample, with a pleasant aromatic odour and a brownish colour, was already quite fragmented and contained some impurities like grass and the remains of bees and other insects that were removed before the following procedures. The sample was stored in glass jars, in the dark, at 4 °C until use.

1.1 Ash content analysis

Ash content was determined adapting the method described by Lopes *et al.* (2017): 1 g of raw propolis was placed in a previously weighed ceramic crucible and then placed in a muffle furnace for 3 h, at 550 °C for the sample to be converted into ash. At the end of this time, the muffle was allowed to cool and the crucible was removed and carefully covered with aluminium foil and left in a desiccator overnight. The following day, the crucible with ash was weighed and the ash content was calculated using **Equation 1** and expressed in percentage (%). The analysis was done in triplicate.

$$\text{Ash content (\%, m/m)} = \frac{\text{Crucible with ash mass} - \text{Empty crucible mass}}{\text{Initial propolis mass}} \times 100 \qquad \textbf{Equation 1}$$

1.2 Water content analysis

Water content was determined according to Woisky and Salatino (1998). In a previously weighed beaker, 4 g of G21 were placed and covered with aluminium foil. The beaker was then placed in an oven for 5 h, at 105 °C to dry the sample. At the end of this time, the beaker was placed in a desiccator overnight and weighed the following day. Water content was calculated using **Equation 2** and expressed as a percentage (%). The analysis was done in triplicate.

$$\text{Water content (\%, m/m)} = \frac{\text{Initial propolis mass} - \text{Dry propolis mass}}{\text{Initial propolis mass}} \times 100 \qquad \textbf{Equation 2}$$

1.3 Wax content analysis

Wax content was determined using an adaptation of the method described by Hogendoorn *et al.* (2013) which is based on the density difference between propolis and beeswax. An amount of 1g G21 was placed in a falcon tube, and 2.5 ml of deionized water was added. The tube was heated in the microwave oven carefully so that the water did not boil and then cooled to room temperature (RT) until 3 layers were observed: propolis (at the bottom), water (middle layer), and beeswax (floating on top). The wax layer was removed with the help of a warm spatula, weighed, and wax content was calculated using **Equation 3** and expressed as a percentage (%). The analysis was done in triplicate.

$$\text{Wax content (\%, m/m)} = \frac{\text{Wax mass}}{\text{Initial propolis mass}} \times 100 \qquad \textbf{Equation 3}$$

1.4 Balsam content analysis

G21 balsamic content was determined using the method described by Popova *et al.* (2007), placing 0.5 g of raw propolis in an Erlenmeyer flask and 15 ml of 70% ethanol (Carlo Erba Reagents). The mixture was kept in the dark at 26 °C and with stirring at 125 revolutions per minute (rpm) for 24 h At the end of this time, the mixture was filtered with a Buchner funnel and filter paper in a Kitasato system connected to a vacuum pump. The filtrate was stored at 4 °C, and the residue was re-extracted under the conditions described. After a second filtration, the two obtained filtrates were pooled, and 70% ethanol was added until the final volume of 50 ml was reached. Finally, 3 aliquots of 2 ml were taken from this mixture and the solvent was evaporated by nitrogen flow to calculate the balsamic content, using **Equation 4** and expressing as a percentage (%). The analysis was done in triplicate.

$$\text{Balsamic content (\%, m/m)} = \frac{\text{Final dry extract mass (g)}}{\text{Initial propolis mass (g)}} \times 100 \qquad \textbf{Equation 4}$$

2. Preparation of propolis ethanol extract

For the extraction of bioactive compounds, an ethanol extraction was done incubating 30 g of G21 with absolute ethanol at a 1:5 ratio in an Erlenmeyer flask under 125 rpm, at 26 °C in the dark. After for 24 h, the suspension was filtered with a Buchner funnel and filter paper in a Kitasato system connected to a Humm-vac vacuum pump. The filtration residues were collected and incubated again with 100 ml of absolute ethanol under the above-referred conditions followed by another filtration. Solvent evaporation was performed in a Büchi RE111 Rotavapor, at 50 mbar, 40 rpm, with Büchi 461 water bath

at 40 °C to obtain the dried propolis ethanol extract (G21.EE). G21.EE was stored at 4 °C in the dark until further use. To calculate the final yield of this extraction, **Equation 5** was used.

$$\text{Yield (\%, m/m)} = \frac{\text{Final dry extract mass (g)}}{\text{Initial propolis mass (g)}} \times 100 \qquad \textbf{Equation 5}$$

For the following assays, a stock solution of this extract was prepared by diluting G21.EE in the same extraction solvent.

3. Chemical characterisation of G21.EE

3.1 Determination of total polyphenols content

The total polyphenols content (TPC) of G21.EE was determined using the Folin-Ciocalteu colourimetric method (Kumazawa *et al.*, 2004), based on the fact that the Folin-Ciocalteau reagent (Folin-C) in the presence of sodium carbonate (Na_2CO_3) reacts with polyphenols changing colour from yellow to blue. This colour change proportional to the polyphenol content can be monitored by absorbance reading at 760 nm (Magalhães *et al.*, 2008).

For this assay, 10 µl of G21.EE, 50 µl of Folin-C (Sigma-Aldrich, 1:10) and 40 µl de Na_2CO_3 (Merck, 7.5%; w/v) were added per well of a 96-well plate, to obtain the concentrations of 50, 100, 150, 200, 250 and 300 µg/ml. For each concentration, a blank was prepared with 10 µl extract and 90 µl ethanol. A control was also used, prepared with 50 µl of Folin-C, 40 µl of Na_2CO_3 and 10 µl of ethanol. After 1 h of incubation in the dark and at RT, the absorbance was read at 760 nm (Spectra Max Plus 384). Gallic acid (GA) (Sigma-Aldrich) was used as standard and was prepared at concentrations 1, 5, 10, 20, and 30 µg/ml. Results obtained for the extract were compared with the calibration curve of GA and the TPC was expressed as gallic acid equivalents (mg GAE/g extract). The assay was performed in triplicate, with three replicates each.

3.2 Determination of total flavonoids content

The total flavonoids content (TFC) of G21.EE was determined using the aluminium chloride ($AlCl_3$) colourimetric method (Woisky and Salatino, 1998), based on the fact that $AlCl_3$ in the presence of flavonoids leads to a colour change of the samples to yellow. This change varies proportionally with flavonoids concentration and can be followed by absorbance reading at 420 nm.

For this assay, in 96-well plates, 50 µl of G21.EE and 50 µl $AlCl_3$ (Acrós Organics, 2 %) were added per well to obtain concentrations of 200, 400, 800, 1000, 1200, 1400 and 1600 µg/ml. For each concentration, a blank was prepared with 50 µl extract and 50 µl ethanol. A control was also used, prepared with 50 µl of $AlCl_3$ and 50 µl of ethanol. After 1 h incubation in the dark and at RT, the absorbance was read at 420 nm. As standard, quercetin (Q) (Sigma-Aldrich), a natural flavonoid, was used. The assay was prepared as described but replacing the extract with quercetin at concentrations of 5, 25, 50, 100, 150 and 200 µg/ml. The results obtained for G21.EE were compared with the calibration curve for quercetin and TFC was expressed as quercetin equivalents (mg QE/g extract). The assay was performed in triplicate, with three replicates each.

3.3 Determination of total *ortho*-diphenols content

The total *ortho*-diphenols content (TOC) of G21.EE was determined using the sodium molybdate (Na_2MoO_4) complexation method described by Garcia *et al.* (2012), with some modifications. This method is based on the complexation of *ortho*-diphenols with molybdate ions resulting in a colourimetric change due to the production of an orange-coloured product which has maximum absorption at 370 nm and can therefore be detected and quantified by absorbance reading at this wavelength (Del Carlo *et al.*, 2012).

For this assay, in 96-well plates, 160 µl of G21.EE and 40 µl of Na_2MoO_4 (Acrós Organics, 50 000 µg/ml, prepared with 50% ethanol) was added per well to obtain concentrations of 25, 50, 100, 150, 200, 250 and 300 g/ml of extract. A blank with 160 µl extract and 40 µl ethanol 50% (v/v) was prepared for each concentration. A control was also used, made with 40 µl of Na_2MoO_4 and 160 µl of 50% ethanol. After 15 minutes incubation in the dark at RT, the absorbance was read at 370. Gallic acid at 10, 20, 40, 60, 80, 100, 140, 160, 180 and 200 µg/ml concentrations replaced the extract for use as standard. Results obtained for the extract were compared with the calibration curve of GA and TOC expressed as gallic acid equivalents (mg GAE/g extract). The assay was performed in triplicate, with three replicates each.

4. Determination of antioxidant activity of G21.EE

4.1 DPPH assay

The method of free radical reduction of DPPH• was used to determine G21.EE antioxidant capacity. DPPH• is a stable radical commonly used to assess the antioxidant potential of compounds

and extracts that changes its colour from purple to yellow when reduced. This reduction is caused by substances described as free radical scavengers which produce an absorbance decrease proportional to their concentration and measurable at 517 nm since DPPH• absorbs at this wavelength (Mitra and Uddin, 2014).

In 96-well plates, were added per well 50 µl of G21.EE and 100 µl of DPPH (Sigma, 0.004%; w/v) to obtain concentrations of 0.5, 1, 5, 10, 25 and 50 µg/ml. For each concentration, a blank was prepared with 50 µl extract and 100 µl ethanol. A control was also used, consisting of ethanol (50 µl) and DPPH (100 µl). After 20 minutes incubation in the dark and at RT, the absorbance was read at 517 nm. The assay was performed in triplicate, with three replicates each. Gallic acid was used as standard at concentrations of 0.2, 0.35, 0.5, 0.75, 1 and 1.5 µg/ml. The antioxidant capacity of the extract was expressed as the percentage (%) reduction of DPPH•, calculated using **Equation 6**:

$$\text{Reduction (\%)} = \frac{\text{Control absorbance} - (\text{Sample absorbance} - \text{Blank absorbance})}{\text{Control absorbance}} \times 100 \qquad \textbf{Equation 6}$$

With the values obtained through **Equation 6** it was possible to obtain the equation of the straight line and estimate the EC_{50} value (µg/ml), that is, the extract concentration necessary to sequester 50% of DPPH•.

