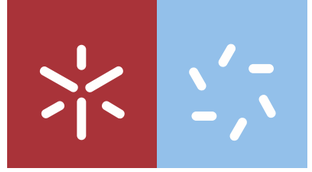
Rafaela Oliveira

Assessing the *in vitro* Antimicrobial and Antitumor Activity of Portuguese Propolis

UMinho | 2022



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Dissertação de Mestrado
Mestrado em Genética Molecular

Trabalho efetuado sob a orientação de
Professora Doutora Cristina Alexandra Almeida Aguiar
e
Professora Doutora Maria de Fátima Monginho Baltazar

junho de 2022

Despacho RT - 31 /2019 - Anexo 3

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ACKNOWLEDGMENTS

Assim terminam os anos mais desafiantes da minha vida. E, como tal, só me resta agradecer a toda a gente que tornou possível a concretização de uma das metas da minha vida.

A realização desta tese contou com apoios e incentivos bastante importantes que permitiram a sua finalização. Primeiramente gostaria de agradecer à Escola de Ciências e ao Instituto de Ciências da Vida e da Saúde por tão bem me acolherem no âmbito da realização deste projeto.

A todas as pessoas que fazem parte do grupo do laboratório do ICVS e do DB, gostaria de agradecer por toda a simpatia, entreajuda e boa disposição. À Sónia deixo um enorme agradecimento por todo o apoio prestado e por toda a ajuda. Foste sem dúvida o meu "anjo da guarda" desta jornada. Estiveste presente em todos os momentos e mesmos naqueles que nos faziam duvidar tu nunca me abandonaste, nem desististe. Remamos um barco juntas e espero que esta miniequipa se mantenha por muito tempo. És linda! À joana, sim também tiveste um lugarzinho aqui para ti, agradeço todas as horas divertidas (humor negro é o teu forte), toda a ajuda com o metabolismo e, espero manter esta ligação (criada em momentos tardios na cultura e nos ovinhos).

À minha família, pelo total apoio e presença constante. Nunca me deixaram desistir, nem quebrar. Ao Diogo, agradeço-te toda a paciência com que aturaste o meu mau humor, todos os fins de semana que esperaste dentro do carro enquanto eu trabalhava, mas principalmente, agradeço-te por todo o amor e carinho que consegues me proporcionar. Sem dúvida mantem-te na minha vida, preciso dessa motivação. Aos meus amigos, ao "Cenas", deixo um agradecimento especial por todos estes anos de amizade e superação conjunta. Agora, e mais importante, um agradecimento especial aos meus pais e ao meu irmão, por serem um exemplo para mim, em todos os aspetos, e por serem modelos de trabalho, esforço, sacrifício e luta. Obrigada por todo o apoio que me deram nesta fase, sem dúvida são os dois pilares fundamentais na minha vida.

E, por fim, queria agradecer de forma particular às minhas orientadoras, Professora Cristina Aguiar e Professora Fátima Baltazar, pela paciência, ajuda constante, palavras de incentivo e por estarem sempre presentes e prontas. Obrigada por me tornarem numa pessoa mais culta, por me permitirem aprender mais a nível laboratorial e da investigação. O sucesso desta tese é um sucesso conjunto. Obrigada por toda a dedicação demonstrada, não podia ter tido melhor orientação. Estarei sempre grata.

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STATEMENT OF INTEGRITY

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

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

RESUMO

Determinação das Propriedades Antimicrobianas e Antitumorais *in vitro* do Própolis Português

O própolis é um produto natural resinoso produzido por abelhas melíferas. Diferentes grupos de compostos têm sido identificados no própolis e associados a diversas bioatividades. Algumas amostras de própolis Português exibem potencial antioxidante e antimicrobiano pelo que, neste trabalho, se avaliou esses atributos numa amostra de própolis obtida no Gerês em 2018. A atividade antitumoral do própolis Português só foi descrita no carcinoma renal, cancro da próstata, colorretal e mama. Até à data, nada se sabe sobre o efeito deste produto em melanoma com mutação *BRAF*, o cancro de pele mais agressivo e letal. Assim, para além da avaliação das propriedades antioxidantes e antimicrobianas do própolis do Gerês, este trabalho teve como objetivo avaliar também o seu potencial contra melanoma.

Primeiramente, as propriedades antioxidantes e antimicrobianas do G18.EE e de micro-extratos etanólicos de própolis do Gerês recolhido em 2020 (G20.miEEs) foram avaliadas, demonstrando atividade antioxidante e antimicrobiana significativas, sendo G18.EE o mais eficaz. Adicionalmente, G18 revelou cumprir os requisitos de qualidade estabelecidos para própolis. Assim, G18.EE foi selecionado e fracionado – *n*-hexano, acetato de etilo (EtOAc) e *n*-butanol (*n*-BuOH) – para uso nos ensaios antitumorais *in vitro*. Quanto à atividade antitumoral, o G18.EE e as suas frações afetam a viabilidade de células de melanoma mutadas em *BRAF*, sendo *n*-BuOH/G18.EE e *n*-BuOH/EtOAc as frações mais ativas para a linha A375 e WM9, respetivamente. O efeito de G18.EE e das frações selecionadas (*n*-BuOH e EtOAc) na proliferação, migração, crescimento, apoptose, metabolismo, produção de ROS e potencial mitocondrial foi avaliado. Conclui-se que os tratamentos diminuem a migração celular e o crescimento e a migração dos esferóides. Também se observou um aumento da produção de lactato, consistente com o aumento da expressão de LDHA e MCT-1, assim como uma acumulação de ROS em células de melanoma tratadas. Por fim, através da avaliação da expressão de proteínas pró e anti-apoptóticas, validou-se a indução da apoptose. Em suma, pela primeira vez, apresentam-se evidências de atividade antitumoral do própolis português em melanoma, sendo que este modula importantes marcadores tumorigénicos – crescimento tumoral, proliferação e migração– e promove a morte celular via apoptose mediada por ROS. Este produto natural é uma potencial fonte de compostos para o desenvolvimento de novas terapias anticancerígenas.

Palavras-chave: Atividade Antimicrobiana, Atividade Antioxidante, Atividade Antitumoral, Melanoma, Melanoma com mutação *BRAF*, Propolis Português.

ABSTRACT

Assessing the *in vitro* Antimicrobial and Antitumor Properties of Portuguese Propolis

Propolis is a natural resinous product produced by *Apis mellifera L.* (worker honeybees). Different groups of compounds have been identified in propolis and associated with different bioactivities. Portuguese propolis is a powerful antioxidant and antimicrobial agent. Thus, in this work, the last properties were evaluated in a propolis sample from Gerês collected in 2018. Regarding its antitumoral activity, it has only been verified in renal cell carcinoma, prostate, colorectal, and breast cancer. Nothing is known about the potential of this national natural product in *BRAF*-mutated melanoma, the most aggressive and lethal type of skin cancer. In light of this, besides the evaluation of the antioxidant and antimicrobial properties from Gerês propolis, this work aims to assess its potential against melanoma.

To start, antioxidant and antimicrobial properties of G18.EE and of ethanol micro-extracts harvested in 2020 in Gerês (G20.miEEs) were evaluated, demonstrating significant antioxidant and antimicrobial activities, with G18.EE showing the highest efficacy. Additionally, it was established that G18 fulfills the quality requirements established for propolis. Considering this, G18.EE was selected and fractionated - *n*-hexane, ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH) fractions - to use in the *in vitro* antitumoral assays. About the antitumoral activity, we first observed that G18.EE and its fractions affect the *BRAF*-mutated melanoma cells viability, being *n*-BuOH/G18.EE and *n*-BuOH/EtOAc the most active fractions for A375 and WM9 cells, respectively. The effect of G18.EE and selected fractions (*n*-BuOH and EtOAc) on proliferation, migration, growth, apoptosis, metabolism, ROS production, and mitochondrial potential was assessed. It was concluded that propolis treatments decrease cell migration and the growth and migration of tumor spheroids. Additionally, we observed an increase in lactate production, which is consistent with the increased expression of LDHA and MCT-1, as well as an accumulation of ROS in melanoma-treated cells. Lastly, the upregulation of pro-apoptotic and the downregulation of anti-apoptotic proteins show a stimulation of apoptosis. To summarize, for the first time, we provided evidence that Portuguese propolis has anticancer activity against melanoma, since it modulates important hallmarks that dictate tumorigeneses, namely cell proliferation, cell migration, and tumor growth; and promotes cancer cell death via ROS-mediated apoptosis. This natural product is a potential source of compounds for the development of new anticancer therapies.

Keywords: Antimicrobial activity, Antioxidant activity, Antitumoral Activity, *BRAF*-mutated melanoma, Portuguese Propolis.

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LIST OF ABBREVIATIONS, SYMBOLS AND ACRONYMS

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

AJCC – American Joint Committee of Cancer

AMPK – AMP-activated protein kinase

AZA – Azacitidine

Bax – Bcl-2 associated X protein

BCC – Basal Cell Carcinoma

Bcl-2 – B-cell lymphoma 2

BRAF – V-Raf murine sarcoma viral oncogene homolog B

BSA – Bovine Serum Albumine

BuOH – Butanol

CAFs – Cancer-associated fibroblasts

CAGR – Compound Annual Growth Rate

CAPE – Caffeic acid phenethyl ester

CAT – Catalase

CDKN1A – Cyclin-dependent kinase inhibitor 1A

CDKN2A – Cyclin-dependent kinase inhibitor 2A

CDK4 – Cyclin-dependent kinase 4

CM – Cutaneous Malignant Melanoma

CPE – Cytopathogenic Effect

CpG – Cytosine-Guanine dinucleotide

CSD – Cumulative Solar Damage

DNA – Deoxyribonucleic acid

DMEM – Dulbecco´s Modified Eagle´s Medium

DMSO – Dimethyl sulfoxide

DPPH – α,α- diphenyl-β-picrylhydrazyl

DTIC – Dacarbazine

ECM – Extracellular Matrix

EE – Ethanol Extract

ESI-MS – Electrospray ionization – mass spectrometry

ESI-MS^n – Electrospray ionization – tandem mass spectrometry

EtOH – Ethanol

EtOAc – Ethyl acetate

FACs – Fluorescence-activated Cell Sorting

FBS – Fetal Bovine Serum

FDA – Food and Drug Administration

FNAP – Federação Nacional dos Apicultores de Portugal

FRAP – Ferric reducing antioxidant power

G – Gerês

G18 – Gerês propolis collected in 2018

G20 – Gerês propolis collected in 2020

GA – Gallic Acid

GC-MS – Gas chromatography-mass spectrometry

G.EEs – Ethanol Extracts of Gerês

GPX – Glutathione peroxidase

HIV – Human immunodeficiency virus

HPLC – High-performance liquid chromatography

IARC – International Agency for Research on Cancer

IHC – International Honey Commission

IR – Ionizing Radiation

LDHA – Lactate dehydrogenase A

MAPK – Mitogen Activated Protein Kinase

MCT – Monocarboxylate transporter

MDS – Myelodysplastic Syndromes

MIC – Minimum inhibitory Concentration

MMP – Matrix metallopeptidase

MRSA – Methicillin-Resistant *Staphylococcus aureus*

MSSA – Methicillin-Sensitive *Staphylococcus aureus*

NaCl – Sodium chloride

NADES – Natural Deep Eutectic Solvents

NCC – Neural Crest Cells

NF-κB – Nuclear factor-κB

NLRP1– NLR Family Pyrin Domain Containing 1

NMR – Nuclear Magnetic Resonance

NRAS – Neuroblastoma RAS viral oncogene homolog

OD – Optical Density

OPO – Orange Peel Oil

OS – Overall Survival

PFS – Progression-free survival

PI3K – Phosphoinositol 3-kinase

RECIST – Response Evaluation Criteria in Solid tumors

RNA – Ribonucleic acid

RNS – Reactive Nitrogen Species

ROS – Reactive Oxygen Species

rpm – Rotations per minute

RT – Room Temperature

SARS-CoV-2 – Severe Acute Respiratory Syndrome Coronavirus 2

SB – Stratum basale

SCC – Squamous Cell Carcinoma

SC – Stratum corneum

SD – Standard Deviation

SG – Stratum granulosum

SS – Stratum spinosum

SOD – Superoxide dismutase

SRB – Sulforhodamine B assay

TCA – Trichloroacetic acid

TME – Tumor Microenvironment

TNM – Tumor, Nodes, Metastases

TP53 – Tumor protein 53

TRIPQ – Technical Regulation of Propolis Identity and Quality

USA – United States of America

USSR – Union of Soviet Socialist Republics

UV – Ultraviolet

WHO – World Health Organization

WLE – Wide Local Excision

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Chapter 1 - Introduction

1.1. Propolis: A Promising Natural Product

Over the years, the interest in plants and plant-based products has increased. Natural products provide a wide range of biomolecules, which is one of the key reasons for the growing interest in this research area. However, this excitement about natural products is far from being recent. In fact, plant extracts and plant-based products have been used in Chinese medicine since ancient times. In 1979, the United States presented the largest retail market for medical plants and drugs containing at least one plant-derived active compound (Phillipson, 1994). Currently, more than 50% of Food and Drug Administration (FDA)-approved drugs are natural products or derivates (Gu *et al.*, 2013; Newman & Cragg, 2016). Therefore, natural products and their compounds are potential sources of new drugs with a high therapeutic value in medicine (Silva-Carvalho, 2013).

Honeybees are ancient flying insects that exist for more than 125 million years. They are perennial species that can explore virtually every ecosystem on the planet. As a result of their evolutionary process, these animals can produce specific and unique products, such as honey, propolis, royal jelly, beeswax, and bee venom (Bankova, 2005b). The scientific community has recently shown a particular interest in animal-modified plant products, particularly one generated by bees – propolis – which has been mostly neglected and wasted by several countries in the past (Sforcin & Bankova, 2011; Silva-Carvalho *et al.*, 2014).

Propolis, or "bee glue", is a brownish resinous material produced by worker honeybees (mainly *Apis mellifera L.)* from resins collected from leaf buds, branches, flowers, and exudates of plants (Ghisalberti, 1979; Silva-Carvalho *et al.*, 2014). In Central Europe, bees collect secretions from buds of poplar (*Populus* spp.) and alder (*Alnus* spp.) trees. Different poplar species are also a source of resin in other European countries, such as Bulgaria, Hungary, Albania, and England. Birch (*Betula* spp.) is the main source of propolis in Northern Europe (Przybyłek & Karpiński, 2019). Bees also produce propolis from beech (*Fagus sylvatica*), horse chestnut tree (*Aesculus hippocastanum*), eucalyptus (*Eucalyptus* spp.), Brazilian rosemary (*Baccharis dracunculifolia*), Brazilian pine (*Araucaria angustifolia*), willow (*Salix* spp.) and ash (*Fraxinus* spp.) (Oliveira, 2015; Silva-Carvalho *et al.*, 2015; Cruz *et al.*, 2016). This "botanical fingerprint" is obtained through the analysis of pollen and plant parts in propolis samples (Salatino *et al.*, 2005). The collected resin is mixed with beeswax, pollen, and salivary bee enzymes, like β-glucosidase, which causes hydrolysis of the glucosyl flavonoids and originates aglycone flavonoids (Ghisalberti, 1979; Moreira *et al.*, 2008; Sforcin, 2016). Studies using Africanized bees in Brazil showed that bees prefer certain

plant sources, in this case Brazilian rosemary, for resin collection, but the factors that drive such preference are still unknown (Menezes, 2005; Teixeira *et al.*, 2005).

Propolis is considered a lipophilic material, whose physical characteristics change with temperature, being hard and brittle when cold, but very sticky and flexible at warmer temperatures (Hausen *et al.*, 1987; Fokt *et al.*, 2010; Silva-Carvalho *et al.*, 2015). It has a very pleasant and characteristic smell, and its color varies from yellowish-green to red and too dark brown, depending on propolis source and age (Ghisalberti, 1979; Marcucci, 1995; Fokt *et al.*, 2010; Silva-Carvalho *et al.*, 2014). Propolis interacts strongly with skin oils and proteins, making its removal from human skin quite difficult (Ghisalberti, 1979).

Etymologically, propolis comes from the Greek words "pro" and "polis" meaning respectively "in defense" and "city" (Ghisalberti, 1979) revealing its function in hive protection. Propolis is used to cover carcasses of intruders who die inside the hive and are too heavy to be thrown off, avoiding their decomposition and the putrefaction-related plagues (Moreira *et al.*, 2008), and to protect the colony from diseases caused by pathogenic microorganisms, due to its antiseptic and antimicrobial properties (Sforcin, 2016). That is why this natural product is also considered the most important "chemical weapon" of bees (Bankova, 2005a). Besides this protective function, propolis is also a building material: bees apply it to seal the walls, strengthen the borders of combs, and repair structural damage, such as eventual cracks or apertures, leading to thermal isolation and, consequently, to the maintenance of hive inner temperature around 35 °C (Bankova, 2005a; Salatino *et al.*, 2005; Moreira *et al.*, 2008; Silva-Carvalho *et al.*, 2015).