4.2 ABTS assay

The ABTS (ABTS•) radical capture method (Re *et al.*, 1999) was also used to determine G21.EE antioxidant capacity. This method is based on the ability of antioxidants to capture the ABTS• cation (Pérez-Jiménez and Saura-Calixto, 2006) causing a decrease in absorbance, which can be monitored at 734 nm.

The concentrated ABTS (Roche) solution was previously prepared and was left for 14 - 16 h to react. Using this solution, it was necessary to find a concentration of diluted ABTS solution with an optical density (OD) read at 734 nm (OD_{734}) of 0.7 to be used in the assay. In 96-well plates, 2.5 µl of G21.EE and 247.5 µl of ABTS were added per well to obtain extract concentrations of 0.5, 1, 5, 10, 15 and 25 µg/ml. For each concentration, a blank was prepared with 2.5 µl of extract and 247.5 µl ethanol. A control with ethanol (2.5 µl) and ABTS (247.5 µl) was also used. After 30 minutes of incubation in the dark and at RT, the absorbance was read at 734 nm. The assay was performed in triplicate, with three replicates each. Trolox (Acrós Organics) was used as standard at concentrations of 0.2, 0.35, 0.5, 0.75, 1 and 1.5 µg/ml.

The antioxidant capacity of the extract was expressed as the percentage (%) reduction of ABTS•, calculated using **Equation 7**:

$$\text{Reduction (\%)} = \frac{\text{Control absorbance} - (\text{Sample absorbance} - \text{Blank absorbance})}{\text{Control absorbance}} \times 100 \qquad \textbf{Equation 7}$$

With the values obtained through **Equation 7**, it was possible to obtain the equation of the straight line and, thus, the EC_{50} value (μg/ml), or the extract concentration necessary to sequester 50% ABTS•.

5. Evaluation of antimicrobial activity of G21.EE

The MIC or Minimum Inhibitory Concentration, is lowest concentration of an agent that can inhibit the growth of a specific strain, was determined to understand the antimicrobial potential of propolis from Gerês using yeast as well as Gram-positive and Gram-negative bacteria as susceptibility indicator strains **(Table 6)** and an adaptation of the agar dilution method (Grange and Davey,1990).

Table 6. Strains used in the antimicrobial assays. Strains were kindly provided by the Department of Biology of the University of Minho.

Strain	Strain reference	Type of Isolate/Origin
Yeasts		
Candida albicans	53B	Clinical isolate/Vagina
Saccharomyces cerevisiae	BY4741/Y00000	Euroscarf
Gram-positive bacteria		
Bacillus cereus	ATCC 7064	Clinical isolate/Blood
Bacillus megaterium	932	Unknown
Bacillus subtilis	48886	Unknown
Propionibacterium acnes	H60803	Clinical isolate/Unknown
Methicillin-sensitive *Staphylococcus aureus*(MSSA)	ATCC 6538	Clinical isolate/Human wound
Methicillin-resistant *Staphylococcus aureus* (MRSA)	M746665	Clinical isolate/Aspiration
Gram-negative bacteria		
Escherichia coli	CECT 423	Unknown

The most suitable growth medium for yeast is YPD medium (Sigma-Aldrich) which is composed of 1% (w/v) of yeast extract, 2% (w/v) of peptone and 2% (w/v) of dextrose/glucose. The solid medium YPDA was made by adding 2% (w/v) of agar (Labchem) to the recipe. For bacterial culture, LB medium (PanReact AppliChem ITW Reagents), composed of 0.5% (w/v) of yeast extract, 1% (w/v) of tryptone and 2% (w/v) of sodium chloride or solid LBA medium (made by supplementing LB recipe with 2% (w/v) agar) were used.

Yeast and bacteria were grown overnight on YPD and LB media, respectively, and at 30 °C or 37 °C with shaking. Growth was monitored by reading OD_{600} and the cultures were diluted in fresh medium to obtain an OD_{600} = 0.1 and incubated in the same conditions until they reached the exponential growth phase (OD_{600} = 0.4 - 0.6). Once this stage was reached, 5 µl-drops of each culture were added to YPDA or LBA plates containing G21.EE at concentrations ranging from 10 µg/ml to 2000 µg/ml. As controls, microbial cultures were also inoculated on extract-free media and on plates with ethanol-containing media at the maximum used volume of G21.EE.

6. Fractionation of G18.EE_*n*-BuOH

A propolis sample collected in 2018 at the Gerês apiary was extracted with absolute ethanol by Freitas *et al.* (2022a) to obtain the ethanol extract G18.EE. G18.EE was further fractionated (Freitas *et al.*, 2022a) and the resulting four fractions - G18.EE_ *n*-hexane, G18.EE_EtOAc, G18.EE_ *n*-BuOH and G18.EE_water - were stored at 4 °C in the dark after evaporation of the respective solvents.

In this work, the *n*-butanol fraction derived from G18.EE partition (G18.EE_ *n*-BuOH) was further fractionated, being applied to silica gel column chromatography, to separate its compounds based on their polarity. Briefly, on a glass column, silica gel 60 (0.035-0.070 mm; Acrós Organics) diluted in *n*-hexane (Fisher Chemical) was placed. G18.EE_ *n*-BuOH (156.6 mg) diluted in ethanol (Fisher Chemical) was deposited on top and the column was eluted with solvents or solvents mixture (Fisher Chemical) increasing gradually the polarity **(Table 7)**.

The 54 subfractions obtained **(Table 7)** were analysed by thin-layer chromatography (TLC) by applying each subfraction as a spot on pre-coated aluminium backed silica plates (TLC silica gel F_{254}, Merk). Plates were developed using as eluent the solvent corresponding to that used in the chromatography column for each fraction. The developed plates were examined under UV light (254 nm and 366 nm), followed by spraying with anisaldehyde-H_2SO_4 (prepared by adding 0.5 ml of anisaldehyde (Acrós Organics) in 10 ml of fluvial acetic acid (Panreac) and 85 ml of MeOH and 5 ml of concentrated H_2SO_4 (Fisher Chemical) (Stahl and Kaltenbach (1961), adapted)) and heated for 5 minutes at 100 °C, to visualise the compounds present in each subfraction. The fractions were pooled according to similarities in their TLC-chromatographic profile, obtaining in the end 17 subfractions, which were assigned a code from A to Q **(Table 7)**. After solvent remotion under nitrogen flow, subfractions were stored at 4 °C, in the dark, until further assays.

Table 7. Subfractions obtained by silica gel column chromatography and respective solvents, or solvents mixture used as eluents.

Eluents	Subfractions	Code	Weight (mg)
Hexane:EtoAc (90:10)	F3 - F7	A	8.3
Hexane:EtoAc (90:10)	F8 - F10	B	7.1
Hexane:EtoAc (80:20)	F11	C	6.9
Hexane:EtoAc (80:20)	F12	D	11.5
Hexane:EtoAc (80:20)	F13	E	11.5
Hexane:EtoAc (70:30)	F15	F	5.5
Hexane:EtoAc (70:30)	F16 and F17	G	7.5
Hexane:EtoAc (70:30)	F18 and F19	H	8.1
Hexane:EtoAc (70:30)	F20 - F23	I	10.2
Hexane:EtoAc (70:30)	F27 - F34	J	6.8
Hexane:EtoAc (50:50)	F35 and F36	K	12.3
Hexane:EtoAc (40:60)	F37 - F39	L	5.4
Hexane:EtoAc (30:70)	F40 - F42	M	6.4
Hexane:EtoAc (10:90)	F43 - F46	N	9.6
EtoAc	F47 and F48	O	8.5
EtoAc:MeOH (90:10)	F49 - F52	P	13.2
EtoAc:MeOH (70:30)	F53 and F54	Q	4.9

EtoAc = Ethyl acetate; MeOH = Methanol

7. Evaluation of anticancer activity of propolis in melanoma

7.1 Sample preparation

G21.EE, G18.EE, the G18.EE_ *n*-BuOH and the seventeen G18.EE_ *n*-BuOH subfractions were dissolved in dimethyl sulphoxide (DMSO) (Honeywell) to obtain the stock solutions **(Table 8)** used to prepare the working solutions at the required concentrations for the assays. The final concentrations of the samples used in the experiments were obtained by diluting the stock solutions in culture medium (PAN-Biotech TM) with 0.5% fetal bovine serum (FBS). DMSO concentrations in the assays were 0.25%.

Table 8. Concentrations of stock solutions of G21.EE, G18.EE, G18.EE_*n*-BuOH fraction and 17 G18.EE_*n*-BOH subfractions.

Origin	Sample	Stock solution concentration (mg/ml)
Ethanol Extracts	G21.EE	152.0
	G18.EE	162.1
G18.EE fraction	G18.EE_*n*-BuOH	168.3
G18.EE_*n*-BuOH subfractions	A	83.0
	B	71.0
	C	69.0
	D	115
	E	115
	F	55
	G	75
	H	81
	I	102
	J	68
	K	123
	L	54
	M	64
	N	96
	O	85
	P	132
	Q	49

7.2 Cell lines and culture conditions

In vitro assays were performed using two distinct human melanoma cell lines: A375 ($BRAF^{V600E}$ mutated) and SK-MEL-23 (wild-type). These human cell lines were established from cutaneous malignant melanoma. A375 cell line was kindly given by Dra. Marta Viana-Pereira (University of Minho, Braga, Portugal) and SK-MEL-23 cell line was gently provided by Dr. Francisco Real (National Oncological Research Centre, Spain). The A375 cell line was grown in Dulbecco's Modified Eagle's Medium (DMEM, PAN-Biotech) and supplemented with 10% FBS. The SK-MEL-23 cell line was grown in Roswell Park Memorial Institute Medium (RPMI, PAN-Biotech) and supplemented with 10% FBS. Both cell lines were incubated at 37 °C in a humidified environment with 5% CO_2.

7.3 Cell viability assay

To determine the effect of the extracts and the G18.EE_*n*-BuOH fraction on cell viability, the Sulforhodamine B (SRB, TOX-6, Sigma-Aldrich) assay was used. This colorimetric assay developed by Skehan *et al.* (1990), was later modified by Vichai and Kirtikara (2006) and is commonly used to measure cytotoxicity induced in cells by treatments. SRB is a pink aminoxanthene dye that can bind to basic amino acid residues of cell proteins fixed with trichloroacetic acid (TCA) in a pH-dependent manner. Under slightly acidic conditions it binds and under basic conditions, it is solubilised allowing absorbance measurement (Voigt, 2005). The binding of SRB to cells is stoichiometric, i.e., the SRB extracted from stained cells is proportional to cell mass and therefore we can indirectly assess the cytotoxicity caused by the treatments (Orellana and Kasinski, 2016). After IC_{50} determination, another SRB assay was carried to evaluate whether storage conditions and continuous handling of the samples cause a decrease in cytotoxic activity. For this, a sample of G18.EE that had been stored at –20 °C and had not been manipulated (162.1 mg/ml) was used. Two concentrations were tested in A375 cell line: the IC_{50} of G18.EE calculated previously by Oliveira (2022) (IC_{50}2021 = 16.98 μg/ml) and the IC_{50} of G18.EE calculated in this work (IC_{50}2022 = 26.87).

In 96-well plates, both cell lines were plated at a concentration 25x10^4 cells/ml in 200 μl of medium and left overnight at 37 °C and 5% CO_2 (humidified atmosphere) to adhere to the plate. The following day, cells were subjected to serum starvation for 2 h using a culture medium without FBS so that all cells are in the same cell cycle phase. At the end of this period, the samples - diluted in DMEM or RPMI supplemented with 0.5% FBS with different concentrations (10 to 50 μg/ml) - were added to the plate. DMSO was used as control. DMSO final concentration was 0.25% in all the wells. For each concentration or control tested, triplicates were made. After the addition of the treatments, the plates were left for 72 h at 37 °C and 5% CO_2. In the final part of the assay, cells were fixed to the plate with 100 μl of cold TCA (10%, Sigma-Aldrich) for 1 h at 4 °C. To remove the TCA, cells were washed four times with deionised water and placed to dry at 37 °C for 1h30 min. The next step was the addition of SRB (0.4% SRB in 0.1% acetic acid) which was left to react for 30 min at RT. To remove unbound SRB, cells were washed with 1% acetic acid (Brand) and left to dry for 30 min at 37 °C. In the end, the dye was solubilised with 100 μl of 10 mM Tris base (PanReact AppliChem ITW Reagents) and the plates were incubated for 10 min at RT. The absorbance was measured at 490 nm (Thermo Scientific Varioskan Flash). With the absorbance values obtained it was possible to calculate the IC_{50} values using GraphPad Software Version 8.0. Three independent experiments were carried out, each one conducted in triplicate.

7.4 Cell viability screening using G18.EE_ *n*-BuOH subfractions

To determine the effect of the G18.EE_*n*-BuOH subfractions on A375 cell viability, the SRB assay was used. The methodology applied was the same described above, with the difference that here only one concentration (25 μg/ml) of each of the 17 subfractions (A to Q) was tested. DMSO was used as control. At least, three independent assays were carried out, each one performed in triplicate.

8. UPLC-DAD-ESI/MS^n subfractions analysis

The phenolic compounds of the subfractions **E**, **G**, **H**, **I**, **J** and **K** were analysed by UPLC-DAD-ESI/MS^n. This analysis was generously performed at the Chemistry Department of the University of Aveiro by Dra. Susana Cardoso as follows: the UPLC-DAD-ESI/MS^n analysis was performed on an Ultimate 3000 (Dionex Co.) apparatus equipped with an ultimate 3000 Diode Array Detector (Dionex Co.) and coupled to a mass spectrometer LTQ XL Linear Ion Trap 2D. The chromatographic system consisted of a quaternary pump, an autosampler, a photodiode-array detector and an automatic thermostatic column compartment. Analysis was run on a Hypersil Gold (Thermo Scientific) C18 column (100 mm length; 2.1 mm i.d.; 1.9 μm particle diameter, end-capped) and its temperature was maintained at 30 °C. The mobile phase was composed of (A) 0.1% of formic acid (v/v) and acetonitrile (B). The solvent gradient started with 20% of solvent (B), reaching 40% at 25 min, 60% at 35 min and 90% at 50 min, followed by the return to the initial conditions. The flow rate was 0.1 ml min−1 and UV-Vis spectral data for all peaks were accumulated in the range 200-500 nm, while the chromatographic profiles were recorded at 280 and 320 nm.

The mass spectrometer used was a Thermo LTQ XL (Thermo Scientific) ion trap MS equipped with an ESI source. Control and data acquisition were carried out with the Thermo Xcalibur Qual Browser data system (Thermo Scientific). Nitrogen above 99% purity was used, and the gas pressure was 520 kPa (75 psi). The instrument was operated in negative-ion mode with ESI needle voltage set at 5.00 kV and an ESI capillary temperature of 275 °C. The full scan covered the mass range from m/z 100 to 2000. CID-MS/MS and MS^n experiments were simultaneously acquired for precursor ions using helium as the collision gas with collision energy of 25-35 arbitrary units.

9. Statistical Analysis

Each assay was performed in triplicate and repeated at least three times independently ($n \geq 3$) and results were expressed as mean ± standard deviation (SD). For IC_{50} determination it was used GraphPad Prism 8.0 software for logarithmic transformation after applying a non-linear sigmoidal dose-response regression. One-way analysis of variance (ANOVA) was used for the comparison of more than two means and Tukey's test for multiple comparisons. The threshold used for statistical significance was $p<0.05$ and differences considered statistically significant were noted with different letters.

CHAPTER III: RESULTS & DISCUSSION

1. G21 meets quality criteria for propolis samples

The potential use of propolis demands a characterization of the target sample and should include the assessment of parameters such as water, ash, waxes, and balsamic contents to obtain information about its quality (Lopes *et al.*, 2017). The values obtained for these parameters for G21 are listed in **Table 9** as well as the values set out in the literature, for comparison.

Table 9. Results for G21 regarding the quality criteria parameters for propolis. Results obtained for G21 sample can be compared with the values described by Oliveira (2022) for G18, Falcão (2013) for Portuguese propolis types I and II and the values established by TRPIQ for Brazilian propolis (Brazil, 2001). The results for G21 and G18 samples are presented as mean ± standard deviation (SD) (n≥3).

Propolis content	G21	G18	Portuguese propolis type I	Portuguese propolis type II	TRPIQ
Ash (%, m/m)	1.00 ± 0.02	0.73 ± 0.06	Max. 2	Max. 4	Max. 5
Water (%, m/m)	2.42 ± 0.14	5.25 ± 0.30	Max. 5	Max. 5	Max. 8
Wax (%, m/m)	15.07 ± 1.27	2.18 ± 0.19	Max. 25	Max. 31	Max. 25
Balsam (%, m/m)	51.67 ± 2.89	56.67 ± 13.12	Min.65	Min.45	-

"-"= value not provided.

The value obtained for ash (1.00 ± 0.02%) is very close to the value obtained for G18 (0.73 ± 0.06%), the sample collected in 2018 from the same region (Oliveira, 2022) and both below the limits established for both Portuguese (Falcão, 2013) and Brazilian propolis (Brazil, 2001). In fact, for poplar type propolis the value of ash content is between 1–2% (Falcão 2013). The high ash content is usually related to the presence of soil and, consequently, a high ash content is associated with a sample with less bioactive compounds (Pereira *et al.*, 2020).

Water content is mainly influenced by handling and storage time (Falcão, 2013; El Menyiy *et al.*, 2021) and in G21 (2.42 ± 0.14%) is lower than the values established in the literature. When compared with the sample of 2018 (5.25 ± 0.30%), G21 presents a lower water content **(Table 9)**. The difference could be explained by the influence of storage time since this parameter was only evaluated in G18 after three years of its storage at 4 °C (Oliveira, 2022), which may have led to increased humidity in the sample, while G21 was analysed in the year of its collection.

Propolis with high amounts of wax is considered to be of inferior value as it will have fewer pharmacologically interesting compounds, hence a lower commercial value (Falcão, 2013; Pereira, 2020). Wax content in G21 - 15.07 ± 1.27% - does not exceed any of the maximum values defined for the two types of Portuguese propolis nor the maximum values accepted for Brazilian propolis **(Table 9)**.

Specifically, for propolis from northern Portugal, as is the case of G21, the wax values fluctuate between 15 and 35% (Falcão, 2013) and therefore G21 fits into these values. However, G21 has a much higher wax content than G18 (2.18 ± 0.19%) which may indicate a higher biological activity in the latter sample.

G21 balsamic content was determined as 51.67 ± 2.89%. This parameter refers to compounds that are soluble in ethanol, such as phenolic compounds, which are largely responsible for the bioactivities of propolis and, therefore, a quality sample must have high balsamic content (Falcão *et al.*, 2019). Regarding this parameter, G21 seems to fit more in the Portuguese type II propolis **(Table 9)**, which was not expected considering its location. However, looking at the balsamic values obtained for propolis samples from the northern regions of Portugal by Falcão (2013) it can be seen that values vary between 37 and 85%. Yet, the value obtained for G21 is lower than that obtained for G18 (56.67 ± 13.12%), which again suggests G18 as the most biologically active sample .

Therefore, G21 meets propolis quality standards, with parameters agreeing with a Portuguese propolis type I, considered to be similar to the poplar type, common in Europe. Besides, it fulfils the parameters defined by the Brazilian legislation which allows its commercialization. With these results, G21 has potential interest for investigating its biological activity.

2. Extraction yield for G21.EE

The yield obtained in the ethanol extraction of propolis from Gerês harvested in 2021 (G21.EE) was 78.6%. This value obtained may be a bit higher than the actual extraction yield, due to the fact that the extract is not fully dry, in a powdered form, for instance, meaning that the ethanol could not have been completely evaporated and this may contribute to an overestimation of the value obtained. The solvents normally chosen to prepare propolis extracts are ethanol, methanol, or chloroform (Fokt *et al.*, 2010). Absolute ethanol was chosen because it is described as a solvent that allows obtaining extracts rich in phenolic compounds (Silva-Carvalho *et al.*, 2014; Kubiline *et al.*, 2015; Chen *et al.*, 2018; Devequi-Nunes *et al.*, 2018). Also in our research group, the use of absolute ethanol has enabled to obtain extracts with high levels of bioactive compounds and various biological properties (Cruz *et al.*, 2016; Freitas *et al.*, 2019; Peixoto, 2018; Pereira, 2021; Passão, 2021; Oliveira, 2022), and therefore it was also used in this work. Some studies (Archaina *et al.*, 2015; Sun *et al.*, 2015) compared the yields obtained with increasing concentrations of ethanol and concluded that the higher the ethanol concentration used in the extraction the higher the yield obtained. However, there are also contradictory studies that state that the use of 70% ethanol allows to be obtain a greater proportion of phenolic compounds (Kara *et al.*, 2022).