1.2. The Use of Propolis: From Ancient to Modern Times

After honeybee domestication, men began to explore the use of natural products for their own benefit. Propolis is a very popular natural remedy that has been extensively used since ancient times, at least since 300 BC (Ghisalberti, 1979; Castaldo & Capasso, 2002; Silva-Carvalho *et al.*, 2015). Indeed, it was used in folk medicine and in other activities, like food preservation (Silva *et al.*, 2012), in many parts of the world. Egyptians knew about the anti-putrefactive properties and embalming capacity of propolis, using it in the mummification of corpses. The medicinal properties of propolis were recognized by Greek and Roman physicians too, such as Hippocrates and Pliny, respectively. They described this natural product as an antiseptic, anti-inflammatory (Moreira *et al.*, 2008), cicatrizing (internal and external), and a disinfectant agent. The Persians discovered that propolis could be used as a medicine to treat eczemas, myalgia, and rheumatism, and the

Incas, the population of the new world, described propolis as an antipyretic agent (Castaldo & Capasso, 2002; Silva-Carvalho *et al.*, 2015). Summing up, the use of propolis was mainly associated with its pharmacological properties (Kujumgiev *et al.*, 1999) Between the 17th and 20th centuries, propolis popularity increased in Europe due to its antibacterial activity. It was used for the treatment of health problems in very important situations and by organizations that marked this period of History. The former Union of Soviet Socialist Republics (USSR) applied this resinous material in human and veterinary medicine, and during World War II (1939-1945) and the Boer war (1899-1902) propolis was used in the healing of wounds and tissue regeneration due to its antiseptic activity (Burdock, 1998; Silva-Carvalho *et al.*, 2015; Sforcin, 2016) The London pharmacopeias of the 17th century listed propolis as an official drug for the first time (Castaldo & Capasso, 2002).

Propolis popularity remained over the years, although it is not considered a therapeutic agent in conventional medicine. Widely used in pharmaceutical, cosmetic, and food products, it is available in different forms for ingestion or topical use, such as capsules (either in pure form or combined with aloe gel and rosa canina or pollen), extracts (hydroalcoholic or glycolic), mouthwash solutions (combined with melissa, sage, mallow and/or rosemary), throat lozenges, creams, powders, dentifrices, gels, cough syrups, wine, cakes, soaps, chewing gums and tablets (Fokt *et al.*, 2010; Anjum *et al.*, 2019). Besides its important role in alternative medicines, propolis is a great varnish and restorer for musical instruments (Burdock, 1998; Silva-Carvalho *et al.*, 2015)

The growing interest in propolis and its related products has culminated in an increase and intensification of pharmacological and chemical studies in the last 30 years (Marcucci, 1995; Burdock, 1998; Bankova, 2005b; Silva-Carvalho *et al.*, 2015; Anjum *et al.*, 2019) So, other activities of this natural product were recognized, as described below.

1.2.1. Global Propolis Market – Growth and Forecasts

The global propolis market was evaluated at USD 607.10 million in 2020. Currently, European Union, Swiss, Japan, and the USA stand out in this market **(Figure 1)** (GPP, 2019). Japan, owning more than 50% of propolis commercial licenses in 2000, is the largest importer of this natural product, with a preference for the Brazilian one. Contrariwise, Brazil is one of the main regions of propolis production and consumption (Salatino *et al.*, 2005; Fokt *et al.*, 2010). Between 2011 to 2016, China became the largest consumer region, with an average consumption growth rate of 11.52% (Mordor Intelligence, 2021). In 2021, Covid-19 lockdowns became a problem for beekeepers and bees. Non-essential vehicles were forced to stop due to the pandemics and,

consequently, movement of bee boxes, bee pollination, and propolis production were negatively affected (Mordor Intelligence, 2021). Europe tends to use more propolis-containing products than the USA (GPP, 2016). This rise in the use of propolis in Europe can be attributed to a growing understanding of the health benefits of this natural product. According to the national association of beekeepers, FNAP (2019), propolis output can achieve 500 g/hive/year, yet a very low rate (GPP, 2019). As a result, there is a deficit of bee products other than honey, and this industry is significantly dependent on imports (Fonte *et al.*, 2017).

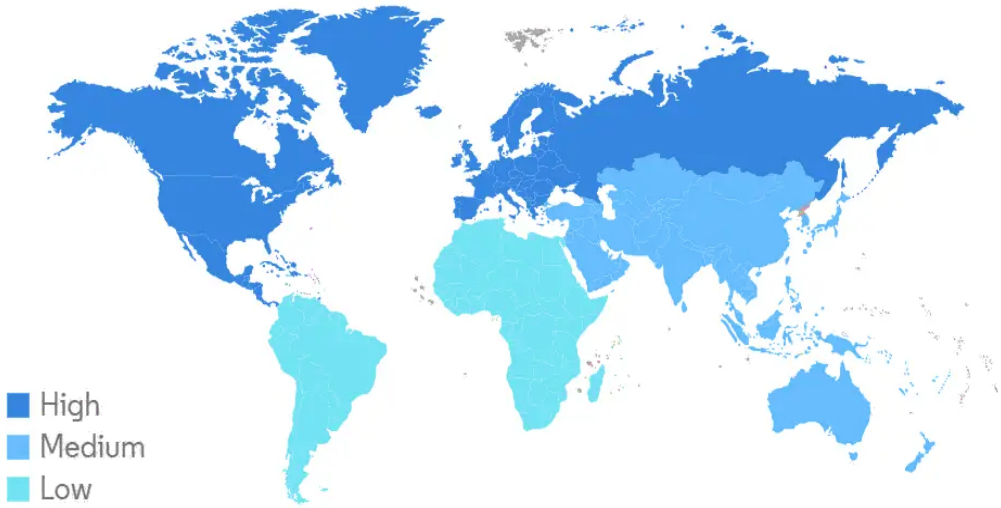

Figure 1 - Propolis Market – Global Market Size by Region, 2020. Colorful representation of the size of the worldwide propolis markets: high ( ), medium ( ) and low ( ) (from Mordor Intelligence, (2021)).

The main challenge of the propolis global market is the lack of standardization among the available brands. The percentage of propolis present in the product label neither is always available nor informs the consumer about propolis quality. The lack of legislation and information transparency can restrict market growth as well as propolis acceptance. The global propolis market is predicted to increase at a compound annual growth rate (CAGR) of 5.48% during the next five years (2021 – 2026), a slightly lower projection than the one made in 2019 (CAGR of 5.63%), which covered the years from 2019 to 2024 (Mordor Intelligence, 2021).

1.3. Origin and Composition of Propolis

Over the years, there was an evolution in the knowledge of propolis, resulting from an intensification of studies on its chemical composition. Propolis is derived from different botanical sources and its chemical composition is very complex (Salatino *et al.*, 2005; Assumpção *et al.*, 2020). The countless number of samples analyzed from different geographic origins showed that the chemical composition of raw propolis is highly variable and difficult to standardize because it depends on macro and micro-geographical factors, such as flora, season, and environmental conditions of the harvesting site. It also depends on the producing bee´ species and on the extraction conditions, such as the applied technique and the chosen solvent (Marcucci, 1995; Silva-Carvalho *et al.*, 2015; Cruz *et al.*, 2016). Propolis is composed of more than 180 different types of chemicals (Kuropatnicki *et al.*, 2013). Marcucci (1995) and Bankova *et al.* (2000) described more than 300 compounds in propolis but at least 500 new compounds have been identified in propolis (Huang *et al.*, 2014). This number is growing every year as new compounds are constantly being discovered (Marcucci, 1995; Bankova *et al.*, 2000; Huang *et al.*, 2014; Šturm & Ulrih, 2020). Compounds never described before, such as polyisoprenylated benzophenones in Cuban propolis and diterpenes in propolis of European origin, were also identified (Falcão *et al.*, 2010; Silva-Carvalho *et al.*, 2014; Anjum *et al.*, 2019).

In general, propolis is composed of resin (50%), wax (30%), essential oils (10%), pollen (5%), and other substances (5%) which include some mineral and organic compounds **(Figure 2)**. These percentages depend on the time of collection and also on the geographical origin (Silva-Carvalho *et al.*, 2015).

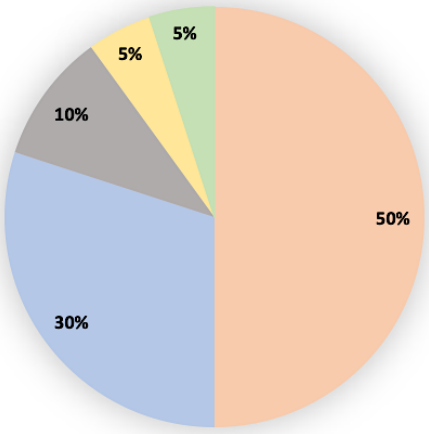

Figure 2 - General composition of propolis. In general, propolis is composed by resin (50%) (), wax (30%) (), essential oils (10%) (), pollen (5%) () and other substances (5%) ().

Organic compounds identified in different propolis samples belong to very different chemical groups, such as phenolic acids (cinnamic and caffeic acid) or their esters; flavonoids (flavones, flavanones, flavonols, and dihydroflavonols chalcones); carbohydrates; aldehydes; amino acids; terpenes; ketones; sesquiterpenes; naphthalene; aromatic aldehydes and alcohols; fatty acids; stilbenes; β-steroids; aliphatic hydrocarbons; chalcones and dihydrochalcones; nicotinic acid; pantothenic acid and vitamins (B_1, B_2, B_3, B_4, B_5, B_6, C, and E) (Silva-Carvalho *et al.*, 2015). Flavonoids are the most abundant compounds. Pollen grains are a very rich source of essential mineral elements (at least 35), such as magnesium, iron, calcium, nickel, and zinc (Dobrowolski *et al.*, 1991; Bankova, 2005a; Barlak *et al.*, 2011; Silva-Carvalho *et al.*, 2015; Anjum *et al.*, 2019). Some groups of substances like glycosides were discovered quite late; other groups such as alkaloids and tannins were found even more recently (Šturm & Ulrih, 2020). The identification of fundamental and specific chemical components ensures the uniqueness of each propolis sample (Ghisalberti, 1979; Falcão *et al.*, 2010; Silva-Carvalho *et al.*, 2015).

1.3.1. Classification and Standardization of Propolis

Propolis can be classified in two different ways. One is typifying the resin according to its "gatherers". In this case, two main types are known: the "normal" propolis which is collected by honeybees, and the "geopropolis" collected by stingless bees (Ghisalberti, 1979; Šturm & Ulrih, 2020). The main difference between honey and stingless bees is that the first are generalist pollinators while stingless bees are specialists that only visit particular flowers (Rao *et al.*, 2016). Another way of classifying propolis is according to the plant source. This is the most widely used system of classification. Certain main types of propolis are now recognized in this context (Silva-Carvalho *et al.*, 2015), such as poplar propolis (Bankova *et al.*, 2000), the most popular and widespread type (Europe, North America, non-tropical regions of Asia); the unique *Baccharis* or Brazilian green propolis (Pereira *et al.*, 2003); *Clusia* or Brazilian red propolis, also found in Cuba, Venezuela and Chile (Alencar *et al.*, 2007); eucalyptus propolis (Abu-Mellal *et al.*, 2012); *Macaranga* or Taiwanese green propolis (Huang *et al.*, 2007); birch propolis (König, 1985); Mediterranean propolis (Popova *et al.*, 2010), a new type of European propolis; "Pacific" propolis; "Canarian" propolis (Silva-Carvalho *et al.*, 2015) and Australian propolis (Ghisalberti, 1979). Still, this system of classification is being updated as more propolis of different plant origins are being discovered and characterized. Poplar and Brazil green propolis are the most commercially available and widely studied of all propolis types, because of their strong pharmacological activity (Šturm & Ulrih, 2020).

Poplar propolis is composed of phenols, phenolic acids, and their esters, clearly different from the ones present in other propolis types **(Table 1)** (Bankova *et al.*, 2000; Silva-Carvalho, 2013; Šturm & Ulrih, 2020). On average, phenols represent 28 ± 9% of the whole poplar propolis mass, of which 8 ± 4% are flavones/flavonols and 6 ± 2% are flavanones/dihydroflavonols (Šturm & Ulrih, 2020). Mediterranean propolis is characterized by a high concentration of diterpenoids and can be found, for example, in Greece, Sicily, and Malta (Popova *et al.*, 2010). Propolis from tropical areas, like Brazil, Cuba, Venezuela, and Chile, has prenylated phenylpropanoids and caffeoylquinic acids as main constituents (Silva-Carvalho *et al.*, 2015), but can also have flavonoids kaempferide and isosakuranetin, diterpenes, lignans, and acetophenone. In addition, red propolis presents high concentrations of phenolic acids and of some flavonoids, such as formononetin, isoliquiritigenin, medicarpin, and biochanin A (Silva-Carvalho *et al.*, 2015; Anjum *et al.*, 2019). Birch propolis, collected from Russia, has flavonols and flavones of *Betula verrucosa* (different from poplar type) (Anjum *et al.*, 2019). In the last years, Cuban propolis (*Clusia* type) has sparked interest in the scientific community because of its peculiar enrichment in polyisoprenylated benzophenones, a composition that differs significantly from the European and Brazilian bee glues (Falcão *et al.*, 2010).

Table 1 – Classification and characterization of some recognized types of propolis. Classification parameters include geographical origin, botanical source, and main bioactive compounds (adapted from Silva-Carvalho *et al.* (2015)).

	Geographic Origin	Plant Source	Main bioactive compounds
Poplar Propolis	Europe, North America, non-tropical regions of Asia and New Zealand	*Populus* spp. (*P. nigra*)	Pinocembrin, pinobanksin, chrysin, galangin, caffeic acid, ferulic acid, cinnamic acid and their esters
Green Propolis	Brazil	*Baccharis* spp. (predominately *B. dracunculifolia*)	Prenylated phenylpropanoids, prenylated *p*-coumaric acids, acetophenones, diterpenic acids, caffeoyl quinic acids, kaempferide and isosakuranetin
Red Propolis	Cuba, Venezuela	*Clusia* spp. (mainly *C. rosea* e *C. minor*)	Polyisoprenylated benzophenones, particularly nemoronose; xanthochymol and guttiferone E
"Pacific" Propolis	Havai, Taiwan, Okinawa	*Macaranga tanarius*	C-prenylflavonones, alk(en)ylresorcinols, cycloartane-type triterpenes, and 27-hydroxyisomangiferolic acid
"Canarian" Propolis	Canary Islands	Unknown	Furofuran lignans
Birch Propolis	Russia	*Betula* spp. (*B. verrucosa*, *B. pendula* e *B. pubescens*)	Cinnamic acids, sesquiterpenoids, phenylpropanoids, acacetin, apigenin, ermanin, rhamnocitrin, kampferide, α-acetoxybetulenol
Mediterranean Propolis	Greece, Sicily, and Malta	Unknown	Main compound: diterpenoids (Isocupressic, pimaric, commonic and imbricatoloic acid)

1.3.2. Propolis Phenolic Characterization: Current Approaches

Propolis presents a very complex structure and composition making the quantification of compounds impossible to perform directly. The analysis and characterization of this natural product demand its extraction with a suitable solvent. Water, ether, acetone, methanol, ethanol, chloroform, and dichloromethane are the most often used (Wagh, 2013; Ahangari *et al.*, 2018). The ideal solvent appears to be ethanol and/or ethanol/water mixtures (like ethanol 70%, 70:30, v/v), a non-toxic solvent apparently especially effective in obtaining dewaxed propolis extracts rich in polyphenols and flavonoids (Miguel *et al.*, 2010; Ahangari *et al.*, 2018). However, the presence of alcohol restricts the use of propolis extracts in medicine (Petkov *et al.*, 2018). So, to protect human health and consider the green economy, the investigation has been directed to the search for new natural, healthier, and green solvents for the extraction of bioactive natural compounds. Natural deep eutectic solvents (NADES) are an example of "green" approaches to propolis extraction, but the selection of NADES with appropriate polarity is required for extraction of propolis bioactive substances such as flavonoids, phenolic acids and their esters (Funari *et al.*, 2019; Trusheva *et al.*, 2019). NADES can potentially extract similar compounds as alcoholic/hydroalcoholic extracts, improving direct consumption. Another example of a green solvent with applicability in propolis extraction is the use of orange peel oil (OPO) ethanol (Keskin, 2020).

In terms of methodologies, a wide range of distinct approaches can be used to extract bioactive compounds from propolis. Maceration is a conventional and effective extractive procedure yet time-consuming (Trusheva *et al.*, 2007). Microwave-assisted (1 min at 140 W) and ultrasonic extraction (15 min at 20 kHz) are two current methods that have been developed to improve extraction efficiency and performance. The benefits of the second technique are assumed to be mostly attributable to the mechanical effects of sonic cavitation (Liu & Wang, 2004; Trusheva *et al.*, 2007; Oroian *et al.*, 2020).

Several techniques can then be used to characterize phenolic compounds in propolis extracts: spectrophotometry (Popova *et al.*, 2004), thin-layer chromatography (Medić-Šarić *et al.*, 2004), gas chromatography-mass spectrometry (GC-MS) (Sahinler & Kaftanoglu, 2005), high-performance liquid chromatography (HPLC) (Bruschi *et al.*, 2003), liquid chromatography-mass spectrometry (LC-MS) (Volpi & Bergonzini, 2006), electrospray ionization – mass spectrometry (ESI-MS) and electrospray ionization – tandem mass spectrometry (ESI-MS^n) (Sawaya *et al.*, 2004), capillary electrophoresis (Volpi, 2004) and nuclear magnetic resonance (NMR) (Watson *et al.*,

2006). MS with ESI is one of the most used to analyze natural products, as it is able to analyze complex mixtures due to its high selectivity and to provide very useful structural information (Falcão *et al.*, 2010). The chemical composition of European propolis was determined using GC-MS, LC-MS, and ESI-MS (Bankova *et al.*, 2000; Volpi, 2004; Sahinler & Kaftanoglu, 2005; Falcão *et al.*, 2010). The identification of more propolis components, including flavonoids, terpenes, phenols, esters, sugars, hydrocarbons, and some minerals results from the application of the above-mentioned methods (Ahangari *et al.*, 2018).

1.4. Propolis Quality: Challenges and Commitments

The increasing popularity of propolis prompted the establishment of quality control criteria for its commercialization. The management of propolis pollutants is important to avoid contaminations with heavy metals and pesticides, such as acaricides, which have a detrimental impact on human health and the environment. However, setting universal quality norms is difficult due to the broad variety of propolis types and chemical components (Woisky & Salatino, 1998; Bankova *et al.*, 2016). Different propolis extracts, on the other hand, show stable and distinct chemical profiles, allowing classification based on key natural ingredient groups, such as bioactive and sensorial compounds (Bankova *et al.*, 2016; Cahango, 2017).

Some approaches for quality control of propolis samples were proposed and discussed over the years. In 1980, Ivanov investigated the saponification, wax level, acidity, and iodine content of propolis samples, specifically of Bulgarian origin (Ivanov, 1980). Later, in 1992, Bankova *et al.* (1992a,b) identified and quantified the main flavonoids of propolis (Bankova *et al.*, 1992a; Bankova *et al.*, 1992b) whereas Woisky and Salatino (1998), proposed some metrics for chemical quality control of crude propolis, such as total phenolic substances, flavonoids, volatile substances, wax, ash, and dry residue free of volatile compounds (Woisky & Salatino, 1998). In 1997, the *International Honey Commission* (IHC) published a document outlining standardized procedures for determining propolis chemical quality. This document was last updated in 2009 and defines quality metrics that can be considered universal, such as ash, wax, and balsamic contents, as well as phenolic composition (IHC, 2009; Lopes, 2017). In addition to the previously mentioned criteria, other quality parameters can be evaluated, such as physical-chemical (density, fusion point, and ethanol solubility) and sensory properties (consistency, odor, taste, and color) (Funari & Ferro, 2006).

Propolis of high quality should be free of impurities, have low percentages of wax, insoluble materials, and ash and present a high content of biologically active compounds. In addition, the botanical origin should be known to define the active compounds (Bankova *et al.*, 2000). The quantification of balsam; total flavone and flavonol content; total phenolic content; total flavanone and dihydroflavonols content; and the concentration of major bioactive components are used to develop standards for different types of propolis **(Table 2)** (Teixeira *et al.*, 2005; Falcão, 2013). The concentration of bioactive compounds in poplar propolis (*Populus* sp.; European type) as well as for Portuguese propolis were defined (Popova *et al.*, 2007; Righi, 2008; Bogdanov & Bankova, 2017). However, legislation concerning such reference values is still lacking.

Table 2 – Quality criteria of propolis. Reference values were established by Technical Regulation of Propolis Identity and Quality (TRPIQ) (from the Ministry of Agriculture, Livestock, and Food Supply in Brazil) for Brazilian propolis, by Bogdanov (2011) for European propolis and by Falcão (2013) for Portuguese propolis types I and II. (Cahango, 2017).

	TRPIQ	European Propolis	Portuguese Propolis Type I	Portuguese Propolis Type II
Dry residue free of volatile substances (%)	≤ 8	-	≤ 5	≤ 5
Ash Content (%)	≤ 5	-	≤ 2	≤ 4
Beeswax Content (%)	≤ 25	≤ 25	≤ 25	≤ 31
Balsamic Content (%)	-	≥ 45	≥ 65	≥ 45
Total phenolics (%)	≥ 5	≥ 21	≥ 18	≥ 6
Total flavones and flavonols (%)	-	≥ 5	≥ 3	≥ 2
Total flavanones and dihydroflavonols (%)	-	≥ 4	≥ 5	≥ 3
Total flavonoids (%)	-	≥ 9	Non-Specified	Non-Specified

Portugal's location in a temperate zone suggests a propolis type from *Populus* species. Indeed, the most common type is poplar propolis but Portuguese propolis can also be collected from *Cistus ladanifer*, a species characteristic of the Mediterranean area (Falcão, 2013). Thus, two different types of Portuguese propolis are considered: type I (North, Central Coast, and Azores) and II (Central Interior, South, and Madeira) **(Table 2)**. Portuguese propolis type II is significantly different from European propolis, presenting a higher percentage of wax and lower phenolic compounds (and concomitantly balsamic) content. These variations make the commercial value of this kind of propolis substantially lower when compared to European propolis (Falcão *et al.*, 2013).

1.5. Propolis Bioactivities: From Biological to Therapeutic Properties

Propolis has become increasingly well-known throughout the years. However, chemical standardization is lacking, though indispensable for its acceptance in the health system (Silva-Carvalho *et al.*, 2015; Sforcin, 2016). Propolis standardization requires the characterization of a wide variety of propolis samples according to their botanical origin and matching chemical profiles. Knowing such information turns feasible to extrapolate the potential activity and mode of action of a propolis under study, which can contribute to the development of novel therapeutic candidates (Bankova, 2005b; Sforcin & Bankova, 2011).

The main chemical compounds responsible for propolis bioactivities are flavonoids, aromatic and diterpene acids and phenolic compounds (Fokt *et al.*, 2010; Silva-Carvalho *et al.*, 2014). Propolis has been extensively studied in the last years, allowing the identification of biomedical properties, such as immunostimulant (Pagliarone *et al.*, 2009); antibacterial (Velazquez *et al.*, 2007); antifungal (Kujumgiev *et al.*, 1999; Silici *et al.*, 2005); anti-inflammatory (Sforcin, 2007); antiviral (Gekker *et al.*, 2005; Schnitzler *et al.*, 2010); antioxidant (Banskota *et al.*, 2001); antitumor/anticancer (Sforcin, 2007; Valente *et al.*, 2011; Silva-Carvalho *et al.*, 2014); anesthetic (Ghisalberti, 1979; Omar *et al.*, 2017); cariostatic (Libério *et al.*, 2009); antiprotozoal (Sforcin, 2016); antihypertensive (Toreti *et al.*, 2013); anti-hepatotoxic/hepatoprotective (Banskota *et al.*, 2001); antineurodegenerative (Chen *et al.*, 2008); antituberculosis (Yildirim *et al.*, 2004); radioprotective (Suarez & Levitt, 1996); genotoxic and anti-genotoxic (Cruz *et al.*, 2016).

1.5.1. Antimicrobial Activity

Propolis is the bee product with the highest antimicrobial activity (Bogdanov, 2016). This biological activity is the most studied and best-documented bioactivity of propolis against different types of microorganisms, such as bacteria (antibacterial activity) (Sforcin *et al.*, 2000); filamentous fungi (Ghaly *et al.*, 1998), and yeast (Sforcin *et al.*, 2001) (antifungal activity); protozoa (Freitas *et al.*, 2006) and virus (antiviral activity) (Gekker *et al.*, 2005; Búfalo *et al.*, 2009). This propolis property is particularly important considering the increased microbial resistance to antibiotics and antifungals, and the consequent need for new treatments against infectious diseases. Antimicrobial action was related to the presence of flavonoids, aromatic acids, and esters **(Table 3)** (Popova *et al.*, 2004). Caffeic acid and ferulic acid have also been associated with this property (Popova *et al.*, 2004). *In vitro*, propolis acts directly on microorganisms and *in vivo* it stimulates the immune system, leading to the activation of mechanisms involved in microbial defense (Sforcin & Bankova, 2011; Silva-Carvalho *et al.*, 2014).

Different approaches can be used to determine the antibacterial/antifungal activity of propolis samples, such as diffusion in agar (using cups, steel cylinders, and paper disks), which employs the diameter of the inhibition zone, also known as the inhibition halo, as a metric for the activity (Sawaya *et al.*, 2011). The dilution method, which includes broth macro or microdilution as well as agar dilution, is another approach used for the same purpose and allows the calculation of the Minimum Inhibitory Concentration (MIC), the lowest concentration that inhibits visible bacterial growth (Fokt *et al.*, 2010; Sawaya *et al.*, 2011). The cytopathogenic effect (CPE) reduction assay is the most commonly used approach to test propolis' antiviral effectiveness, measuring the concentration of antiviral that lowers the virus-induced cytopathic effect (Kujumgiev *et al.*, 1999; Fokt *et al.*, 2010).

Table 3 - Compounds responsible for the antimicrobial activity of different propolis types (adapted from Silva-Carvalho *et al.* (2015)).

	Poplar Propolis	Green Propolis	Red Propolis	"Pacific" and "Canarian" Propolis
Antimicrobial Activity	Flavanones, flavones, phenolic acid and their esters, terpenes	Phenolic acid and their esters, flavonoids, artepillin C and prenylated phenylpropanoids	Flavonoids, polyisoprenylated benzophenones and phenolic compounds	Di- and Triterpenes lignanes, flavonoids and phenolic acids and their esters

1.5.1.1. Antibacterial Activity

Antibacterial activity, one of the most important propolis bioactivities, was the first biological activity to be recognized (Kujumgiev *et al.*, 1999) and to be demonstrated against a wide spectrum of bacteria (including Gram-negative and Gram-positive bacteria), such as *Bacillus cereus*, *Bacillus mesentericus*, *Corynebacterium* spp., *Corynebacterium diphtheriae*, *Diplococcus pneumonae*, *Enterococcus* spp. (*Enterococcus faecalis*), *Mycobacteria* sp., *Mycobacterium tuberculosis*, *Staphylococcus* spp. (*S. auricularis*, *S. capitis*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. mutans*, and *S. warneri*), *Staphylococcus aureus*, *Streptococcus* spp. (*S. cricetus*, *S. faecalis*, *S. pneumioniae*, *S. pyogenes*, *S. β-haemolyticus*, *S. mutans*, *S. sobrinus*, and *S. viridians*), *Branhamella catarrhalis*, *Escherichia coli*, *Helicobacter pylori*, *Klebsiella ozaemae*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella* spp. (Fokt *et al.*, 2010; Bogdanov, 2016).

Propolis has a strong effect against Gram-positive bacteria, such as *Enterococcus* spp. and *S. aureus* (Bogdanov, 2016), acting as a bactericidal agent, to stop cell division and protein synthesis and destroy cell wall and bacterial cytoplasm. It also inhibits bacterial mobility and enzyme activity and exhibits bacteriostatic activity against different bacterial genera (Fokt *et al.*, 2010; Anjum *et al.*, 2019). Ethanol extracts (EE) of propolis completely inhibit the growth of *S. aureus*, *Enterococcus* spp., and *B. cereus*, and partially inhibit *E. coli* (Fokt *et al.*, 2010). Sforcin *et al.* (2000) demonstrated that low concentrations of an EE inhibit the growth of *S. aureus*, whereas higher concentrations were needed to inhibit some Gram-negative bacteria such as *P. aeruginosa*, *E. coli,* and *Salmonella typhimurium*. Antibacterial activity has been related to some specific active compounds present in propolis extracts, such as aromatic acids, flavonoids, galangin, pinocembrin, pinostrobin, and pinobanksin (Grange & Davey, 1990; Fokt *et al.*, 2010; Anjum *et al.*, 2019).

Propolis has a synergistic effect with commercial antimicrobial drugs, allowing dose reduction of selected drugs and potentiating their effect (Sforcin & Bankova, 2011; Freitas *et al.*, 2022). This association with commercially disposable drugs is a field of interest in the development of new products by the pharmaceutical industry (Sforcin & Bankova, 2011). Research in this area has shown that propolis decreases bacteria cell wall resistance to antibiotics and exerts synergetic effects with antibiotics, like chloramphenicol, that act on ribosomes; ampicillin, ceftriaxone, and doxycycline, that are effective against *S. aureus* and gentamicin, that is used against severe or serious bacterial infections (Stepanović *et al.*, 2003; Freitas *et al.*, 2022). Fernandes *et al.* (2005) demonstrated a synergistic effect between propolis and antimicrobial drugs against *S. aureus*, mainly for agents that interfere with bacterial protein synthesis. A synergetic effect with bactericidal anti-tuberculosis drugs, such as streptomycin, rifamycin, and isoniazide, was also reported (Scheller *et al.*, 1999). However, propolis does not appear to interact with antibiotics that act on deoxyribonucleic acid (DNA) or folic acid synthesis, such as ciprofloxacin and cotrimoxazole (Orsi *et al.*, 2006; Sforcin & Bankova, 2011; Orsi *et al.*, 2012a; Orsi *et al.*, 2012b).

1.5.1.2. Antiviral Activity

Propolis extracts display antiviral activity against a plethora of DNA and ribonucleic acid (RNA) viruses, including Herpes simplex types 1 and 2, adenovirus type 2, poliovirus type 2, Influenza type A and B, Parainfluenza virus, Coronavirus, Newcastle disease virus (Bogdanov, 2016; Bachevski *et al.*, 2020) and, more recently, human immunodeficiency virus (HIV) (Gekker *et al.*, 2005), among others (Amoros *et al.*, 1992; Schnitzler *et al.*, 2010; Sartori *et al.*, 2012).

This bioactivity was identified and described for the first in the 1960s and is related to high flavonoid content, particularly galangin, acacetin, chrysene, kaempferol, and quercetin, as well as caffeic acid phenethyl ester (CAPE), a well-known antiviral molecule (Búfalo *et al.*, 2009; Bogdanov, 2016; Bachevski *et al.*, 2020; Ripari *et al.*, 2021). Propolis antiviral action is mediated through a complex mechanism that culminates in the inhibition of viral replication (60-99% of inhibition) (Yildirim *et al.*, 2016; Berretta *et al.*, 2020): propolis blocks viruses from entering the cell and inhibits protein synthesis and enzymatic activity, interfering with viral replication stages (Amoros *et al.*, 1992; Búfalo *et al.*, 2009; Yildirim *et al.*, 2016). It is also responsible for viral RNA or DNA degradation (Amoros *et al.*, 1992; Búfalo *et al.*, 2009).

Acyclovir, a nucleoside derivate, is the most potent antiviral drug currently used to treat some viruses, such as Herpes simplex viruses. Yildirim *et al.* (2016) reported a synergetic effect between this drug and Hatay propolis, which allowed a dose reduction and drug effect potentiation.

Recently and in the middle of the pandemic crisis, Refaat *et al.* (2021) highlighted the promising potential of Egyptian propolis liposomes as a treatment approach for Covid-19. Propolis components presented a higher affinity to COVID 3-CL protease and spike protein when compared to potent antivirals commonly used, such as favipiravir, hydroxychloroquine, and Remdesivir (Refaat *et al.*, 2021).

1.5.1.3. Antifungal Activity

The antifungal activity of propolis has been well documented in the literature. This bioactivity is particularly important in the control of several fungal diseases. The spectrum of action of propolis includes yeast belonging to the *Candida* genus, such as *Candida albicans* (Hegazi *et al.*, 2000; Trusheva *et al.*, 2006), *Candida guillermondii, Candida parapsilosis* and *Candida tropicalis* (Bogdanov, 2016); as well as other clinically significant species, like *Aspergillus* sp., *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Madurella mycetomatis*, *Microsporum audouinii, Microsporum canis*, *Microsporum cepillo*, *Microsporum distortum*, *Microsporum ferrugineum*, *Microsporum gypseum*, *Piedraia hortae*, *Phialophora jeanselmei*, *Penicillium italicum*, and *Trichosporon cutaneum* (Bogdanov, 2016). This natural bee product is also effective against *Saccharomyces cerevisiae*, which was isolated from onychomycosis (Oliveira *et al.*, 2006). Propolis also showed good results on species of the genera *Trichophyton* (*Trichophyton mentagrophytes* and *Trichophyton rubrum*) and *Fusarium* as well as other skin infecting fungi (Castro, 2006).

According to Koç *et al.* (2011), poplar propolis displayed the strongest antifungal effect against *C. albicans*, *Candida glabrata*, *Candida krusei*, and *Trichosporon* spp. (Koç *et al.*, 2011), with *C. albicans* being the most susceptible species (De Castro *et al.*, 2013). Synergistic effects with nystatin, an antifungal agent that acts against *C. albicans*, were also reported (Stepanović *et al.*, 2003).

Propolis antifungal action is mediated by complex mechanisms that are associated with genes involved in the mitochondrial electron transport chain, vacuole acidification, regulation of macroautophagy associated with protein targeting to vacuoles, and cellular response to starvation (De Castro *et al.*, 2011). According to De Castro *et al.* (2013) research, the propolis antifungal effect is also linked to its ability to induce apoptosis via metacaspase and Ras signaling.

Chemical diversity displays a significant influence on the antifungal activity of propolis. However, research has shown that key propolis components involved in this bioactivity include 3-acetylpinobanskin, pinobanksin-3-acetate, pinocembrin, *p*-coumaric acid, and caffeic acid (Anjum *et al.*, 2019). Pinocembrin shows specific activity against *P. italicum*, reducing the levels of phosphorylated adenosine nucleotides in the hyphae (Peng *et al.*, 2012; De Castro *et al.*, 2013) and destroying the hyphae and cell membrane, resulting in ionic leakage and soluble protein (Peng *et al.*, 2012).