These differences can be explained by the highly variable chemical composition of propolis since different solvents solubilise different compounds (Watanabe *et al.*, 2011).

The yield values obtained for ethanol extractions of propolis from Gerês made by our research group since 2011 are between 66 and 88% (Carvalho, 2012; Pereira, 2013; Araújo, 2014; Freitas, 2015; Oliveira, 2022), so the value obtained for G21.EE is within this range of those values and is in line with what is expected for propolis from this geographical location and the solvent and extraction technique used.

3. G21.EE presents high phenolic content

Propolis is a combination of several compounds, whose composition differs mainly according to the type of plants the bees visit, therefore the composition of propolis is highly variable (Bankova *et al.*, 2000). As mentioned earlier, the pharmacological properties of propolis are often attributed to its phenolic compounds (Miguel *et al.*, 2010; Wieczorek *et al.*, 2022). Thus, to characterize a propolis sample and determine its quality/value, it is common to assess its phenolic content (Lopes *et al.*, 2016). Phenolic compounds are the most abundant class of compounds in propolis and are a wide group that can include at least ten types of compounds. In propolis of European origin, flavonoids and phenolic acids are the most relevant phenolic classes (Bankova *et al.*, 2000; Falcão *et al.*, 2010; Miguel *et al.*, 2010). With this in mind, total polyphenols content (TPC), total flavonoids content (TFC) and total *ortho*-diphenols content (TOC) were determined for G21.EE **(Table 10)**.

Table 10. Phenolic content of G21.EE. Total polyphenols content (TPC), total flavonoids content (TFC) and total *ortho*-diphenols content (TOC) were determined for G21.EE and compared with the same parameters in G11.EE, G12.EE, G13.EE, G14.EE, G15.EE and G18.EE (A.S. Freitas, personal communication; Peixoto *et al.*, 2022). The results are presented as mean ± SD (n≥3). Statistical analysis was performed by one-way ANOVA followed by Tukey test for significance. For each variable, same letters mean no statistically significant differences between values and different letters mean statistically significant differences between values.

Propolis content	G11.EE	G12.EE	G13.EE	G14.EE	G15.EE	G18.EE	G21.EE
TPC (mg GAE/g extract)	143.0 ± 1.9^{a}	198.0 ± 25.4^{b}	205.8 ± 3.5^{b}	165.4 ± 4.1^{a}	207.9 ± 7.5^{b}	224.60 ± 10.86^{b}	136.33 ± 2.23^{a}
TFC (mg QE/g extract)	31.6 ± 0.9^{a}	34.6 ± 2.7^{a}	32.6 ± 0.8^{a}	31.0 ± 1.3^{a}	51.7 ± 0.9^{b}	44.74 ± 1.26^{c}	40.94 ± 1.32^{c}
TOC (mg GAE/g extract)	-	-	-	-	-	263.05 ± 15.19	332.27 ± 9.58

"- "= not determined.

For TPC, gallic acid was used as a standard and therefore the results are expressed in gallic acid equivalents (GAE). G21.EE presented a TPC of 136.33 ± 2.23 mg GAE/g extract. This value is within the range of values that have been obtained in recent years, 143.0 ± 1.9 to 224.60 ± 10.86 mg GAE/g

extract **(Table 10)**. However, it is significantly lower than the value obtained for G18.EE (224.60 ± 10.86 mg GAE/g extract) anticipating that G21.EE may be less bioactive. Comparing to values obtained for other regions of Portugal, a propolis sample from the Northeast of the country showed a TPC of 329.00 ± 0.01 mg GAE/g extract while a sample from the central region displayed 151.00 ± 0.01 mg GAE/g extract (Moreira *et al.*, 2008), and for an ethanol extract of propolis sample of Beira Alta the TPC obtained was 160.40 ± 16.56 mg GAE/g extract (Cruz *et al.*, 2016) whereas 150.39 ± 6.29 mg GAE/g extract was the TPC of an ethanol extract prepared with propolis from Caramulo (Central region) (Pereira, 2021), the latter being the most similar to the G21.EE TPC. Falcão (2013) evaluated the phenolic content of several samples from different regions of the country, concluding that the values ranged between 200 - 400 mg/g. These values are higher than the one obtained for G21.EE, however analysing the results for the Central region (Penamacor, 127.1 ± 0.2 mg/g extract), G21.EE once again presented a TPC similar to this region of the country. An explanation for the difference registered among TPC values can be the standard(s) used in the assays - a mixture of caffeic acid, galangin, and pinocembrin instead of gallic acid. In fact, Miguel *et al.* (2010), have already reported that the use of gallic acid as a standard resulted in lower values compared to the results obtained using pinocembrin. Furthermore, gallic acid is not very representative of samples from temperate zones and is more common in propolis from tropical zones (Miguel *et al.*,2010) despite its identification in propolis from Gerês (Freitas *et al.*, 2019)

Regarding the flavonoid content, the values are presented in quercetin equivalents (QE) as quercetin was chosen as standard. G21.EE presented a TFC of 40.94 ± 1.32 mg QE/g extract, similar o the values obtained for propolis from Gerês in previous years (31.0 ± 1.3 to 51.7 ± 0.9 mg QE/g extract) **(Table 10)** and is only significantly lower than the TFC value obtained by our research group for G15.EE (51.7 ± 0.9 mg QE/g extract). It also appears to be lower than the results described for an ethanol propolis extract from Caramulo (65.44 ± 1.61 mg QE/g extract; Pereira, 2021) and for ethanol extracts of propolis collected in different years (2011 to 2015) from Guarda area (TFC ranging from 44.7 ± 12.0 to 101.8 ± 4.2 mg QE/g) (Peixoto, 2018). Although samples are from the same country, their content differs depending on the type of flora around the hives and therefore G21.EE could not present neither the same amount nor the same compounds, being actually poorer in flavonoids content. Also, the method used to determine TFC can influence the results, as the $AlCl_3$ method identifies mostly flavones and flavonols and therefore leaves out important compounds such as flavanones and dihydroflavonols (Chang *et al.*, 2002). Even so, the TFC of G21.EE (40.94 ± 1.32 mg QE/g extract) is within the range of values determined for several locations in Portugal (5.2 ± 0.3 to 114.2 ± 0.1 mg/g extract; Falcão, 2013). Searching the literature, it can be noticed that the TFC value of G21.EE is very similar to the value obtained

for poplar type propolis from Slovakia (40.42 ± 1.07 mg QE/g extract) (Widelski *et al.*, 2018) and falls within the range of values obtained for extracts of propolis from different parts of the world (2.5 to 176 mg QE/g extract) (Kumazawa *et al.*, 2004).

Regarding the TOC, a value of 332.27 ± 9.58 mg GAE/g G21.EE was obtained for this content, also expressed as GAE as gallic acid was again used as a standard in this assay. *Ortho*-diphenols are a parameter still poorly evaluated in propolis, although it is reported to be linked to antioxidant activity mainly in olive and olive oil (Gouvinhas *et al.*, 2014; Soufi *et al.*, 2014). The calculated TOC for G21.EE seems to be substantially higher than that of G18.EE (263.05 ± 15.19 mg GAE/g extract) **(Table 10)** and apparently more similar to TOC value obtained by Pereira (2021) for the ethanol extract of Caramulo propolis (314.17 ± 13.09 mg GAE/g extract).

Together, these results confirm that G21.EE is rich in phenolic compounds, with the most representative group being *ortho*-diphenols. It should also be reinforced the need to standardise the analytical methods for assessing these parameters as well as the compounds used as standards for calibration in order to obtain comparable results and to be able to define universal quality criteria.

4. G21.EE presents antioxidant activity

Oxidative stress caused by ROS found in the organism is the cause of several pathologies and therefore there is a frequent demand for antioxidant compounds. The antioxidant activity of propolis is well documented (Banskota *et al.*, 2000; Kumazawa *et al.*, 2004; Farooqui and Farooqui, 2012; Anjum *et al.*, 2018) and is mostly associated with its phenolic compound-rich composition (Wieczorek *et al.*, 2022). Since G21.EE exhibit considerable levels of phenolic compounds it is important to evaluate its antioxidant potential as well. For a substance to be considered an antioxidant, it has to be able to eliminate free radicals. Therefore, two *in vitro* methods were used to evaluate the antioxidant capacity of propolis extract by assessing its ability to scavenge free radicals, in this case DPPH• and ABTS•. **Table 11** presents the EC_{50} obtained for G21.EE using the two methods as well as the EC_{50} of the two standard antioxidants (gallic acid and trolox) and the results obtained by Oliveira (2022) for G18.EE.

Table 11. Antioxidant activity of G21.EE Results refer to DPPH• and ABTS• scavenging activities and are compared with the values obtained by Freitas (2015) for G11.EE, G12.EE, G13.EE, G14.EE and by Oliveira (2022) for G18.EE. Gallic acid and trolox were used as standards for the DPPH and ABTS assays, respectively. The results are presented as mean ± SD (n≥3). Statistical analysis was performed by one-way ANOVA followed by Tukey test for significance. For each variable, same letters mean no statistically significant differences between values and different letters mean statistically significant differences between values.

EC_{50} (μg/ml)	G11.EE	G12.EE	G13.EE	G14.EE	G18.EE	G21.EE
DPPH	17.77 ± 0.78^{a}	14.41 ± 0.56^{b}	25.24 ± 2.45^{c}	16.47 ± 0.75^{a,b}	10.90 ± 0.34^{e}	10.81 ± 0.18^{e}
ABTS	-	-	-	-	9.83 ± 0.21	9.36 ± 0.45

"- "= not determined.

The EC_{50} values determined through both methods for G21.EE are relatively similar **(Table 11)**. Using the DPPH free-radical-scavenging method, the EC_{50} value was 10.81 ± 0.18 μg/ml while for the standard used in this assay, Gallic acid, an EC_{50} of 0.80 ± 0.01 μg/ml was obtained. Regarding ABTS radical scavenging activity, 9.36 ± 0.45 μg/ml was the EC_{50} value of G21.EE and 3.46 ± 0.22 μg/ml the one shown by Trolox, the used standard. Thus, G21.EE is a potential natural antioxidant since displays DPPH• and ABTS• scavenging activity (Sheng *et al.*, 2007). These results are in line with those previously described for G18.EE by Oliveira (2022) **(Table 11)**.