1.5.2. Antioxidant Activity

Oxidative stress is a phenomenon generated by an imbalance between the production and accumulation of reactive oxygen species (ROS) in cells and tissues. This stress is amplified by the disruption of antioxidant defense mechanisms that include endogenous antioxidant species, such as superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) (Pizzino *et al.*, 2017). ROS - which include ozone (O_3), superoxide anion ($O_2^{\cdot}$), hydroxyl radical ($OH^{\cdot}$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) – and reactive nitrogen species (RNS) - such as nitric oxide ($NO^{\cdot}$) (Finkel & Holbrook, 2000; Silva-Carvalho *et al.*, 2015) - are generated as metabolic by-products of cell metabolism (leakage in enzymatic reaction of the mitochondrial electron transport chain, among others) or by the exposure to different exogenous agents and events, such as ultraviolet (UV) and ionizing radiation (IR); cytokines; growth factors; chemotherapeutic drugs; macrophages, during the inflammatory response; environmental toxins; heat shock/hyperthermia; and dehydration (Costa & Moradas-Ferreira, 2001; Salmon *et al.*, 2004).

Depending on their levels, ROS can be beneficial or harmful to the cells. When low to moderate amounts are present in the cell, they can be beneficial, being involved in biological

processes such as signaling and redox control (Fridovich, 1999; Freitas, 2015). In normal settings, cells remove ROS from the environment via enzymatic and non-enzymatic molecules (antioxidant defense mechanism), ensuring a balance between antioxidant defense and radical generation. When this equilibrium is compromised, an accumulation of ROS occurs, resulting in severe and damaging oxidative stress (Ferreira *et al.*, 2007; Sá *et al.*, 2013). Elevated levels of ROS are responsible for the occurrence of a wide variety of human diseases, such as cancer, neurodegenerative and cardiovascular diseases, aging processes, diabetes, and atherosclerosis. In addition, high concentrations of reactive species in breast cancer cells are a metastasis-promoting condition (Malins *et al.*, 1996; Finkel & Holbrook, 2000; Cooke *et al.*, 2003; Gonçalves, 2017).

The interest in determining the antioxidant activity of natural products has grown exponentially in the last years (Viuda-Martos *et al.*, 2008). Propolis is a rich source of natural antioxidants such as polyphenols, flavonoids, phenolic acids, and terpenoids, as well as other phytochemicals **(Table 4)** (Durazzo *et al.*, 2021), being polyphenols reported to be the main bioactives responsible for the antioxidant potential in different propolis samples. This bee product works by blocking key enzymes, which are responsible for ROS generation; by scavenging activity, interrupting lipid peroxidation; and by chelating metal ions (generally iron and cooper) (Silva-Carvalho *et al.*, 2014). The antioxidant action of propolis samples can be determined through different spectrophotometry methodologies, such as α,α- diphenyl-β-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazolin-6-sulphonate) (ABTS) or ferric reducing antioxidant power (FRAP), among others (Amorati & Valgimigli, 2015; Apak, 2019).

Table 4 - Compounds responsible for the antioxidant activity of different propolis types (adapted from Silva-Carvalho *et al.* (2015)).

	Poplar Propolis	Green Propolis	Red Propolis	"Pacific" and "Canarian" Propolis
Antioxidant Activity	Flavonoids, phenolic acids and their esters	Flavonoids, prenylated *p*-coumaric acids	Flavonoids, polyisoprenylated benzophenones	Prenylated benzophenones

Antioxidant activity is affected by propolis type, phenolic profile, apiary location, and harvesting time (year and season). Fabris *et al.* (2013) reported an association between higher phenolic contents and higher antioxidant activity in several types of propolis. This correlation was also confirmed in samples of propolis EE from Transylvania (Mihai *et al.*, 2011). Chen *et al.* (2009) described CAPE as another component with an essential antioxidant role.

1.5.3. Antitumor and Cytotoxic Activity

Natural products are a rich source of substances that can be used in cancer treatment, particularly chemotherapy. Indeed, the large range of natural substances can be a basis of innovative revolutionary approaches to diseases like cancer, whether as monotherapy or in combination with approved drugs. The anticancer molecules are mostly natural or derived from natural compounds, which lends support to cancer-propolis research (Karikas, 2010). Propolis has anticancer properties, including cytotoxicity against breast, colon, uterine cervix, lung, skin, and kidney cancer cell lines (Watanabe *et al.*, 2011; Valença *et al.*, 2013; Silva-Carvalho *et al.*, 2014), being regularly referred in scientific literature as an antitumor and immunomodulatory agent (Bankova, 2005a; Sforcin, 2007).

Propolis presents a high variety of phytochemicals that act through multiple pathways to reduce the development and other malignant characteristics of cancer cells **(Table 5)**. CAPE and artepillin C are involved in cell cycle arrest (G2/M phase), inhibition of matrix metalloproteinases, anti-angiogenesis effect, and metastases remission (Castaldo & Capasso, 2002; Chan *et al.*, 2013; Sforcin, 2016; Anjum *et al.*, 2019). CAPE, one of the most important compounds of propolis, presents antitumor and chemoprotective activities, without being cytotoxic to normal cells (Fokt *et al.*, 2010; Silva-Carvalho *et al.*, 2014). Many of its effects have been shown to be mediated through inhibition of nuclear factor-κB (NF-κB) (Silva-Carvalho *et al.*, 2014). More recently, the CAPE effect on genes related to tumor cell growth and survival was partially associated with its role as a histone deacetylase inhibitor. Another CAPE effect is the reversion of UV-mediated epigenetic modifications in human dermal fibroblasts by inhibiting the activity of several histone acetyltransferases (Chan *et al.*, 2013; Assumpção *et al.*, 2020). Other components of propolis, such as galangin, cardanol, nemorosone, and chrysin, are responsible for the prevention of rapid division of tumor cells (Banskota *et al.*, 2001; Sforcin, 2016).

Table 5 - Compounds responsible for the antitumoral activity of different propolis types (adapted from Silva-Carvalho *et al.* (2015)).

	Poplar Propolis	Green Propolis	Red Propolis	"Pacific" and "Canarian" Propolis
Antitumoral Activity	Flavonoids, phenolic acids, and their esters, like caffeic acid phenethyl ester	Flavones, flavanones, phenolic acids and their esters (*p*-coumaric and cinnamic acid), cinnamic acid derivates (artepillin C, drupanin, baccharin)	Prenylated benzophenones	Prenylflavanones

Propolis can block specific oncogenic signaling pathways, which in turn leads to a decrease in cell proliferation and growth. This bee product can also act by decreasing the cancer stem cell population, increasing apoptosis, exerting antiangiogenic effects, and modulating the tumor microenvironment, suppressing the invasion and migration (Sawicka *et al.*, 2012; Chan *et al.*, 2013; Silva-Carvalho *et al.*, 2014). Furthermore, propolis or its isolated compounds can modulate the expression of cancer-related genes, such as tumor protein 53 (*TP53*) and cyclin-dependent kinase inhibitor (*CDKN1A*) (Ishihara *et al.*, 2009), which are involved in controlling cell growth and cycle progression; and proteins like matrix metallopeptidase 2 (MMP2), that is involved in the breakdown of extracellular matrix; TIMP metallopeptidase inhibitor 2 (TIMP2), which functions as both MMP inhibitor and activator; B-cell lymphoma 2 (Bcl-2) and Bcl-2 associated X protein (Bax), which are involved in the regulation of cell death by apoptosis (Peng *et al.*, 2012; Sulaiman *et al.*, 2012).

The plethora of studies associated with propolis cytotoxicity and its compounds *in vitro* and *in vivo* allows us to unravel the potential of this natural product for the development of new antitumor agents and indicates promising usefulness (Sforcin & Bankova, 2011; Chan *et al.*, 2013; Silva-Carvalho *et al.*, 2014).

1.6. Portuguese Propolis: Characterization and Applications

Portuguese propolis has been highly neglected over the years, both by beekeepers and by the scientific community. Only in 2008, national propolis was the subject of a study (Moreira *et al.*, 2008). Since then, it has been established that Portuguese propolis contains the same phenolic acids and flavonoids as European samples (poplar type), and some specific components, such as

new methylated, esterified, and hydroxylated derivates of flavonoids and pinocembrin/pinobanksin peculiar derivates, like phenyl propanoic acid derivate moiety and p-coumaric ester derivate (Falcão *et al.*, 2010; Silva-Carvalho *et al.*, 2014). Silva-Carvalho *et al.* (2014) showed that Portuguese propolis from Pereiro (district of Guarda, Beira Alta) has a high concentration of phenolic compounds.

The properties of this Portuguese natural product have not been studied and explored to its full potential. Still, known bioactivities of Portuguese propolis are antioxidant activity (Moreira *et al.*, 2008; Miguel *et al.*, 2010; Valente *et al.*, 2011; Freitas *et al.*, 2019), antitumor activity mainly on renal cell carcinoma (Valente *et al.*, 2011; Freitas *et al.*, 2022), human colorectal cancer (Valença *et al.*, 2013), breast and prostate cancer (Silva-Carvalho *et al.*, 2014); antimicrobial activity, mainly antibacterial and antifungal bioactivities (Freitas *et al.*, 2019), and genotoxic activity (Moreira *et al.*, 2008; Miguel *et al.*, 2010; Valença *et al.*, 2013; Silva-Carvalho *et al.*, 2014; Cruz *et al.*, 2016). National propolis seems to be a powerful antioxidant agent that can be used against oxidative stress and be beneficial to human health, probably because is an important source of total phenols, flavones, and flavonols (Moreira *et al.*, 2008; Miguel *et al.*, 2010). It can also protect human erythrocytes from free radical damage by decreasing lipid peroxidation (Valente *et al.*, 2011).

Portuguese propolis is active against *S. aureus*, *P. aeruginosa,* and *E. coli*, and presents greater activity against Gram-positive than against Gram-negative bacteria. This is a relatively common and generalized trait for all types of propolis (Vardar-Ünlü *et al.*, 2008; Kim & Chung, 2011) that can be explained by the structural differences in bacterial cell walls between both bacterial types (Silva *et al.*, 2012). Portuguese propolis from the North (Bragança county) and Center of Portugal presents high antifungal activity against *T. rubrum* and *C. albicans* and low activity against *Aspergillus fumigatus* (Sawaya *et al.*, 2011). More recently, Silva *et al.* (2012) demonstrated that these propolis samples are more active against *S. aureus* than against *C. albicans* and *T. rubrum*.

The first study related to the antitumor activity of Portuguese propolis used methanol extracts, which exhibited selective toxicity against malignant cells of renal cell carcinoma, *in vitro* (Valente *et al.*, 2011). Antitumor activity against human colorectal cancer cells was reported for a propolis sample from Azores (Valença *et al.*, 2013) and was related not only to the decrease in cell proliferation and induction of cell death but also to the disturbance of cancer cells' glycolytic metabolism. Another propolis sample from Pereiro (district Guarda, Beira Alta) appears to be an

excellent candidate for the development of a new antitumor drug, as it affects cell proliferation, migration, and angiogenesis and promotes cell death in breast and prostate cancer cell lines (Silva-Carvalho *et al.*, 2014). Ethanol extracts of a propolis sample from Côa (Beira Alta, Portugal) exhibited unique dual genotoxic and antigenotoxic effects using the yeast *S. cerevisiae* eukaryotic model (Cruz *et al.*, 2016). In 2018 and for the first time, Alves *et al.* (2018) showed that Portuguese propolis has necrosis-mediated cytotoxicity in yeast cells (Woisky & Salatino, 1998).

Studies related to Portuguese propolis have been contributing to adding value to this natural resource and opened new perspectives for its exploitation in the pharmacological and food areas through the conception of new propolis-based products that will improve health (Silva-Carvalho *et al.*, 2014).

1.6.1. Portuguese Propolis from Gerês: What Do We Know So Far?

Portuguese propolis samples collected in an apiary sited at Gerês (G) have the same phenolic compounds (Freitas *et al.*, 2019) previously described in other Portuguese and European propolis samples (Bankova *et al.*, 2002; Falcão *et al.*, 2010; Cruz *et al.*, 2016). Chrysin, caffeic acid, isoprenyl ester, and pinocembrin are the most prevalent phenolic compounds, and pinobanksin and phenolic acid derivates minor ones. The chemical composition of propolis samples collected over a four-year period showed only minor differences, which is a significant finding considering the difficulty of propolis standardization and subsequent acceptance and commercialization. This consistency between different Gerês propolis samples can lead to a variety of applications for this propolis (Freitas *et al.*, 2019).

Ethanol extracts of Gerês (G.EEs) propolis collected over a four-year period in the same apiary exhibited consistent antibacterial and antioxidant properties. Antibacterial activity is more evident against Gram-positive bacteria, particularly spore-forming bacteria of the genus *Bacillus*, while *E. coli* and Methicillin-Resistant *Staphylococcus aureus* (MRSA) remained non-susceptible. A strong synergistic effect with the broad-spectrum antibiotic gentamicin, which interferes with protein synthesis, was detected, a very interesting finding considering the current worldwide problem of antibiotic resistance (Freitas *et al.*, 2019; Freitas *et al.*, 2022). Pinocembrin, ferulic acid, galangin, quercetin, caffeic acid cinnamyl ester, and CAPE are the main compounds identified that can be responsible for antimicrobial activity. Yet, the mechanism of action for antibacterial activity remains to be understood (Freitas *et al.*, 2019).

Gerês propolis plays a protective role against yeast oxidative stress, either *in vivo* or *in vitro*, and its ethanol extracts also demonstrate iron chelating activity. This is a crucial function because

iron, an essential ion for health, is extremely reactive and causes the generation of ROS (Freitas, 2015).

Portuguese propolis research is growing. However, though promising, the results are still scarce and further studies will be necessary for a safe and effective application of Gerês propolis in several areas of interest, namely for medical applications.

1.7. A General Overview of Cancer

1.7.1. Cancer Epidemiology

Cancer is the world's second leading cause of death and a severe public health issue, with approximately 10 million deaths registered in 2020, despite the efforts observed over the past few decades to improve different potential treatments. Each year, the number of new cases reported increases exponentially, with 19.3 million cases recorded in 2020 (Ferlay *et al.*, 2021). Population growth and aging are the largest contributors to the rise of cancer incidence. The countries' socioeconomic development is also a significant cancer-related risk factor (Sung *et al.*, 2021). According to the World Health Organization (WHO) (Lewandowska *et al.*, 2019), 35% of deaths caused by this malignant neoplasia are lifestyle associated, including modifiable risk factors like cigarette smoking, alcohol consumption, dietary factors, obesity, hormone replacement therapy, physical inactivity, and UV light exposure, among others. COVID-19, a global pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), resulted in delays in diagnosis and treatment, and a reduction in healthcare access, which will be reflected in cancer prevalence and morbidity statistics in the next years (Omran, 1971; Sung *et al.*, 2021). The cancer burden is expected to increase, with a predicted number of new cases around 24.6 million by 2030 (Thun *et al.*, 2009; Bray *et al.*, 2012).

1.7.2. The Hallmarks of Cancer

Cancer is usually characterized by rapid proliferation and abnormal cell growth. This malignant disease is caused by multiple changes in gene expression, which results in dysregulated cell proliferation, cell death, and, lastly, the evolution into a population that can invade nearby tissues and spread from the primary site to other body areas (metastasis) (Sarkar *et al.*, 2013). Tumors can be classified as malignant (cancerous) or benign (noncancerous). The ability of cancer to proliferate, invade, disseminate, and metastasize are the characteristics that distinguish these types of tumors from benign ones (Patel, 2020).

Tumorigenesis is a very complex and highly dynamic process, involving a variety of molecular events that provide support and advantage to the malignant transformation (Grizzi & Chiriva-Internati, 2006; Hanahan, 2022). In 2000, Hanahan and Weinberg proposed an organized principle to rationalize and understand the complexities of neoplastic diseases, which included six distinct biological processes essential for tumor growth and metastatic spreading (The Hallmarks of Cancer) **(Figure 3A)** (Hanahan & Weinberg, 2000). This number was updated ten years later **(Figure 3B)** (Hanahan & Weinberg, 2011) and, currently, the Hallmarks of Cancer encompass eight hallmark capabilities and two enabling characteristics: (i) promotion of tumor inflammation, (ii) evasion to immune destruction, (iii) deregulation of the cellular energetics, (iv) genome instability and mutation, (v) evasion from growth suppressors, (vi) invasion and metastasis activation, (vii) induction of angiogenesis, (viii) replicative immortality, (ix) resistance to cell death and, lastly, (x) sustained proliferative signaling. More recently, Hanahan (2022) proposed some new biological parameters such as "unlocking phenotypic plasticity", "nonmutational epigenetic reprograming", "polymorphic microbiomes" and "senescent cells" **(Figure 3C)**. These cancer-enabling characteristics, however, lack validation. So, regarding all this information, malignancy development is directly related to the deregulation of a plethora of cellular mechanisms for neoplasia profit.

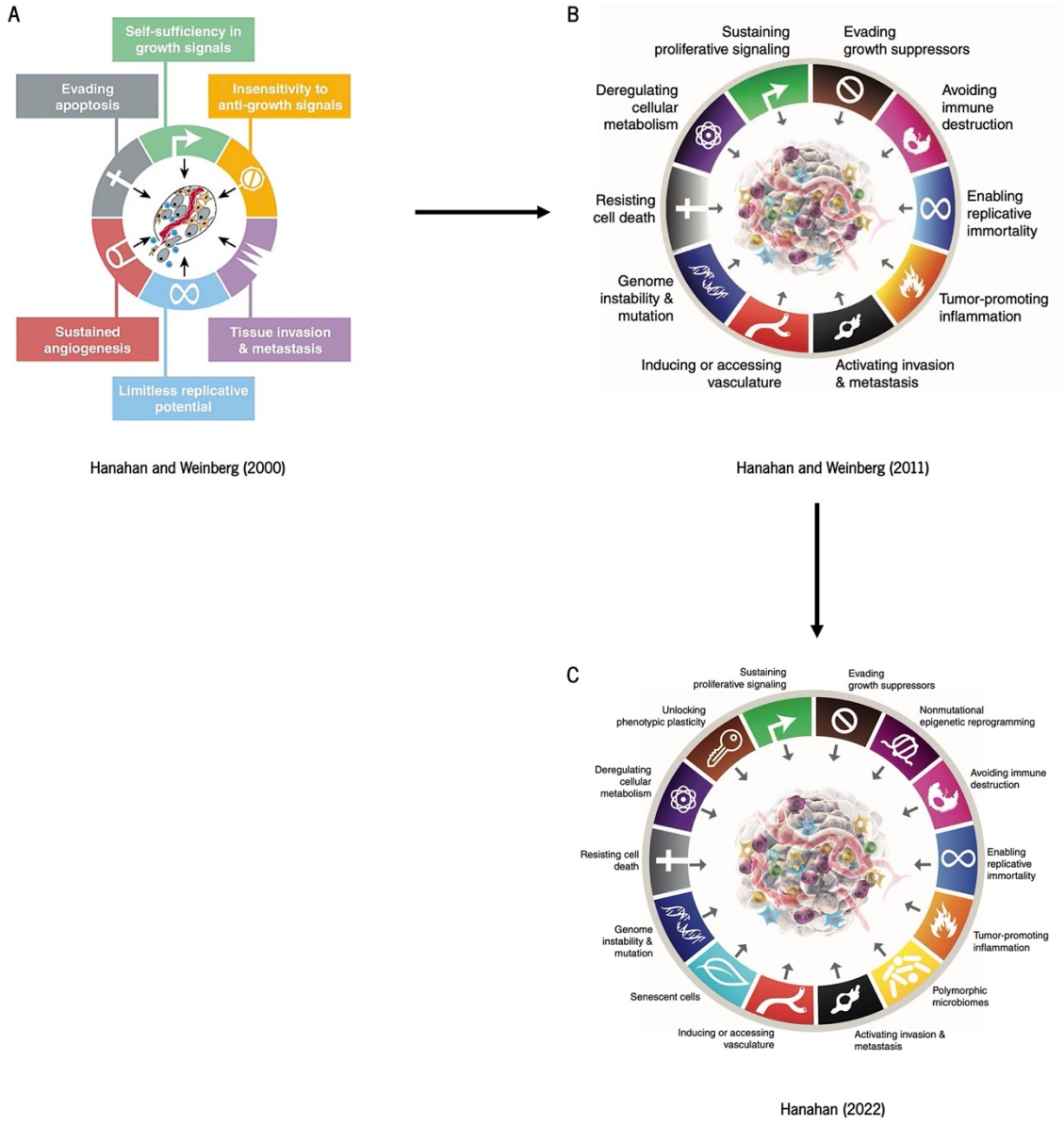

Figure 3 - Evolution of the Hallmarks of Cancer. (A) Hanahan and Weinberg proposed in 2000 six hallmarks essential for tumor growth; **(B)** The same authors updated this number to ten some years later, considering eight hallmark capabilities and two enabling characteristics; **(C)** The new dimension of the Hallmarks of Cancer suggested by Hanahan (2022) that compasses four new enabling characteristics. However, this last formulation lacks validation. (Adapted from Hanahan and Weinberg (2000), Hanahan and Weinberg (2011), and Hanahan (2022)).

Tumors are a very complex and rapidly evolving system. Cancer complexity is primarily associated with the heterogenicity of neoplastic cells, which is acquired through evolutionary processes such as mutations, genetic drift, and selection involving heterotypic interactions with other surrounding agents from the tumor microenvironment (TME) (Hanahan & Weinberg, 2011; Turajlic *et al.*, 2019). As a result of the accumulation of cancer-promoting genetic and epigenetic changes, many cellular biological regulatory processes and functions are affected – copy number

variation (deletion and amplification), chromosomal rearrangements (translocation, inversion, and deletion), mutations in the DNA and epigenetic alterations (DNA methylation/hydroxymethylation and histone methylation/acetylation) (Kinzler & Vogelstein, 1996; Brait & Sidransky, 2011; Hanahan & Weinberg, 2011). The inactivation of tumor-suppressor and stability genes and the activation of oncogenes are common outcomes of these modifications. At the physiologic level, these tumor-suppressor and oncogene alterations operate on the same principle, causing an increase in neoplastic cells by stimulating cell growth and proliferation and inhibiting cell death or cell-cycle arrest (Vogelstein & Kinzler, 2004).

One of the most well-studied epigenetic modifications in cancer is DNA methylation. Normal DNA methylation normally occurs through the covalent addition of a methyl group (CH_3) in regions rich in CpG dinucleotide islands. This mechanism is vital to the maintenance of the genomic structure and the regulation of gene expression (Craig & Bickmore, 1994; Brait & Sidransky, 2011). Aberrant methylation, on the other hand, is essential for tumor development and can consist of either hyper or hypomethylation (Morgan *et al.*, 2018). Hypermethylation is the accumulation of methylation in DNA promoter regions, that results in a decreased gene expression. The transcriptional mechanism is suppressed due to transcription factor unbinding (Kulis & Esteller, 2010; Pan *et al.*, 2018). The inversion of this epigenetic mechanism (hypomethylation or demethylation) indicates a lack of DNA methylation. This abnormality causes oncogene activation and compromises genomic stability, making it an interesting cancer cell indicator (Smet & Loriot, 2010; Brait & Sidransky, 2011; Pan *et al.*, 2018). DNA Methyltransferase inhibitors (DNMTi) (hypomethylation drugs), such as azacitidine (AZA), decitabine, and zebularine, have been discovered. According to Khan *et al.* (2012), low doses of AZA have been shown to improve survival and quality of life in individuals with myelodysplastic syndromes (MDS). The FDA approved azacitidine as the first hypomethylating drug for the treatment of MDS (Khan *et al.*, 2012; Pan *et al.*, 2018).

The TME is an emerging area of research due to its importance in tumor development, therapeutic response, and clinical outcome (Wu & Dai, 2017). Scientists have mostly focused on oncogenic mutations, tumor cell heterogeneity, and epigenetic modifications in the past few years. However, currently, it is known that TME is crucial for tumor progression, metastasis, immune system suppression, and drug resistance (Vaidya *et al.*, 2020; Ni *et al.*, 2021). The TME comprises multiple cell types, both malignant and non-malignant ("stromal" cells: infiltrating immune cells, cancer-associated fibroblasts (CAFs), endothelial cells, and pericytes), as well as extracellular

elements (cytokines, hormones, growth factors, extracellular matrix, among others) that are located around neoplastic cells and are nourished by a vascular network **(Figure 4)** (Hanahan & Coussens, 2012; Wu & Dai, 2017). Tumor cells can circumvent apoptosis and develop resistance to treatment due to the multiple interactions between malignant cells and the TME, encouraging cancer cell growth and proliferation (Meads *et al.*, 2009). Furthermore, because targeting TME-associated cells can benefit cancer management or tumor progression suppression, TME has become a prominent topic of research. Thus, TME-based therapeutics is the most recent scientific strategy for cancer treatment (Arneth, 2019).

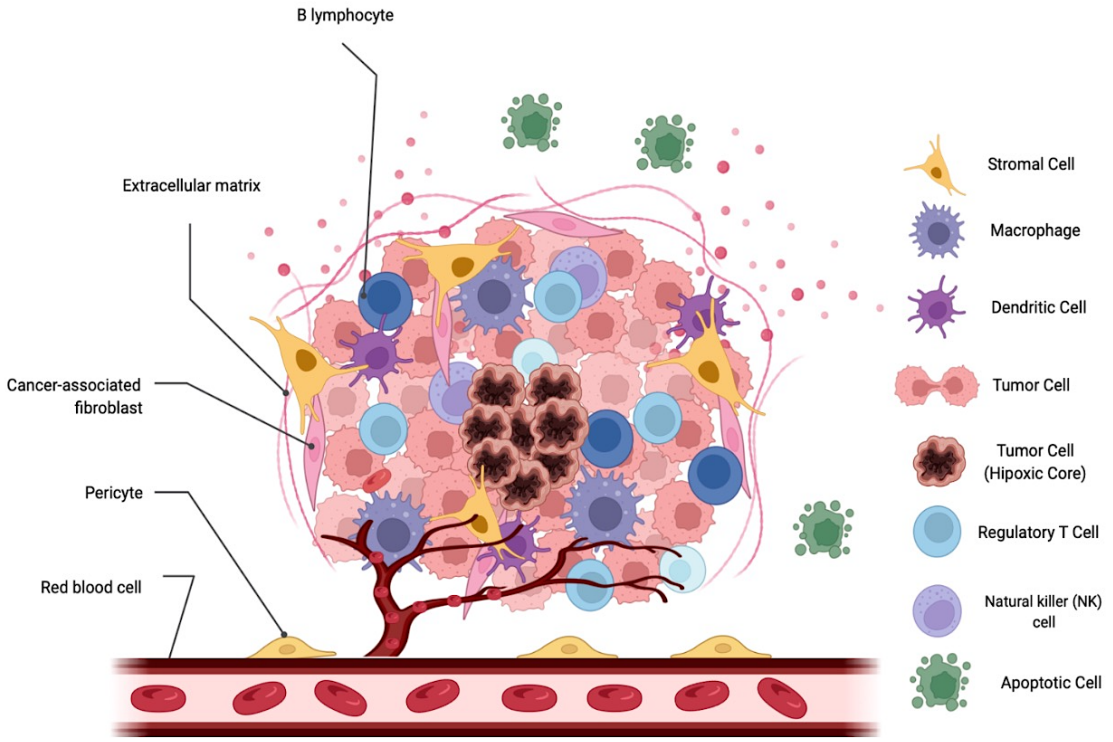

Figure 4 – Components of the TME. TME is a complex system of heterogenic tumor cells, stromal and immune system cells, and extracellular elements residing in a vascular network. Tumor-infiltrating immune cells of myeloid and lymphoid lineage are found within the TME. Figure created with BioRender.com upon adaptation from Fernández *et al.* (2019).

1.7.3. Effectiveness and Resistance to Cancer Therapies

Surgery, radiation, chemotherapy, targeted therapy, and immunotherapy are the most common cancer treatments nowadays. However, these procedures have side effects that have a detrimental effect on the patient's quality of life. Thus, the search for innovative anticancer therapies that are both effective and tolerable remains a current challenge (Mun *et al.*, 2018).

Furthermore, drug resistance is also a key health problem that restricts the effectiveness of anticancer drug treatments. Tumors may be intrinsically drug-resistant or can acquire resistance during treatment. Acquired resistance becomes a special concern since tumor cells become resistant to the original treatment and can also develop cross-resistance to other therapies that act through different mechanisms (Longley & Johnston, 2005). This type of resistance can arise from mutations during treatment or through other adaptative responses, such as increased expression of the therapeutic target, activation of other compensatory alternative pathways, and therapy-induced selection (Holohan *et al.*, 2013). In patients with metastatic cancer, this condition is responsible for 90% of therapy failure (Longley & Johnston, 2005). As a result, if drug resistance mechanisms could be overcome and understood, the impact on treatment efficacy and consequent cancer patient survival would be significant.

1.8. Morphology and Function of the Skin

Skin is the human body's largest and most complex organ, accounting for approximately 8% of its total mass and covering an area of 1.8 m^2 (Naves *et al.*, 2020). This organ is responsible for a multitude of functions, including homeostasis, barrier, immunity, and sensory feedback (Knox & O'Boyle, 2021). Skin is structured in three distinct layers: epidermis, a superficial keratinocyte-based structure with melanocytes located in the basement membrane; the dermis, a middle layer composed of a fibrous extracellular matrix (ECM) generated by resident fibroblasts; and hypodermis, the innermost layer, mostly comprised of fatty tissue (Wong *et al.*, 2016; Knox & O'Boyle, 2021). The skin's physical barrier is mostly found in the epidermis while the immunological barrier is found in both the dermis and the epidermis **(Figure 5)** (Orsmond *et al.*, 2021).

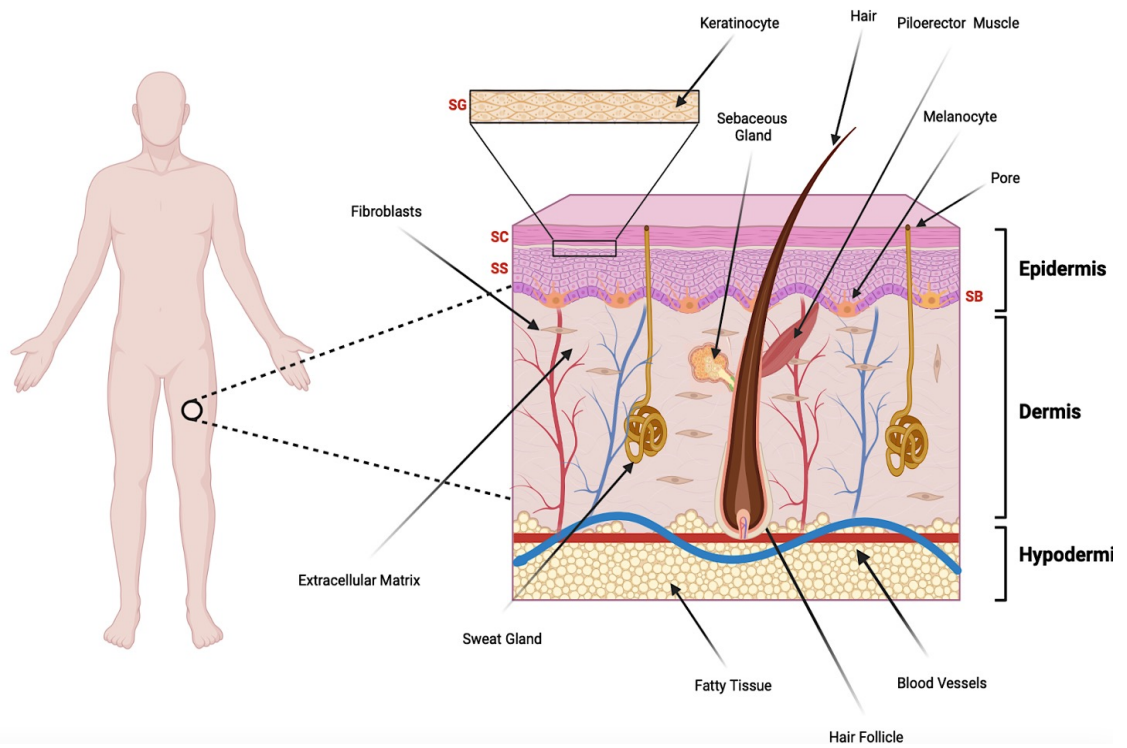

Figure 5 – Schematic Representation of the Skin Ultrastructure. Skin is a very complex organ that is composed of three layers: epidermis, dermis, and hypodermis. The epidermis is the most superficial and physiologically active skin layer and is divided into four different strata, such as **(SC)** stratum corneum, **(SG)** stratum granulosum, **(SS)** stratum spinosum, and **(SB)** stratum basale. Figure created with BioRender.com upon adaptation from Orsmond *et al.* (2021).

The epidermis is the most superficial and physiologically active epithelium since the epidermis' basal layer is continuously renewing (Wong *et al.*, 2016). The stratum corneum (SC), stratum granulosum (SG), stratum spinosum (SS), and stratum basale (SB) are the four distinct strata that comprise this skin layer **(Figure 5)** (Baroni *et al.*, 2012; Orsmond *et al.*, 2021). This last stratum is responsible for the physical separation between the epidermis and dermis (Choi, 2019). Keratinocytes, which are responsible for the production of keratin, are the most predominant cell type in the epidermis. Other cells, such as melanocytes, which produce skin pigment and corneocytes can also be found in this epithelium (Baroni *et al.*, 2012; Choi, 2019). The epidermis comprises the physical, chemical/biochemical (antimicrobial, innate immunity), and adaptative immunological barriers (Proksch *et al.*, 2008).

The dermis is the skin's middle layer, positioned beneath the epidermis. This skin layer is formed by extracellular matrix (ECM), a variety of connective tissues, hair, glands, lymphatic system, nerves, many types of cells, and blood vessels (Elias & Menon, 1991; Boughton & Mclennan, 2013). The dermis has two distinct areas: the superficial papillary dermis and the deeper reticular dermis (Wong *et al.*, 2016). The predominant resident cells in this skin's middle

layer are dermal fibroblasts, classified as mesenchymal cells and responsible to produce ECM and hair follicle initiation and cycling. (Boughton & Mclennan, 2013; Thulabandu *et al.*, 2018). The dermis acts as a barrier, regulating water retention and heat loss as well as providing protection from external infections. It also provides structure, strength, and flexibility to the skin (Naves, 2018).