The antioxidant activity of Portuguese propolis has been reported on numerous occasions. For ethanol extracts of Gerês propolis from four consecutive years (2011 to 2014), Freitas (2015) reported EC_{50} values ranging from 14.41 ± 0.56 to 25.24 ± 2.45 μg/ml **(Table 11)**. Later, Peixoto (2018) presented an EC_{50} value of 19.7 ± 8.8 μg/ml for a Gerês propolis extract from 2015 and of 10.3 ± 1.7 to 22.0 ± 0.47 μg/ml for propolis extracts from Guarda. Samples from the South of Portugal (Algarve) showed EC_{50} values of 27 and 31 μg/ml, using DPPH assay (Miguel *et al.*, 2010), substantially higher than those obtained for G21.EE **(Table 11)**, hence a more limited antioxidant activity. The ethanol extract of propolis from Caramulo, with an EC_{50} value of 9.58 ± 0.66 μg/ml (Pereira, 2021), is extremely similar to that exhibited for G21.EE **(Table 11)**. The EC_{50} values of G21.EE are still within the lower limit of the values obtained by Falcão (2013) for samples from different locations in Portugal (8 to 93 μg/ml). Furthermore, they agree with the results presented for the North region of Portugal, being especially similar to the value determined for Montalegre propolis (10 μg/ml). G21.EE also fulfils the requirement established in the same publication for Portuguese propolis type I (similar to poplar propolis) which should have a maximum EC_{50} value of 20 μg/ml **(Table 4)**. With this information, it is possible to conclude that G21.EE presents a strong capacity to scavenge free radicals, being therefore a potential natural antioxidant with one of the lowest EC_{50} for Portuguese propolis.

5. G21.EE inhibits the growth of several microorganisms

Currently, there is a demand for substances with antimicrobial capacity, predominantly due to the high levels of drug resistance and the lack of new antibiotics and antifungals. Moreover, most of the newly approved antibiotics are derived from existing antibiotics which leads to a rapid emergence of resistance. Therefore, it is necessary to find new mechanisms of action to avoid this situation (Burki, 2021). Propolis antimicrobial activity gains particular importance in this context of the current problem of antibiotic resistance, considered by the WHO as one of the major risks to world health (WHO, 2019).

Antimicrobial activity of G21.EE was assessed by the agar dilution method and MIC values were defined against each microorganism as the lowest concentration for which no microbial growth was observed **(Table 12)**.

Table 12. MIC values (µg/ml) of Gerês propolis extracts against various microorganisms. Determined MIC values for G21.EE can be compared to those obtained by Oliveira (2022) for G18.EE and for G.EEs (G11.EE, G12.EE, G13.EE and G14.EE) by Freitas (2015).

	MIC (µg/ml)		
Strains	**G21.EE**	**G18.EE**	**G.EEs**
Yeasts			
Candida albicans	2000	>2000	>2000
Saccharomyces cerevisiae	2000	>2000	>2000
Gram-positive bacteria			
Bacillus cereus	50	50	50
Bacillus megaterium	100	50	50
Bacillus subtilis	50	50	50
Propionibacterium acnes	200	500	-
MSSA	200	500	200
MRSA	>2000	>2000	>2000
Gram-negative bacteria			
Escherichia coli	>2000	>2000	>2000

"-"= not determined. *G.EEs MIC values were the ones obtained for G11.EE, G12.EE, G13.EE and G14.EE.

The yeast tested exhibited the same response to G21.EE (MIC value of 2000 µg/ml) whereas for bacteria different organisms present different susceptibilities to G21.EE. In general, Gram-positive bacteria were more susceptible than the only Gram-negative bacteria tested *(Escherichia coli*), as expected since the higher resistance of Gram-negative bacteria to propolis extract is well documented (Bankova *et al.*, 2000; Sforcin *et al.*, 2000; Silva-Carvalho *et al.*, 2015; Rufatto *et al.*, 2018; Freitas *et al.*, 2019). The genus *Bacillus* was the most susceptible of the microbial panel, more specifically the species *B. cereus* and *B. subtilis* (MIC = 50 µg/ml), followed by *B. megaterium* (MIC = 100 µg/ml).

Propionibacterium acnes and MSSA (methicillin sensitive *Staphylococcus aureus*) showed a similar response to G21.EE (MIC = 200 µg/ml). Finally, the only Gram-positive bacteria whose growth was not affected by the G21.EE concentrations tested (MIC >2000 µg/ml) was MRSA.

The anti-yeast activity of G18.EE was also previously evaluated for the same microorganisms´ panel but the concentrations tested in that assay did not inhibit growth (MIC >2000 µg/ml; Oliveira, 2022), unlike G21.EE **(Table 12)**. In addition, G18.EE also has higher MIC values (500 µg/ml) compared to those determined for G21.EE (200 µg/ml) against *Propionibacterium acnes* and MSSA though it was more effective against *Bacillus megaterium* (50 µg/ml). In common, G18.EE and G21.EE share the fact that the concentrations tested did not affect the growth of MRSA and *Escherichia coli* **(Table 12)**. For other Gerês propolis samples from previous years (2011, 2012, 2013 and 2014), Freitas (2015) concluded that there was a constancy in MIC values against each of the strains regardless of the sample harvesting year **(Table 12)**. Also, this set of extracts was not effective against the tested yeast, unlike G21.EE, which suggests that there might exist some different compounds and/or the same compounds but in different proportions that establish different interactions giving G21.EE an antimicrobial spectrum different from the ones of other samples from the same geographical region. For bacteria, the MIC values of G21.EE agree with those obtained for other G.EEs, except for *Bacillus megaterium* **(Table 12)**. G21.EE also follows the tendency already demonstrated by other ethanol extracts of Gerês propolis (G15, G17, G19 and G20) for antibacterial activity that proved to be most effective against Gram-positive bacteria and within this panel the species of the genus *Bacillus* were the most sensitive (Gonçalves, 2017; Gomes, 2019; Cardoso, 2021; Oliveira, 2022).

The susceptible of this panel of strains to propolis extracts had already been reported several times (Stepanović *et al.*, 2003; Muli and Maingi 2007; Bittencourt *et al.*, 2015; Jang *et al.*, 2015; Al-Ani *et al.*, 2018; Rufatto *et al.*, 2018). For example, AL-Ani *et al.* (2018) evaluated the antimicrobial activity of ethanol extracts of propolis from three European countries (Germany, Ireland and Czech) against various microorganisms, including *Staphylococcus aureus*, MRSA, *Bacillus subtilis*, *Escherichia coli* and *Candida albicans*. For the German extract, *Bacillus subtilis* and MRSA showed similar behaviour (MIC = 300 µg/ml) while *Staphylococcus aureus* was less susceptible (MIC = 1200 µg/ml), being the least sensitive strains *Escherichia coli* and *Candida albicans* (MIC = 5000 µg/ml). As for the Irish extract, *Bacillus subtilis* and *Staphylococcus aureus* were the most susceptible strains (MIC= 80 µg/ml), the MIC value against *Bacillus subtilis* is similar to that obtained for G21.EE **(Table12)** but more active against *Candida albicans* (MIC = 600 µg/ml) as well as MRSA and *Escherichia coli* (MIC= 1200 µg/ml). Finally,

the Czech propolis extract exhibited the ability to inhibit *Escherichia coli* and MRSA growth (MIC = 600 µg/ml), unlike G21.EE, as well as *Candida albicans* (MIC = 600 µg/ml), however the MIC value (300 µg/ml) for *Bacillus subtilis* and *Staphylococcus aureus* is higher than the observed for G21.EE (50 and 200 µg/ml, respectively).

For *Propionibacterium acnes*, associated with the development of acne, the information in the literature is scarcer. However, for two propolis samples, one from Korea and another from Brazil, higher antibacterial activity was reported, with MIC values of 1 and 4 µg/ml, respectively.

The variability of the antimicrobial effect of propolis extracts is related to its highly variable chemical composition which gives it different activities depending on the type, quantity and quality of the compounds present in the propolis sample (Santos *et al.*, 2002; Sawaya *et al.*, 2004; Dezmirean *et al.*, 2017). The fact that different techniques are frequently applied to determine MIC values and microbial susceptibility, as well as the fact that different concentration ranges and strains, even those belonging to the same species, are tested, make it difficult to compare results from different research. Yet, G21.EE was able to inhibit most of the microorganisms tested. For the strains in which growth was not inhibited, it is possible to extend the range of concentrations tested in a next trial to find the concentration of G21.EE is capable of avoiding the growth of MRSA and *Escherichia coli.*

6. G21.EE, G18.EE and G18.EE_*n*-BuOH decrease melanoma cell viability

Melanoma is the most lethal type of skin cancer, and its incidence has been increasing (Forsea, 2020). Some therapeutic options already exist for this pathology, but their effectiveness is still limited, mainly due to acquired resistance (Sousa *et al.*, 2010). Furthermore, conventional therapies have associated adverse effects and therefore research in this area continues to search for new forms of treatment (Livingstone *et al.*, 2014; Gastaldello *et al.*, 2021). Propolis has proven anti-cancer activity against several types of cancer (He *et al.*, 2006; Cogulu *et al.*, 2009; Missima *et al.*, 2010; Wu *et al.*, 2011; Markiewicz-Żukowska *et al.*, 2013; Silva-Carvalho *et al.* 2014; Patel, 2015; Frión-Herrera *et al.*, 2020; Rodrigues *et al.*, 2021; Freitas *et al.* 2022a) and although few studies exist, some researches proved its anti-melanoma activity (Patel, 2015; Cisilotto *et al.* 2018; Pereira *et al.*, 2021; Popova *et al.*, 2021; Oliveira *et al.*, 2022).

The cytotoxic effect of G21.EE, was evaluated against two human melanoma cell lines, the A375 (*BRAF*-mutated) and SK-MEL-23 (wild-type) through the SRB assay. Other two propolis samples - G18.EE

and G18.EE_*n*-BuOH - previously described with cytotoxic activity for A375 melanoma cells (Oliveira, 2022) were used for control purposes. The three samples tested were effective in decreasing the cell biomass of both lines in a dose-dependent manner **(Figure 7)**. For each cell line, the treatments under study exhibited very similar effects on cell viability. In fact, both A375 and SK-MEL-23 cells seem to be more sensitive to G18.EE_*n*-BuOH. Still, while A375 and cells tend to be less sensitive to G18.EE **(Figure 7A)** SK-MEL-23 cells tend to be less sensitive to G21.EE **(Figure 7B)**. On the other hand, A375 cell line appears to be more sensitive to any of the samples than SK-MEL-23 cell line according to IC_{50} values **(Table 13)**.

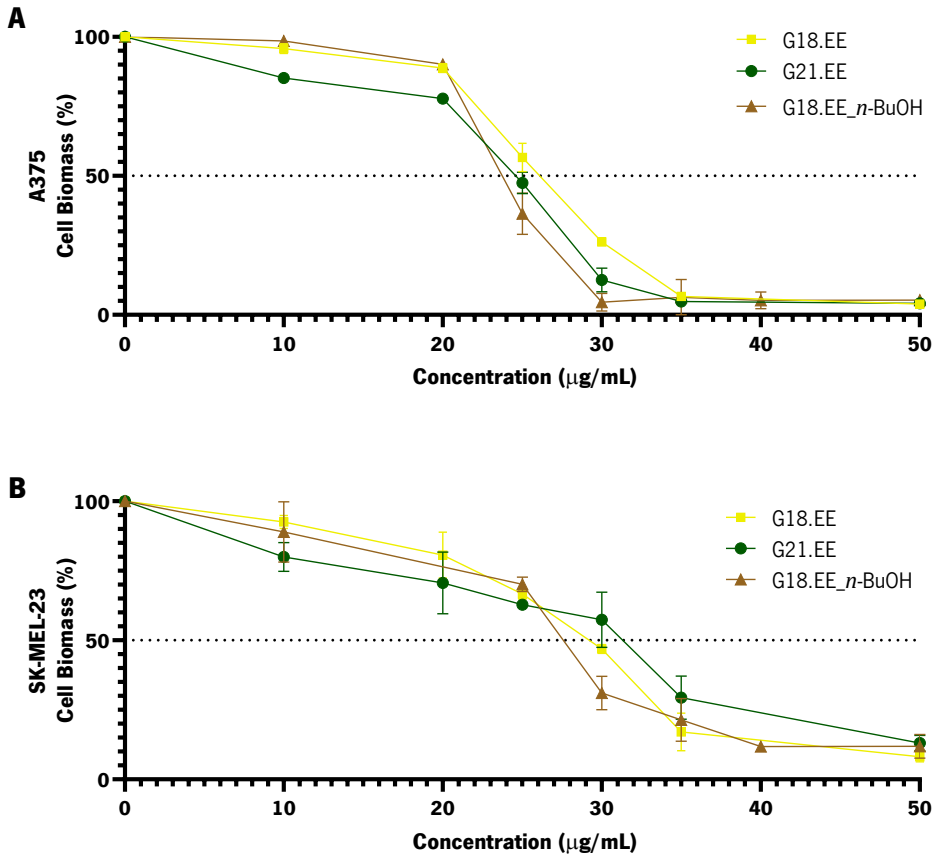

Figure 7. Effect of G21.EE, G18.EE and G18.EE_*n*-BuOH on total cell biomass of melanoma cells. (A) A375 and **(B)** SK-MEL-23 cell lines were treated with a range of concentrations (10 to 50 µg/ml) of G21.EE, G18.EE and G18.EE_*n*-BuOH for 72 h to determine the IC_{50} concentrations. Cell biomass was measured by the SRB assay. Data were normalized for total biomass. The results are presented in the form mean ± SD (n≥3).

Table 13. IC_{50} values of G21.EE, G18.EE and G18.EE_*n*-BuOH melanoma cell lines. A375 and SK-MEL-23 cells were treated for 72 h with 10 to 50 μg/ml of each sample. The results are presented in the form mean ± SD (n≥3). Statistical analysis was performed by one-way ANOVA followed by Tukey test for significance. For each variable, same letters mean no statistically significant differences between values.

	IC_{50} (μg/ml)		
	G21.EE	G18.EE	G18.EE_*n*-BuOH
A375	24.52 ± 3.5[a]	26.87 ± 1.73[a]	23.53 ± 1.55[a]
SK-MEL-23	32.21 ± 4.57[a]	29.51 ± 1.29[a]	27.54 ± 5.37[a]

Previous studies had already described the cytotoxic effect of propolis on melanoma. For example, a Chinese propolis ethanol extract had an anti-proliferative effect on A375 (IC_{50} = 112 μg/ml) (Zheng *et al.*, 2018), but much less evident than the one presented here for Gerês propolis against A375 **(Table 13)**. An ethanol extract with propolis from Poland demonstrated decreased viability of Me45 melanoma cells (Kubina *et al.*, 2015) as well as Dutch propolis isolated compounds (cinnamic acid derivatives, flavonoids and glycerol derivatives) in murine B16-BL6 melanoma (Banskota *et al.*, 2012). A hydroalcoholic extract (70%) of Brazilian propolis has a cytotoxic effect in SK-MEL-28 (35 to 197 μg/ml reduces cell number by 50%) (Cisilotto *et al.*, 2018) and methanol extract of propolis from Thailand showed with a similar effect in this cell line (IC_{50} from 33.38 to 153.38 μg/ml). The ethanol extract of Algerian propolis as well as compounds isolated from green propolis (namely Baccarin and *p*-Coumaric acid) decreased murine melanoma tumour progression (Benguedouar *et al.*, 2016; Gastaldello *et al.*, 2021). In general, Gerês propolis extracts and fraction show lower IC_{50} values than those described in the literature for propolis effects in melanoma and can therefore be considered more active. However, these comparisons are difficult to establish since, different cell lines are used that present different genetic profiles were used in different researches.

Considering the results obtained for A375 cell line, it seems that G18.EE, G18.EE_*n*-BuOH and G21.EE have similar anticancer activity, but surprisingly these results do not fit with the ones previously reported by Oliveira (2022). In fact, the IC_{50} values obtained in this work for G18.EE and G18.EE_*n*-BuOH against the A375 cell line are higher than the ones obtained by Oliveira (2022), possibly indicating that propolis has lost some activity. Thus, although G21.EE and G18.EE look similar in what concerns the cytotoxic activity herein evaluated, the same cannot be said when comparing the effect of G21.EE against A375 melanoma cell line with the effect described by Oliveira (2022) for G18.EE (IC_{50} = 16,98 μg/ml) questioning the constancy of activity claimed for Gerês propolis. This putative loss of activity of G18.EE is probably related to handling and storage conditions. Actually, the stock solutions used here for the viability

assays were already used in several previous works. Samples are dissolved in DMSO and were stored at –20 °C which implies several freezing and defrosting cycles to perform the assays. To confirm this hypothesis, a sample of G18.EE that had been stored at –20 °C and was not manipulated was tested against A375 cell line using the IC_{50} values obtained for G18.EE: the one previously calculated by Oliveira (2022) - IC_{50}2021 = 16,98 µg/ml - and the calculated in this work - IC_{50}2022 = 26,87 µg/ml **(Figure 8A)**. Results using this non-manipulated G18.EE sample exhibited a 33.03 ± 8.90% viability using the IC_{50}2021 and a 5.87 ± 3.52% viability using the IC_{50}2022 values **(Figure 8B)**. The tested concentrations (IC_{50}2021 and IC_{50}2022) with the non-manipulated sample exhibited cell viability lower than 50% and indicated that the non-manipulated sample is more active, and that the continuous manipulation of G18.EE led to loss of propolis activity over time. The effect of this continued manipulation on the biological activities of propolis is not described in the literature. Raw Gerês propolis samples (Araújo *et al.*, 2022) as well as ethanol extracts of Gerês propolis stored (at –20 °C) for long periods of time are known to maintain antimicrobial and antioxidant properties (Freitas, 2015; Oliveira, 2022). Nevertheless, some authors have reported the effect of temperature and light on phenolic compounds over time. They all report that samples subjected to sun exposure and room temperature for long periods show a higher degradation of phenolic compounds and decreased antioxidant activity when compared to samples kept in cold (4/5 °C) and protected from light (Srivastava *et al.*, 2007; Ali *et al.*, 2018; Deng *et al.*, 2018).

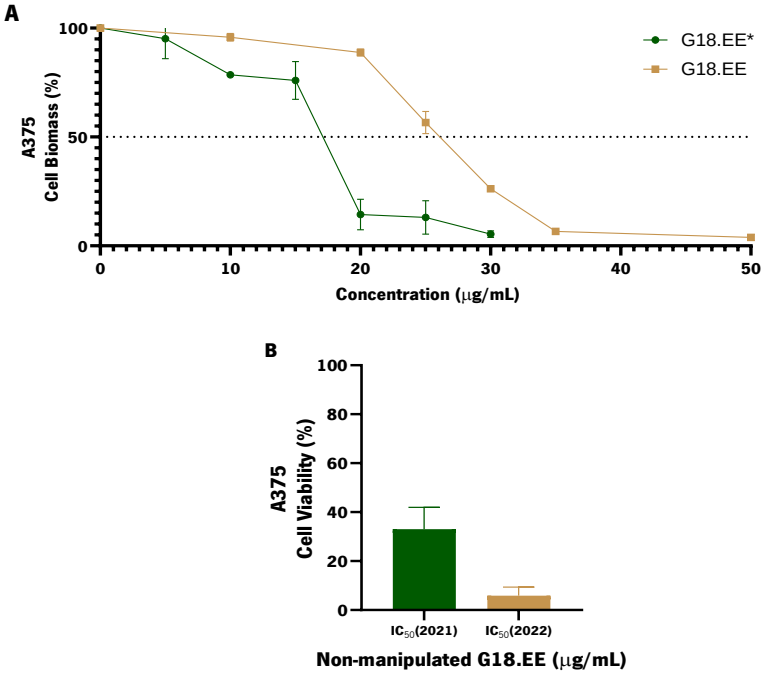

Figure 8. Manipulation effect on propolis antitumoral activity in A375 cell line. (A) Effect of G18.EE* (Oliveira, 2022) and G18.EE on total cell biomass of A375 melanoma cells. **(B)** Effect of non-manipulated G18.EE sample at two concentrations: IC_{50}2021 (16,98 µg/ml) (Oliveira, 2022) and IC_{50}2022 (26,87 µg/ml) on A375 cell biomass. Cell biomass was measured at 72 h by SRB assay after the addition of treatments. The results are presented in the form mean ± SD (n≥3). G18.EE* correspond to the effect previously described by Oliveira (2022) for G18.EE against A375 cell line.