The hypodermis, also known as the innermost layer, is the third layer of the skin and is composed of loose connective tissue that generates adipose tissue pockets (Wong *et al.*, 2016). Fibroblasts, adipose cells, and macrophages are the most prevalent cell types in this layer of the skin (Gordon, 2013; Wong *et al.*, 2016). The hypodermis is responsible for the skin's thermoregulation and mechanical characteristics. It also protects the skin and supports nerves, vessels, and lymphatics, which are responsible for supplying the region (Chu *et al.*, 2012; Boughton & Mclennan, 2013).

1.9. Skin cancer

Skin cancer is an eminent public health issue. This neoplasia is highly prevalent and can be divided into three major types: basal cell carcinoma (BCC), squamous cell carcinoma (SCC), and cutaneous malignant melanoma (CM) or melanoma, all of which have distinctive precursor cells (Gordon, 2013; Simões *et al.*, 2015; Cheng *et al.*, 2021). This classification is based on clinical behavior as well as the origin of the cells that cause skin cancer (Simões *et al.*, 2015). Non-melanocytic skin cancer (NMSC), more specifically BCC and SCC, has a high incidence rate (almost 2-3 million new cases each year) and is relatively treatable since it remains at the primary site (WHO, 2017; Hogue & Harvey, 2019).

1.9.1. Melanoma

Melanoma is the most aggressive, life-threatening, and invasive type of skin cancer (Bandarchi *et al.*, 2010; Rastrelli *et al.*, 2016) caused by an uncontrolled melanocyte proliferation (Ward & Farma, 2017). Over the past years, the incidence of this malignant skin cancer has risen steadily and significantly, with a predominance among white people (Bandarchi *et al.*, 2010). According to the International Agency for Research on Cancer (IARC), 324 635 new melanoma cases and 57 043 patient deaths were reported in 2020 (Ferlay *et al.*, 2021; Khaddour *et al.*, 2021). Melanoma has a 5-year survival rate of more than 90% in its early stages, and it can be successfully treated with surgery alone (Luke *et al.*, 2017; Allemani *et al.*, 2018). However, as the tumor spreads to other body areas, the 5-year survival rate drops to 15% (Tas, 2012).

Melanoma is generated by the aberrant proliferation of melanocytes that grow in an irregular pattern (Shain & Bastian, 2016). The human skin contains around 3 billion cutaneous melanocytes, which are concentrated in the epidermis at a density of 1500 melanocytes/mm^2 (Kanitakis, 2010). Melanocytes are epidermal cells that arise from neural crest cells (NCC), which are a type of embryonic cell (Cichorek *et al.*, 2013). The biosynthesis of melanin (melanogenesis), the skin pigment, within membrane-bound organelles known as melanosomes is its primary function (Costin & Hearing, 2007). Melanin is stored in melanosomes and subsequently transmitted to surrounding keratinocytes via dendrites (phagocytic process) in normal cells, where it plays a crucial role in photoprotection against UV radiation **(Figure 6)** (Quevedo, 1972; Costin & Hearing, 2007). This melanocyte-keratinocyte melanin trafficking is carried out during UV light exposure. Melanoma cells maintain melanin traffic, although tumor-associated keratinocytes have a heterogeneous and irregular melanin distribution. Melanin produced in skin malignant neoplasia is darker than melanin observed in normal tissue (Lazova & Pawelek, 2009).

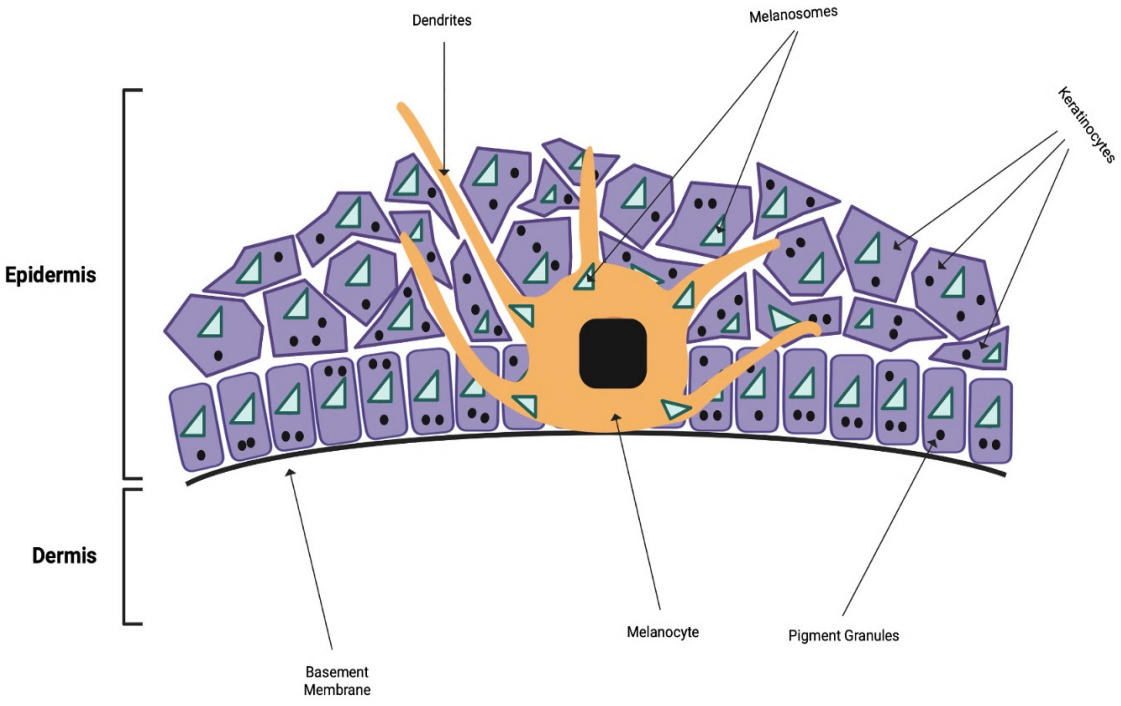

Figure 6 – Melanocyte Structure and Function. Melanocytes are in the epidermis basis layer (stratum basale) and produce melanin, which is stored in melanosomes. Dendrites deliver these tissue-specific organelles to the surrounding keratinocytes. Figure created with BioRender.com upon adaptation from Paluncic *et al.* (2016).

1.9.1.1. Melanoma Risk Factors

Malignant melanoma can develop from a pre-existing benign melanocytic nevus or healthy skin, also known as *de novo* melanoma. There is no precursor lesion in this last case (Longo *et al.*,

2011). Environmental exposure, genetic predisposition, and phenotypic traits are all risk factors implicated in the development of melanoma tumors (Longo *et al.*, 2011; Lugović-Mihić *et al.*, 2019).

UV radiation exposure is responsible for nearly 60 to 70% of cutaneous malignant melanomas, being one of the most important and modifiable environmental risk factors (Rastrelli *et al.*, 2016; Sample & He, 2018). UV radiation is split into three types: UVA (315-400 nm), UVB (280-315 nm), and UVC rays (100-280 nm). Only UVA and UVB are genotoxic and cause carcinogenic skin damage because UVC radiation is absorbed by the ozone layer (Watson *et al.*, 2016; Sample & He, 2018). Long-term or recurrent UV radiation exposure causes chronic inflammation, immunological suppression, and inefficient DNA mutation repair (DNA damage), resulting in uncontrolled cell proliferation and mutations that promote malignant skin cancer development (Mancebo & Wang, 2014). Controlling UV light exposure and environmental changes that promote UV transmission is an essential strategy for reducing health and tumor growth risks.

The risk of contracting skin cancer is strongly influenced by genetic predisposition. Inherited melanoma represents approximately 10% of all malignant skin cancer cases and is particularly related to cell cycle dysregulation (Lugović-Mihić *et al.*, 2019). The most prominent genetic anomalies are in the cyclin-dependent kinase inhibitor 2 (*CDKN2A*) and cyclin-dependent kinase 4 (*CDK4*) genes. These genes play a role in cell cycle control and are identified as high-risk and high penetrance melanoma genes (Meyle & Guldberg, 2009; Lugović-Mihić *et al.*, 2019). Pigmentation phenotype is also considered a risk factor. In fact, populations with naturally fair skin, poor tanning response, blond and red-colored hair, and freckles are more predisposed to develop malignant skin cancer, when compared to darkly pigmented people (Bliss *et al.*, 1995; Meyle & Guldberg, 2009). Melanocytic nevi, a benign accumulation of melanocytes or nevus cells, are a major risk factor for the development, progression, and evolution of melanoma. The number, size, and type of this structure are the key aspects to take into consideration (Rastrelli *et al.*, 2016). Melanoma is more prone to develop in larger (>5 mm) or giant (>20 cm) nevi, as well as in atypical nevi (large, flat component, varied pigmentation, irregular asymmetric form, and indistinct boundaries) (Watt *et al.*, 2004; Tannous *et al.*, 2005). More than 100 nevi are associated with a seven-fold increased chance of developing malignant skin cancer (Gandini *et al.*, 2005).

1.9.1.2. Mutational Landscape and Classification of Melanoma

Skin malignant melanoma genomes have the highest mutation rate (Lo & Fisher, 2014). V-Raf murine sarcoma viral oncogene homolog B (*BRAF*), *CDKN2A*, *TP53*, and neuroblastoma RAS

viral oncogene homolog (*NRAS*) are the most prevalent and significant gene mutations in cutaneous melanoma (The Cancer Genome Atlas Network, 2015; Hayward *et al.*, 2017). The advancement of genetic and molecular techniques has allowed the detection of *BRAF* gene mutation in 40-60% of melanoma patients (Kim & Cohen, 2016). More than 90% of *BRAF* mutations occur at the codon 600 (*BRAF*^{V600E}) (Tas & Erturk, 2020). The substitution of valine by glutamic acid in codon 600 of the *BRAF* gene results in hyperactivation of the RAS-RAF-MEK-ERK mitogen-activated protein kinase (MAPK) signaling cascade, which is interconnected to basic cellular functions like proliferation, migration, apoptosis inhibition, and tumor growth **(Figure 7)** (Ascierto *et al.*, 2012; Lo & Fisher, 2014; Tas & Erturk, 2020). *BRAF*-mutated melanomas are extremely associated with high levels of aggressiveness and proliferation, being more predisposed to metastasize, particularly to the brain (severe condition) (Tas, 2012; Alqathama, 2020).

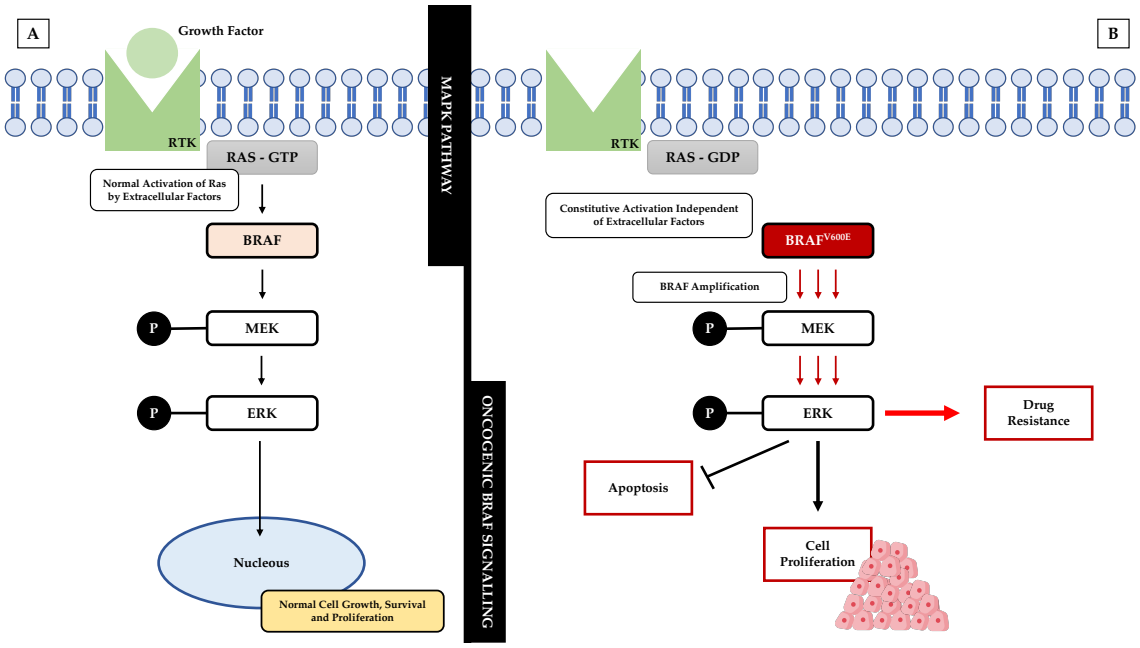

Figure 7 - Schematic overview of the role of BRAF^{V600E} mutation in melanoma. RKT: Receptor Tyrosine Kinase**; (A)** Normal pathway**; (B)** Oncogenic BRAF signaling (Adapted from Ascierto *et al.* (2012)).

NRAS mutations are less prevalent in melanoma than *BRAF* mutations, occurring in 15-30% of cases (Alqathama, 2020). The most common modifications in the *NRAS* gene occurs in codons at position 12, 61, and 13 (Muñoz-Couselo *et al.*, 2017). These three modifications have distinct effects on the NRAS protein, but the outcome is the same: NRAS GTPase activation and consequently hyperactivation of MAPK and phosphatidylinositol 3-kinase (PI3K) pathways. *NRAS* mutation is mutually exclusive with *BRAF* genetic mutation (Muñoz-Couselo *et al.*, 2017; Sun *et*

al., 2020). This driver mutation is strongly connected to aging and long-term UV exposure (Lee *et al.*, 2011). Thus, *NRAS* and *BRAF* driver mutations are two important markers of melanoma.

The progression of malignant skin cancer is a complex process that results in increased tumor aggressiveness and a predisposition to metastasize. Rapid systemic dissemination is commonly verified to target sites, such as liver, bone, lung, skin, muscle, and brain. Brain metastasis is the most severe clinical condition (representing 75% of melanoma cases) with a low survival rate, causing 95% of patients' death (Alqathama, 2020).

Melanoma tumors can be categorized using two different systems: the WHO classification system for skin malignancies and the Tumor-nodes-metastasis classification system (TNM). Melanoma tumors are divided into nine subtypes by the WHO, depending on their epidemiological, clinical, and histological features **(Table 6)** (Elder *et al.*, 2020). Each melanoma subtype is placed at the end of an evolutionary pathway that is anchored in its respective precursor (based on current and available data) (Elder *et al.*, 2020).

Table 6 – WHO Classification of Melanoma (Modified from 2018 WHO Classification). Adapted from Elder *et al.* (2020).

A.	**Melanomas typically associated with CSD***	
	Pathway I	Superficial spreading melanoma/low-CSD melanoma
	Pathway II	Lentigo maligna melanoma/high-CSD melanoma
	Pathway III	Desmoplastic melanoma
B.	**Melanomas not consistently associated with CSD* (no CSD)**	
	Pathway IV	Spitz melanomas
	Pathway V	Acral melanoma
	Pathway VI	Mucosal melanomas
	Pathway VII	Melanomas arising in congenital nevi
	Pathway VIII	Melanomas arising in blue nevi
	Pathway IX	Uveal melanoma
C.	**Nodular melanoma** (may occur in any or most of the pathways)	

* CSD– Cumulative Solar Damage

The American Joint Committee on Cancer proposed the TNM melanoma classification system, which is also known as the melanoma stage system (Balch *et al.*, 2009; Keung & Gershenwald, 2018). This system has been updated over the years and its main objective is to improve staging and prognostication, risk stratification, and selection of patients for clinical trials

(Keung & Gershenwald, 2018). The TNM melanoma classification **(Table 7)** considers some parameters, such as tumor thickness (T category), presence/absence of lymph nodes metastasis (N category), or presence/absence of distant metastasis (M category) (Keung & Gershenwald, 2018; Ogata *et al.*, 2021)

Table 7 – TNM staging classification system of melanoma proposed by AJCC. Adapted from Keung and Gershenwald (2018).

T Category	Thickness	Ulceration status
TX	N/A	N/A
T0	N/A	N/A
Tis	N/A	N/A
T1	≤1.0 mm	Unknown or unspecified
T1a	<0.8 mm	Without ulceration
T1b	<0.8 mm	With ulceration
	0.8–1.0 mm	With or without ulceration
T2	>1.0–2.0 mm	Unknown or unspecified
T2a	>1.0–2.0 mm	Without ulceration
T2b	>1.0–2.0 mm	With ulceration
T3	>2.0–4.0 mm	Unknown or unspecified
T3a	>2.0–4.0 mm	Without ulceration
T3b	>2.0–4.0 mm	With ulceration
T4	>4.0 mm	Unknown or unspecified
T4a	>4.0 mm	Without ulceration
T4b	>4.0 mm	With ulceration

N Category	Number of tumor-involved regional lymph nodes and nodal metastatic burden	Presence of in-transit, satellite, and/or microsatellite metastases
NX	Regional nodes not assessed (e.g. SLNB not performed, regional nodes previously removed for another reason) **Exception:** pathological N category is not required for T1 melanomas, use cN.	No
N0	No regional metastases detected	No
N1	1 tumor-involved node or in-transit, satellite, and/or microsatellite metastases with no tumor-involved nodes	
N1a	1 clinically occult (i.e. detected by SLNB)	No
N1b	1 clinically detected	No
N1c	No regional lymph node disease	Yes
N2	2 or 3 tumor-involved nodes or in-transit, satellite, and/or microsatellite metastases with 1 tumor-involved node	
N2a	2 or 3 clinically occult (i.e. detected by SLNB)	No
N2b	2 or 3, at least 1 of which was clinically detected	No
N2c	1 clinically occult or clinically detected	Yes
N3	≥4 tumor-involved nodes or in-transit, satellite, and/or microsatellite metastases with ≥2 tumor-involved nodes, or any number of matted nodes without or with in-transit, satellite, and/or microsatellite metastases	
N3a	≥4 clinically occult (i.e. detected by SLNB)	No
N3b	≥4, at least 1 of which was clinically detected, or presence of any number of matted nodes	No
N3c	≥2 clinically occult or clinically detected and/or presence of any number of matted nodes	Yes

M Category	Anatomic Site	LDH Level
M0	No evidence of distant metastasis	Not applicable
M1	Evidence of distant metastasis	
M1a	Distant metastasis to skin, soft tissue including muscles, and/or nonregional lymph node	Not recorded or unspecified
M1a(0)		Not elevated
M1a(1)		Elevated
M1b	Distant metastasis to lung with or without M1a sites of disease	Not recorded or unspecified
M1b(0)		Not elevated
M1b(1)		Elevated
M1c	Distant metastasis to non-CNS visceral sites with or without M1a or M1b sites of disease	Not recorded or unspecified
M1c(0)		Not elevated
M1c(1)		Elevated
M1d	Distant metastasis to CNS with or without M1a, M1b, or M1c sites of disease	Not recorded or unspecified
M1d(0)		Not elevated
M1d(1)		Elevated

Abbreviations: N/A, not applicable; TX, primary tumor thickness cannot be assessed (e.g., diagnosis by curettage); T0, no evidence of primary tumor (e.g., unknown primary or completely regressed melanoma); Tis, melanoma *in situ*; SLNB, sentinel lymph node biopsy; LDH, lactate dehydrogenase.

1.9.1.3. Melanoma Treatment

Melanoma treatment is selected based on tumor features such as location, stage, and genetic profile (Domingues *et al.*, 2018). The therapeutic options currently available are surgical resection, chemotherapy, radiotherapy, photodynamic therapy, targeted therapy, and immunotherapy (Davis *et al.*, 2019). The primary treatment option for cutaneous melanoma is surgical resection using the wide local excision (WLE) technique, which may be the only therapeutic option in situations of stage I-III (TNM system). This strategy is critical for reducing local recurrence and melanoma-related mortality (Domingues *et al.*, 2018; Hartman & Lin, 2019). Adjuvant therapies such as immunotherapy and targeted therapy are recommended to increase patients´ survival rate (Austin *et al.*, 2017; Van Zeijl *et al.*, 2017). Metastasectomy is used to treat patients with solitary melanoma metastases (TNM stage IV), and chemo, immunological, and targeted therapies can be used too (Batus *et al.*, 2013; Austin *et al.*, 2017). Despite radiotherapy being rarely used in the treatment of primary tumors, it can be beneficial in the treatment of skin, bone, and brain metastases (Garbe *et al.*, 2016; Domingues *et al.*, 2018).

Nearly 70% of cutaneous melanoma patients carry mutations in genes implicated in critical signaling pathways. Small-molecule inhibitors or antibodies are used in targeted therapy for mutated proteins and, as a result, cancer pathways (Flaherty, 2012; Domingues *et al.*, 2018). Due to the highly resistant nature of skin tumor cells, the need for this sort of melanoma therapy has increased significantly in recent years (Mishra *et al.*, 2018). Different targeted treatments are proposed for melanoma, such as BRAF inhibitors (BRAFi), MEK inhibitors (MEKi), KIT inhibitors, VEGF inhibitors, PI3K-AKT-mTOR pathway inhibitors, cyclin-dependent kinase (CDK) inhibitors, Erb84 inhibitors, among others (Domingues *et al.*, 2018; Mishra *et al.*, 2018).

BRAF is the most frequently mutated oncogene in skin malignant tumors, as previously stated. The RAS–RAF–MEK–ERK signal transduction cascade, generally known as the MAPK pathway, is constitutively activated when this gene is mutated (Ascierto *et al.*, 2012; Tas & Erturk, 2020). Dacarbazine (DTIC) is an alkylating chemotherapeutic drug used to treat metastatic melanoma, with a median survival of 5–11 months and a one-year survival rate of only 27% (DeVita & Chu, 2008; Rebecca *et al.*, 2012; Lee *et al.*, 2013). This alkylating agent is the only chemotherapeutic treatment approved by FDA for metastatic melanoma (Davis *et al.*, 2019). *BRAF*-mutated patients do not respond well to conventional chemotherapy, but MAPK target inhibitors appear to be a promissory treatment (Liu *et al.*, 2020). Vemurafenib (PLX403) and Dabrafenib (GSK2118436), specific inhibitors of $BRAF^{V600E}$, were approved by FDA in 2011 and 2013,

respectively (Bollag *et al.*, 2012; Gouravan *et al.*, 2018). Vemurafenib outperformed dacarbazine treatment in phase III studies, improving the median overall survival (OS) rate of *BRAF*^{V600E} patients (84% vs 64%) (Chapman *et al.*, 2011; Garbe & Eigentler, 2018). Dabrafenib's clinical benefits over standard chemotherapeutic treatment were also verified in *BRAF*-mutant melanoma (Hauschild *et al.*, 2012). Overall, BRAF inhibitors cause rapid remission of melanoma metastases, and according to response evaluation criteria in solid tumors (RECIST), 50-60% of patients with this neoplasm showed a positive response (Menzies & Long, 2014; Davis *et al.*, 2019). Resistance and toxicity associated with these treatments, on the other hand, remain a major public health issue (Mishra *et al.*, 2018).

The paradoxical reactivation of the MAPK/ERK signaling cascade is primarily responsible for BRAFi resistance (Kakadia *et al.*, 2018; Savoia *et al.*, 2020). Thus, combined therapies with MEK/ERK inhibitors, a MAPK/ERK target downstream of BRAF, is an implemented and very intuitive strategy to overcome resistance (Flaherty, 2012; Savoia *et al.*, 2020). Trametinib, a selective inhibitor of MEK1/2, was approved by FDA in 2013 for the treatment of metastatic melanoma, which includes *BRAF*^{V600E} mutations. When compared to chemotherapy, the clinical activity of single-agent MEK inhibition on BRAF-mutated patients revealed that trametinib improves OS and median progression-free survival (PFS) (Flaherty, 2012; Rajakulendran & Adam, 2014). The FDA-approved combination therapy of dabrafenib (BRAFi) and trametinib (MEKi) (CombiDT) exhibited superior clinical benefits when compared to chemotherapy (Flaherty, 2012). This combination is currently the gold standard treatment for advanced melanoma patients (Manzano *et al.*, 2016). However, even with this treatment patients develop resistance (Hegedũs *et al.*, 2017; Mishra *et al.*, 2018).

Cobimetinib, a kinase inhibitor, was approved by the FDA in 2015 for the treatment of unresectable or metastatic melanoma with BRAF mutations in combination with vemurafenib (Boespflug & Thomas, 2016). This treatment showed clinical improvements in terms of PFS, objective response, and OS, although it had a slightly increased toxicity profile (Larkin *et al.*, 2014).

The resistance to targeted therapies can be achieved through the activation of other biological molecules (Domingues *et al.*, 2018). Recently, the TME was classified as essential to the development of drug resistance (Vaidya *et al.*, 2020; Ni *et al.*, 2021). The stimulation of MAPK/ERK and PI3K/AKT signaling cascades by fibroblasts secretion of stromal hepatocyte growth factor (HGF), which activates the receptor kinase MET, contributes to the development of tumor cell resistance to melanoma treatments (Straussman *et al.*, 2012; Ni *et al.*, 2021).

Immunotherapies are also employed in the treatment of advanced-stage melanoma. Because of the increased median OS and PFS, this kind of therapy seems to be a potential adjuvant treatment for metastatic melanoma (Van Zeijl *et al.*, 2017; Domingues *et al.*, 2018). Primary and acquired resistance is also common in these treatments (Gide *et al.*, 2018). Based on CTLA-4 and PD-1 blockers, immunotherapy resistance occurs in 70% and 40–65% of metastatic melanoma patients, respectively. Besides that, of the initial responders, 20-30% also develop resistance (Gide *et al.*, 2018).

Regarding all this evidence, research focused on alternative medicines, such as phytochemicals from plants and natural extracts, offers a great potential for therapeutic innovation. In fact, a high number of natural compounds have demonstrated immunomodulatory and anti-cancer activity, creating favorable conditions for the development of novel treatments.

1.10. Melanoma and Propolis

During the last years, natural compounds have re-emerged as an excellent source for the discovery of potential new drugs due to the emergence of new therapeutic approaches. This interest in the use of natural compounds is also due to their biodegradability and the fact that because they are mixtures, resistance is unlikely to develop. As melanoma incidence has increased dramatically and abruptly in recent years, the search for new treatments or adjuvant drugs has accelerated (Bandarchi *et al.*, 2010). A few studies demonstrated that propolis and its active compounds may display anti-proliferative, cytotoxic, anti-angiogenic, and immunomodulatory activities in various skin cancer or melanoma cell lines (Kudugunti *et al.*, 2010; Kubina *et al.*, 2015; Pelinson *et al.*, 2019). Chinese propolis can inhibit MMP's and induce apoptosis, autophagy, and cell cycle arrest in the A375 human melanoma cell line by targeting an inflammatory signaling cascade - NLR Family Pyrin Domain Containing 1 (NLRP1) pathway (Zheng *et al.*, 2018). According to Zhang *et al.* (2013), galangin, an active flavonoid present in propolis, decreases the proliferation of B16F10 melanoma cells and promotes apoptosis via the mitochondrial pathway by p38 MAPK upregulation and the tyrosinase activity (anti-melanogenesis) deregulation. In A375 and B16-F1 melanoma cell lines, chrysin had a similar impact to galangin, and apoptosis (Bax activation) is stimulated through the upregulation of p38 MAPK and downregulation of the ERK1/2 signaling pathway (Pichichero *et al.*, 2011). Other propolis components display antitumor activity against melanoma cells too, such as CAPE (Ozturk *et al.*, 2012), artepillin C and caffeic acid (Takahashi *et al.*, 2017), and propolin (present in high concentration in Taiwanese propolis) (Chen *et al.*, 2004; Chen *et al.*,

2007). Cisilotto *et al.* (2018) demonstrated that hydroalcoholic propolis extract increases ROS levels in the SK-MEL-28 melanoma cell line, leading to DNA damage and apoptosis. Furthermore, this natural product decreased mitochondrial membrane potential, cell migration, and invasion. Some of these chemotherapeutic properties were confirmed through animal studies (Chiu *et al.*, 2020).

Despite the demonstration of antitumor efficacy of different types of propolis, the activity of Portuguese propolis is unknown at this time because national samples were not included in any of the studies recorded and published. As a result, research on Portuguese propolis opens new perspectives for the development of new propolis-based therapies, with a positive impact on human health.

Chapter 2 – Objectives

Propolis is one of the most explored mixtures of natural compounds. This natural mixture has a wide range of beneficial bioactivities, including antibacterial and anticancer activity (Falcão *et al.*, 2010). Therefore, worldwide propolis is recognized as a valuable natural product capable of stimulating research and therapeutic innovation. On the other hand, Portuguese propolis has been largely neglected by beekeepers and scientists due to a lack of knowledge about its potential. In fact, propolis is produced in small quantities, and producers fear that this production may have a negative impact on honey production, the main outcome of national apiculture, which is a major component of the Portuguese economy. This scenario is shifting, with recent studies highlighting the promising biological features of national propolis, like antioxidant, antibacterial, and anticancer (Valença *et al.*, 2013; Silva-Carvalho *et al.*, 2014; Freitas *et al.*, 2019; Freitas *et al.*, 2022).

Thus, the first aim of this work is to evaluate the antimicrobial activity of propolis from Gerês, starting with a sample collected in 2020. The exploration of this bioactivity is critical given the current and pressing need for new therapeutics for infectious diseases. The influence of propolis on microorganisms was investigated, as this activity has been described for other samples of propolis from Gerês.

Melanoma is the most aggressive, lethal, and invasive form of skin cancer, and its incidence has increased over the last years (Bandarchi *et al.*, 2010). The melanoma genome is highly mutagenic, and the most common mutation is $BRAF^{V600E}$ (The Cancer Genome Atlas Network, 2015). Despite all the advances and research in melanoma treatment, finding effective therapies remains a challenge. Acquired resistance and the adverse effects of standard therapies are the root causes of the current therapeutic problem (Gastaldello *et al.*, 2021). As a result, interest in natural products has increased, due to the broad range of bioactivities that can be applied in the pharmaceutical industry to replace some standard molecules (Freitas *et al.*, 2022).

Taking this into account, the second aim of this work is to evaluate the melanoma antitumoral activity of propolis from Gerês. The study of this bioactivity is important due to the need for new pharmacological agents selective to melanoma cells since classical chemotherapy compromises the physiological homeostasis of several organs and the targeted therapy is prone to rapidly induce cancer cell resistance. The proposed work will be the first attempt to link propolis and melanoma.

We hope that by completing this project, we will be able to contribute to the advancement of knowledge on the use of Portuguese propolis for therapeutic applications and, as a result, increase the value of this bee product.

Chapter 3 – Material and Methods

3.1. Propolis Samples Preparation

3.1.1. Propolis Samples

The propolis samples used in this work, kindly provided by the beekeeper Amadeu Fortunas, from Casa do Couto, were obtained in an apiary next to the Cávado River, more precisely between the villages Paradela and Sirvozelo, in Montalegre, Gerês, Portugal (41045'41.62'' N; 7058'03.34'' W) **(Figure 8)**. These samples were collected in 2018 and 2020. They were identified with the capital letter G (referring to its origin: Gerês) followed by two digits referring to the harvest years: G18 and G20, according to the nomenclature criteria adopted by our research group.

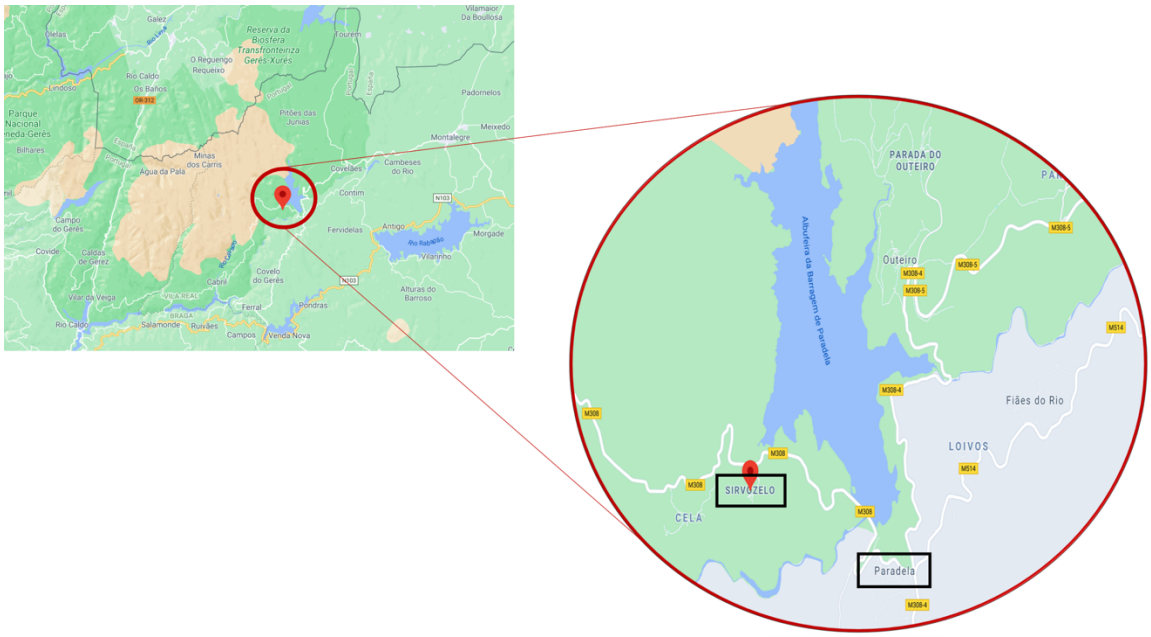

Figure 8 - Localization of the apiary where propolis samples G18 and G20 were harvested.

Samples of Portuguese propolis from Gerês, namely G18, are usually obtained by mixing propolis from three different locations - Bugalho (b), Toutelo (t), and Felgueiras (f) (Freitas *et al.*, 2018). This mixture has consistently displayed identical chemical and biological profiles over time, constituting this consistency a particular and noteworthy feature of Gerês propolis (Freitas *et al.*, 2019). However, due to an environmental problem, the apiary located in Bugalho was discontinued. Thus, the samples of G20 were only harvested from Toutelo (t) and Felgueiras (f) and, for this reason, we decided to study $G20_t$, $G20_f$, and the $G20_{tf}$ combinations.