7. Effects of G18.EE_*n*-BuOH subfractions on melanoma cell viability

Given that G21.EE presented lower cytotoxic activity when compared to the activity previously described for G18.EE and its fraction G18.EE_*n*-BuOH (Oliveira, 2022), the assays proceeded with G18.EE_*n*-BuOH. G18.EE_*n*-BuOH is a promising source of therapeutic compounds for melanoma since this fraction proved to be the most cytotoxic sample tested against *BRAF*-mutated melanoma cells (Oliveira, 2022). The subfractions to be obtained would have a less complex composition and would possibly allow the identification of putative bioactive compounds. Thus, the anti-melanoma activity of these subfractions was tested **(Figure 9)** to understand if they have activity against melanoma cell lines.

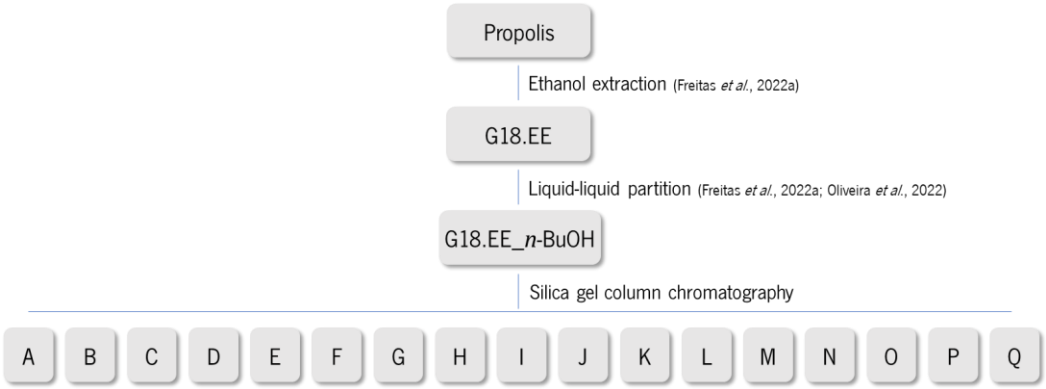

Figure 9. Methodologies used to obtain fraction G18.EE_n-BuOH and the 17 subfractions resulting from its fractionation.

As mentioned several times, propolis has a highly variable chemical composition which is a major barrier to its use in the pharmaceutical industry and traditional medicine (Bankova, 2005a). From this perspective, it is important to isolate and identify which compounds or mixtures of compounds allow G18.EE_*n*-BuOH to have this so prominent anti-melanoma activity. The cytotoxic capacity of all subfractions was tested at 25 µg/ml, concentration corresponding to an intermediate value between IC_{50} obtained for both G18.EE and G18.EE_*n*-BuOH.

These subfractions were obtained using a silica gel column chromatography and therefore, were separated based on their polarity. The most active fractions were E, G, H, I, J and K, while fractions C, D and F demonstrated a behaviour similar to the fraction they were obtained from. The remaining fractions had a less noticeable effect as they are less cytotoxic than the upstream fraction **(Figure 10)**. In the fractions most effective in reducing A375 cell biomass **(Figure 10)**, it is expected that compounds with anti-melanoma activity are present.

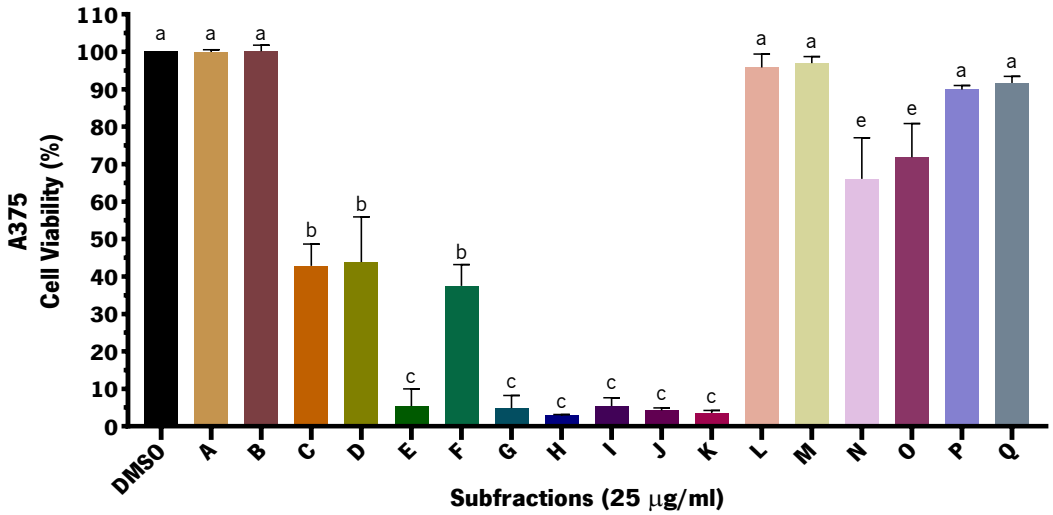

Figure 10. Effect of 25 µg/ml of the subfractions obtained from G18.EE_n-BuOH on A375 cell biomass. Cell biomass was measured at 72 h by SRB assay after treatment whit the 17 subfractions. Data was normalized to the control condition. The results are presented in the form mean ± SD (n≥3). Statistical analysis was performed by one-way ANOVA followed by Tukey test for significance. Different letters mean statistically significant differences between values.

8. Chemical composition of G18.EE_*n*-BuOH subfractions

The subfractions **E**, **G**, **H**, **I**, **J** and **K** were analysed by UPLC-DAD-ESI/MS^n as they demonstrate to be the most cytotoxic subtractions against A375 melanoma cell line. The UHPLC-DAD-ESI/MS^n chemical analysis allows the identification of compounds in mixtures, being indicated for the chemical study of phenolic compounds present in propolis (Alday *et al.*, 2016). **Figure 11** demonstrates the chromatographic profiles at 280 nm for G18.EE_*n*-BuOH and subfractions **E**, **I** and **K**, and the phenolic composition is detailed in **Table 14**. Compounds were identified based on a literature comparison of the data (Oliveira *et al.*, 2022). Subfractions G, H and I showed a chromatogram with very low signals and therefore identification of phenolic compounds was not possible and therefore the assay should be repeated for these subfractions.

The compounds found agree with what is described for other Gerês propolis samples (Freitas *et al.*, 2015; Freitas *et al.*, 2019; Gonçalves, 2017; Oliveira *et al.*, 2022). Moreover, some of the compounds identified here are present in other European propolis samples (Falcão *et al.*, 2010). Indeed, the phenolic compound identified in G18.EE_*n*-BuOH include phenolic acids like ellagic acid and flavonoids such as kaempferol and galagin. Pinobanksin, pinocembrin, chrysin and acacetin were identified too, belonging to the class of flavanones and dihydroflavonols. Flavones were also found, such as apigenin.

Despite the similar anti-melanoma activity found in these subfractions **(Figure 10)**, the chemical composition of the subfractions showed differences. Subfraction **E** exhibited pinocembrin, *p*-coumaric acid isoprenyl ester, pinobanksin-3-O-acetate, pinobanksin-3-O-butyrate, pinobanksin-3-O-propionate and pinobanksin-3-O-pentenoate in its composition. Subfraction **I** has no detectable phenolic compounds in common with **E** subfraction, presenting pinobanksin, acacetin, caffeic acid isoprenyl ester, kaempferide and CAPE. Subfraction **K** presents also acacetin, caffeic acid isoprenyl ester and CAPE but unlike the other sub-fractions it shows apigenin, kaempferol and quercetin-dimethyl-ether.

Some of these compounds, such as pinobanksin 3-O-acetate and pinocembrin present in subfraction **E** were also identified in a sample of Chinese propolis that proved to contribute to a decreased melanoma progression (Zheng *et al.*, 2018b). Besides these compounds, CAPE is a phenolic acid present in propolis of various geographical origins and is associated with significantly chemoprotective and anticancer properties (Castaldo and Capasso, 2002; Bankova, 2005a; Slavov *et al.*, 2013; Huang *et al.*, 2014) including against melanoma cells (Pramanik *et al.*, 2013). Apigenin found in the fraction **K** is a flavonoid with documented anticancer activity. This property is associated to multiple biological processes, including induction of cell cycle arrest, activation of cell apoptosis and autophagy, inhibition of cell migration and invasion, and initiating an immunological response (Yan *et al.*, 2017; Imran *et al.*, 2020). Acacetin identified in subfraction **I** and **K** exhibited anticancer effects in diverse types of cancer such as breast cancer, lung cancer, prostate cancer and colon cancer (Singh *et al.*, 2020). Also, kaempferol present in subfraction **K** proved to have anticancer properties associated with mechanism that induce cancer cells apoptosis (Imran *et al.*, 2019).

Regarding the cytotoxic effects of these subfractions **(Figure 10)**, these compounds are potential targets for the development of new drugs against cancer and the investigation of possible synergism between these compounds should be further investigated. However, in this study it was not possible to identify all phenolic compounds, with some peak values not found in the literature. In addition, it is known that there are other classes of compounds present in propolis with associated bioactivities such as terpenes (Aminimoghadamfarouj and Nematollahi, 2017) that cannot be detected through this method. Thus, this class of compounds should also be identified in subfractions as they may also be a source of compounds with therapeutic properties.

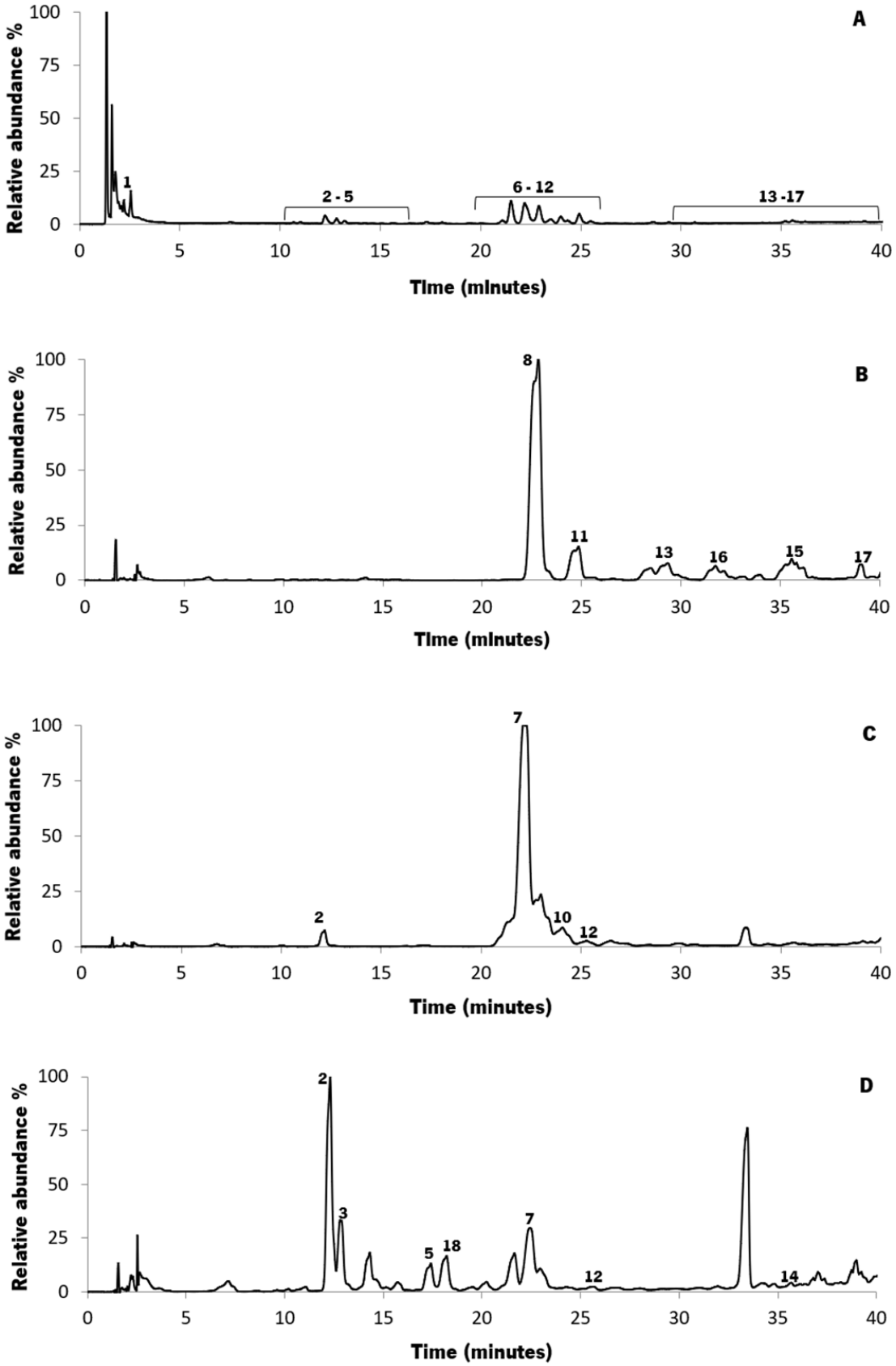

Figure 11. Chromatographic profile of (A) G18.EE_*n*-BuOH; (B) Subfraction E; (C) Subfraction I and (D) Subfraction K obtained by UHPLC-DAD-ESI/MS^n (280nm). Each peak in the figure represents a different compound identified in **Table 14**.

Table 14. Chemical composition of G18.EE_*n*-BuOH and subfractions E, I and K according to UHPLC-DAD-ESI-MS^n analysis.

Peaks	T_R (min)	λmax (nm)	$[M - H]^-$ m/z	Probable compounds	Samples			
					G18.EE_ *n*-BuOH	E	I	K
1	2.5	253	301	Ellagic Acid	+	-	-	-
2	12.2	267, 291, 334	269	Apigenin	+	-	-	+
			271	Pinobanksin	+	-	+	-
3	12.8	265, 364	285	Kaempferol	+	-	-	+
4	13.2	255, 368	315	Isorhamnetin	+	-	-	-
5	17.3	310	313	Unknown	+	-	-	+
6	21.5	268	253	Chrysin	+	-	-	-
			247	Caffeic acid isoprenyl ester	+	-	-	-
7	22.2	268, 330	283	Acacetin	+	-	+	+
			247	Caffeic acid isoprenyl ester	+	-	+	+
8	23.0	289	255	Pinocembrin	+	+	-	-
9	23.5	263, 291	269	Galagin	+	-	-	-
10	24.0	263, 364	299	Kaempferide	+	-	+	-
11	24.9	293	313	Pinobanksin-3-O-acetate	+	+	-	-
12	25.5	325	283	Caffeic acid phenylethyl ester (CAPE)	+	-	+	+
13	29.3	308	231	*p*-Coumaric acid isoprenyl ester (isomer)	+	+	-	-
14	35.5	290	417	Methylated Pinobanksin-3-O-phenylpropionate	+	-	-	-
15	36.2	291	341	Pinobanksin-3-O-butyrate or isobutyrate	+	+	-	+
16	31.7	293	327	Pinobanksin-3-O-propionate	-	+	-	-
17	39.3	292	355	Pinobanksin-3-O-pentenoate or 2-methylbutyrate	-	+	-	-
18	18.1	354, 367	329	Quercetin-dimethyl-ether	-	-	-	+

"+" = compound detected; "-" = compound not detected.

CHAPTER IV: CONCLUSIONS & FUTURE PERSPECTIVES

The use of natural products in several areas such as the food, agricultural, cosmetics, or pharmaceuticals industry is more and more common. This type of products, besides having medicinal properties and, as a rule, low toxicity levels for human beings, are also a sustainable and less aggressive option for the environment. Among the natural products from beehive, propolis is one of the best known. The use of propolis is known for centuries. Propolis research has increased over the years and several biological properties have been attributed to it, making it a valuable product. In Portugal, the rate of exploration and commercialization of this bee product is still very low but recent reports have confirmed that Portuguese propolis is a product with several biological properties, being the antibacterial and antioxidant actions the most documented. In this context, our research group has been working with Portuguese propolis, including the propolis from Gerês (G), which was used in this dissertation, namely a sample harvested in 2021 (G21).

The ash, water, waxes and balsam contents of raw propolis G21 were all within the limits of quality requirements that allow its application and commercialization. Also, the contents of polyphenols, flavonoids and *ortho*-diphenols of the ethanol extract of G21 (G21.EE) confirmed its richness in phenolic compounds, and predicted its high quality, since it is to this class of compounds that the bioactivities described in propolis have been ascribed.

A composition rich in phenolic compounds is associated with high antioxidant activity. The antioxidant activity of G21.EE was evaluated *in vitro* by determining its capacity to scavenge free radicals (DPPH• and ABTS•). Both assays revealed that this propolis sample is a powerful antioxidant agent, presenting a low EC_{50} value. However, other methods, like FRAP or cupric reducing antioxidant capacity (CUPRAC) would be interesting complementary approaches to further confirm this property. Gerês propolis has presented over the years a constant antioxidant activity, therefore it can be applied in the food industry (Gonçalves, 2017) or even in the textile industry since textiles functionalized with Gerês extracts showed antioxidant activity resistance to washes (Cardoso, 2021).

The ability to inhibit microbial growth is perhaps the most widely recognized propolis bioactivity. Antimicrobial properties of this extract are similar to the previously described in literature for most propolis samples, being more effective against Gram-positive bacteria more specifically against species of the genus *Bacillus* and having no effect on the only Gram-negative bacteria tested, *E.coli*. When compared with other extracts of propolis from Gerês, G21.EE presents higher anti-yeast activity, being important to assess its antifungal activity, namely filamentous fungi, with the perspective of applicability in agriculture. In the future, it would be interesting to analyse the chemical composition of G21.EE and compare it with

other Gerês propolis extracts that have already been analysed (Freitas *et al.*, 2019; Oliveira *et al.*, 2022), to identify compounds with possible anti-yeast activity.

The last bioactivity that was evaluated in this work was anticancer activity. G21.EE was effective in decreasing cell biomass of two melanoma cell lines (A375 and SK-MEL-23) in a dose-dependent manner. This is the first time that this propolis activity has been described for the SK-MEL-23 cell line. As a future work, it would be necessary to test these samples on normal melanocytes in order to understand their selectivity towards cancer cells. The cytotoxic effect against A375 melanoma cell line found for G21.EE is considerably lower than that described for G18.EE (Oliveira, 2022), therefore the anti-melanoma activity is not similar for two extracts of propolis from Gerês collected in different years.

In the development of this work, it was found that G18.EE lost some of its cytotoxic activity over time due to high sample manipulation. The loss of propolis bioactivities due manipulation (freezing and defrosting) is something never described before. In the future, it is especially important to evaluated and defined the best conditions for handling and storage of propolis in order to prevent its loss of properties. It would also be essential to evaluate if these two samples (unmanipulated and continuously manipulated) would also have different behaviours for other biological properties, such as antioxidant or antimicrobial capacity.

Although the anti-melanoma effect of the G18.EE_*n*-BuOH did not stand out from the extracts, G18.EE_*n*-BuOH was fractionated based on the promising previous results (Oliveira, 2022). Of the 17 subfractions (A to Q) obtained from silica gel chromatography - E, G, H, I, J and K - showed the highest activity against the *BRAF*-mutated melanoma cell line, A375. This effect should be tested in another *BRAF*-mutated cell line, like WM9 to see if the activity is maintained and it would also be interesting to test the effect of these subfractions in a non-*BRAF*-mutated melanoma cell line, such as SK-MEL-23.

In an attempt to understand which phenolic compounds could account for anti-melanoma activity, the most active subfractions were analysed by UHPLC-DAD-ESI-MS. Some of the compounds identified have already been linked with antitumour activity and deserve more research regarding the development of new drugs against cancer. Propolis has an extremely variable chemical composition, hence the use of isolated compounds, which ensures standardisation, would facilitate its use in traditional medicine. Other chemical analysis like GC-MS (Gas chromatography–mass spectrometry) would be advisable to identify compounds of other classes as terpenes.

The Portuguese propolis is a very promising product for application in several areas. In this study, more health-related properties were highlighted and Gerês propolis emerges as an alternative to conventional therapies that are not always effective and, therefore, deserve greater attention.

CHAPTER V: REFERENCES

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