3.2. Evaluation of Quality Parameters for Portuguese Propolis from Gerês

3.2.1. Dry residue free of volatile substances

The water content of raw propolis collected in 2018 (G18) was evaluated as previously described by Woisky & Salatino (1998). Briefly, 4 g of G18 **(W_0)** were heated in an oven at 105 °C for 5 h. The resulting powder sample was cooled to room temperature (RT) and placed in a desiccator overnight to reach a constant weight **(W_1)**. Analysis of water content was performed in triplicate and expressed in percentage (%) **(Equation 1)**.

$$\text{Water Content (\%)} = \frac{(W_o - W_1)}{W_0} \times 100\% \qquad \textbf{Equation 1}$$

3.2.2. Ash Content

The ash content of G18 raw propolis was assessed using the method of Lopes *et al.* (2017), with some modifications and adjustments. Briefly, 1 g of G18 **(A_1)** was added to a calcined and previously weighed melting pot **(A_2)**. This crude propolis sample was incinerated for 3 h at 500 °C in a muffle furnace and then desiccated until a constant weight was achieved **(A_3)**. The ash content was weighed in triplicate and represented as a percentage (%) **(Equation 2)**.

$$\text{Ash Content (\%)} = \frac{(A_3 - A_2)}{A_1} \times 100\% \qquad \textbf{Equation 2}$$

3.2.3. Beeswax Content

The beeswax content of G18 was analyzed through the method reported by Hogendoorn *et al.* (2013), which is based on specific density differences between water, wax, and propolis free of wax. A volume of 2.5 ml of de-ionized water was added to 2 g of G18 and the tubes were placed vertically in a microwave, set on medium, and heated bellow 100 °C to avoid water boiling. After cooling the samples to RT, a three-layer system was revealed: beeswax at the top layer, water in the middle layer, and de-waxed propolis at the bottom **(Figure 9)**. The beeswax upper layer was removed and weighed. Wax content was measured from three replicates and expressed as a percentage (%) **(Equation 3)**.

$$\text{Wax Content (\%)} = \frac{Wax\ Weight\ (g)}{Sample\ Weight\ (g)} \times 100\% \qquad \textbf{Equation 3}$$

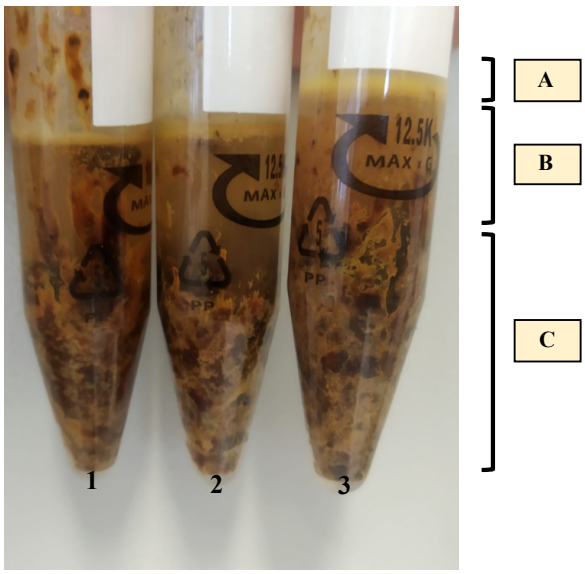

Figure 9 - The three-layers system with **(A)** beeswax, **(B)** water, and **(C)** de-waxed propolis - formed after a heating and cooling cycle of the mixture of G18 propolis and de-ionized water that allow to measure beeswax contents.

3.2.4. Balsam Content

The balsam content of crude samples of G18 propolis was determined using the method reported by Popova *et al.* (2007). In short, 15 ml of ethanol 70 % ($EtOH_{70\%}$) were added to 0.5 g of G18, and the mixture was orbitally stirred for 24 h at 125 rotations per minute (rpm) in the dark at a temperature of 25 °C. This mixture was then filtered under vacuum using Macherey-Nagel filter papers, the filtrate was stored at 4 °C in the dark and the solid residues were extracted once more. After a second filtration, $EtOH_{70\%}$ was added to the filtrate until it reached a final volume of 50 ml, and 2 ml of the final filtrate solution was dried under N_2 flow until achieving a constant weight. The mean of three replicates was calculated and the percentages of balsam content (% p/p) were determined as the ethanol-soluble fraction.

3.3. Propolis Samples Extraction

3.3.1. Ethanol Microextraction

The G20 propolis sample mentioned above was subjected to ethanol microextraction (mi) with absolute ethanol (ethanol 100%; Carlo Erba Reagents) following the protocol proposed by Cao *et al.* (2017). For this extraction, about 1 g of propolis, previously grounded into small pieces and cleaned, was incubated with 7 ml of absolute ethanol at room temperature, in the dark. After this incubation, the sample was submitted to ultrasounds (2510 Branson Ultrasonic Cleaner) over 15 minutes (min), followed by vigorous shaking (VWR W3 Vortex) for 2 min, and, finally, placed on ice

for 1 min. This ultrasound-vortex-ice cycle was repeated four times. Each sample of propolis - $G20_t$; $G20_f$ and $G20_{tf}$ – was subsequently placed, with an aluminum foil coating, in a dark place, at 4 °C, overnight. The solutions were subjected to vigorous vortexing for 2 min and then centrifuged (Eppendorf Centrifuge 5804R) for 5 min at 5000 rpm. The resultant supernatant was collected and subsequently kept under N_2 flow until a constant weight was reached, generating the dried ethanol micro-extracts $G20_t$.miEE, $G20_f$.miEE, and $G20_{tf}$.miEE. The weights of the raw sample (W_s) and of the dried ethanolic extract (W_{EE}) were used to determine ethanol extract (EE) yield (%) **(Equation 4)**.

$$\text{EE yield} = \frac{W_{EE}}{W_s} \times 100\% \qquad \textbf{Equation 4}$$

The ethanol micro-extracts mentioned above were then preserved at 4 °C, in the dark, until further use. Stock solutions for subsequent experiments were prepared by diluting the dry extracts in absolute ethanol at the required concentrations.

3.3.2. Ethanol Extraction

The G18 propolis sample was extracted with absolute ethanol, as previously reported by Freitas *et al.* (2022). Briefly, 80 ml of absolute ethanol was added to 15 g of raw propolis previously degraded into small fragments, and the mixture was kept under orbital agitation at 100 rpm, for 24 h in the dark, at RT (Orbital Shaker SO1). The mixture was filtered under vacuum (Uniweld Humm•Vac Vacuum Pump) using Macherey-Nagel filter papers, and the filtrate was stored at 4 °C in the dark. After this initial filtration, the solid remains were extracted once again with 50 ml of absolute ethanol. The filtrates were pooled, and the solvent evaporated in a Büchi Rotavapor RE 121 at 40 rpm and 38–40 °C, generating the dried ethanol extract G18.EE. This extract was then reserved at 4 °C, in the dark, until further use. Stock solutions used in subsequent assays were prepared by diluting the dried extract in absolute ethanol for fractionation, DPPH and ABTS methods, and agar dilution method; or dimethyl sulfoxide (DMSO) for *in vitro* antitumoral assays.

3.4. Fractionation of the Ethanol Extract of Propolis from Gerês 2018

The ethanol extract of propolis G18 was partitioned by Freitas *et al.* (2022). 4 g of G18.EE were first dissolved in 20 ml of absolute ethanol and, after obtaining a homogeneous solution, 200 ml of distilled water were added. This mixture was successively partitioned with *n*-hexane (Cas Number:110-54-3), ethyl acetate (EtOAc) (Cas Number:141-78-6), and *n*-butanol (BuOH) (Cas

Number:71-36-3) (3x400 ml of each). The fractionation procedure was the same for all the organic solvents: in a separatory funnel, 200 ml of *n*-butanol or *n*-hexane or EtOAc were added to the G18.EE - ethanol mixture, which was then slowly stirred (4x), and the mixture was allowed to settle until the two phases were completely separated. This method was repeated twice. The resulting organic layers were collected and dried over sodium anhydrous sulfate (Sigma-Aldrich). EtOAc, *n*-butanol, and *n*-hexane fractions were evaporated in a Büchi Rotavapor RE 121 at 40 rpm and 38–40 °C, generating the G18.EE-*n*-hexane, G18.EE-EtOAc and G18.EE-*n*-BuOH dried fractions. The solvent used in each fraction was completely evaporated. The water layer was subsequently deep-frozen at -80 °C and lyophilized (Bioblock Scientific Christ Alpha 2-4 LD Plus) for 3-5 days to sublimate the water, generating G18.EE-H_2O dried fraction. All G18.EE-fractions were stored at 4 °C in the dark until further use and dissolved in DMSO to prepare stock solutions for further antitumoral experiments.

3.5. *In vitro* Evaluation of Propolis Antioxidant Properties

3.5.1. DPPH Radical Scavenging Activity Assay

DPPH• (2,2-diphenyl-2-picry-lhydrazyl) is a stable free radical of organic nitrogen which has an unpaired valence electron and is characterized by a typical deep purple color and a maximum wavelength of 517 nm (Blois, 1958; Locatelli *et al.*, 2009). The DPPH radical scavenging assay, one of the most widely used methods to determine the scavenging capacity of several compounds with antioxidant power, is a technically simple method and is based on the reduction of DPPH• radical by antioxidants (hydrogen/electron donor species). This reaction can be followed spectrophotometrically by measuring the decrease in the absorption, caused by the reduction of DPPH• to the non-radical form (DPPH-H), which leads to a change in the color of the solution: from deep purple to yellow (Brand-Williams *et al.*, 1995; Mitra & Uddin, 2014) **(Figure 10)**.

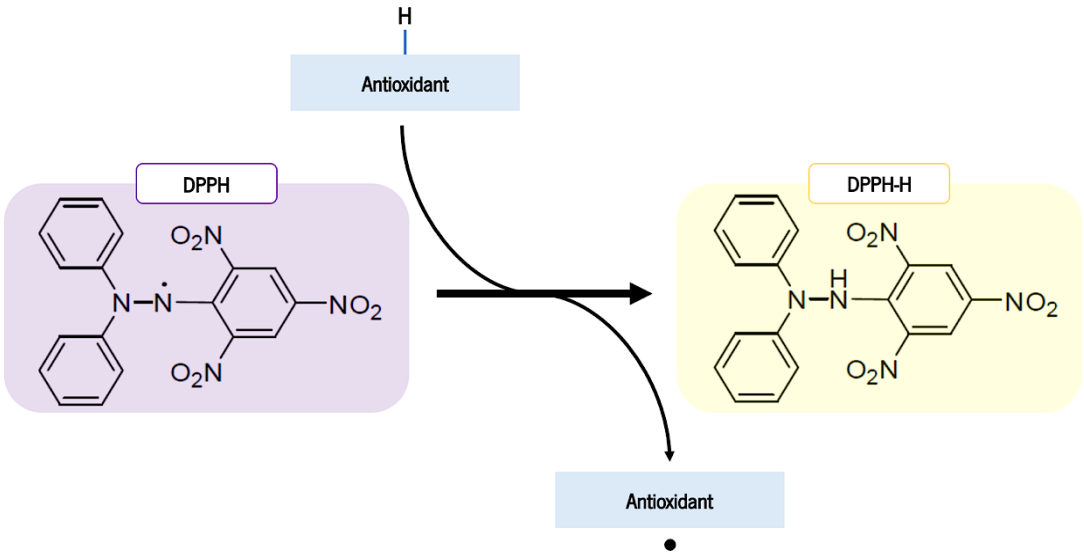

Figure 10 - DPPH• reduction reaction to the non-radical form, DPPH-H, by antioxidants. This reaction is characterized by an absorbance loss when the yellow form (DPPH-H) is produced during the reaction of a hydrogen-donating antioxidant (adapted from Liang & Kitts (2014)).

To evaluate the scavenging activity of propolis extracts from 2018 and 2020, the DPPH colorimetric assay was performed as previously described (Mitra & Uddin, 2014; Freitas *et al.*, 2019). Briefly, propolis extracts were diluted in absolute ethanol to obtain concentrations in the range of 0.5 to 50 $\mu g ml^{-1}$. Then, 50 µl of each dissolved extract was added to 100 µl of DPPH• (Sigma-Aldrich; CAS:1898-66-4) ethanol solution 0.004% (w/v). The control was prepared with DPPH• and ethanol. The mixtures were incubated in the dark, at RT, for 20 min. As previously stated, absorbance was measured at 517 nm (Spectramax Plus 384 Microplate Reader) and ethanol was used as blank. The scavenging activity of ethanol extracts of propolis from Gerês (G18.EE and G20.miEEs) was calculated using the following equation:

$$\text{Reduction } (\%) = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100\% \qquad \textbf{Equation 5}$$

Where $A_{control}$ is the absorbance of the control, obtained from the mixture of DPPH• and ethanol, and A_{sample} is the absorbance of the sample (G18.EE, $G20_{t}$.miEE, $G20_{f}$.miEE and $G20_{tf}$.miEE) after 20 min of reaction. The EC_{50}, defined as the concentration of an extract needed to scavenge 50% of the initial DPPH•, was calculated and expressed as the average value of three independent experiments with 3 replicates. A solution of gallic acid (GA) (Sigma-Aldrich; CAS:149-91-7) was used as a standard with concentrations ranging from 0.2 to 1.5 $\mu g ml^{-1}$.

3.5.2. ABTS radical scavenging activity

To evaluate the radical-scavenging activity of G18.EE, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•) cation radical decolorization assay was carried out with minor modifications (Chun *et al.*, 2005; Yang *et al.*, 2011). Briefly, propolis extracts were diluted in absolute ethanol to obtain concentrations in the range of 0.5 to 25 $\mu g ml^{-1}$. ABTS• cation radical was obtained by reacting 7 mM of ABTS aqueous solution with 140 mM of potassium persulphate (Sigma-Aldrich) for 14 to 16 h in the dark at RT **(Figure 11)**. After that, the ABTS• working reagent was diluted in 100% ethanol, yielding a 734 nm absorbance of 0.70. Then, 2.5 µl of propolis solution was added to 247.5 µl of ABTS• working reagent and incubated for 30 minutes in the dark. Absorbance was measured at 734 nm against a blank prepared with 247.5 µl of 100% ethanol. A solution of Trolox (Sigma-Aldrich) was used as standard. The scavenging activity of G18.EE was calculated using the same equation used in DPPH assay **(Equation 5)**. The EC_{50}, defined as the concentration of an extract needed to scavenge 50% of the initial ABTS radicals, was calculated and expressed as the average value of three independent experiments with 3 replicates.

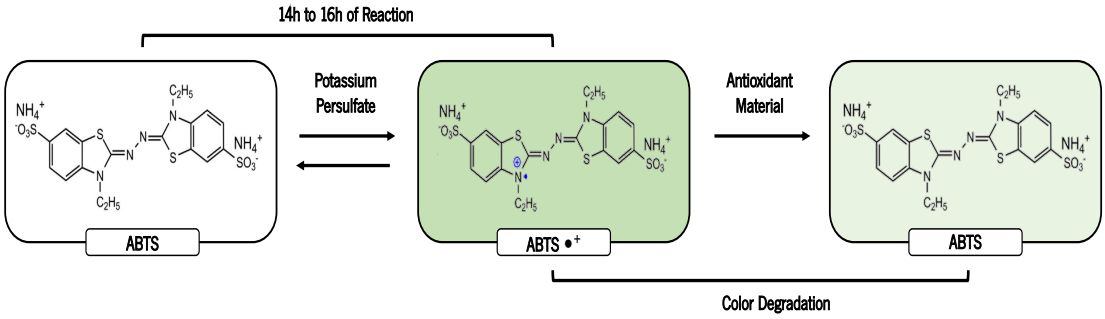

Figure 11 - Schematic representation of the ABTS radical scavenging method. The ABTS• is formed by reacting the ABTS salt with a strong oxidizing agent, namely potassium persulphate, for 14 to 16 h. In the presence of an antioxidant agent, the color of the blue-green ABTS• degrades, which can be detected by variations in wavelength. (Adapted from ÜSTÜNDAŞ *et al.* (2018)).

3.6. *In vitro* Evaluation of Propolis Antimicrobial Potential

3.6.1. Strains and Culture Conditions

The antimicrobial potential of propolis ethanol extracts (G18.EE and G20.miEEs) was determined against a panel of specific microorganisms from the collection of the Department of Biology: seven strains of bacteria - six Gram-positive and one Gram-negative - and two yeast strains **(Table 8)**.

Table 8 - Bacteria and yeast used in this work as susceptibility indicator strain in the antimicrobial assays.

Strains			Strain code
bacteria	Gram-positive	*Bacillus cereus*	ATCC7064
		Bacillus megaterium	932
		Bacillus subtilis	48886
		Methicillin Sensitive *Staphylococcus aureus* (MSSA)	ATCC6538
		Methicillin Resistant *Staphylococcus aureus* (MRSA)	M746665
		Propionibacterium acnes	H60803 (2961351)
	Gram-negative	*Escherichia coli*	CECT423
yeast		*Candida albicans*	53B
		Saccharomyces cerevisiae	BY4741WT/Y000000

3.6.1.1. Bacterial Culture Media and Growth Conditions

Bacteria cell cultures were prepared in liquid LB medium (PanReact AppliChem ITW Reagents) which is composed by 0.5% (w/v) of yeast extract, 1% (w/v) of tryptone and 2% (w/v) of sodium chloride (NaCl). The solid medium LBA, used for bacterial culture maintenance, was made by adding 2% (w/v) of agar (Biolife) to the recipe.

Overnight cultures of bacteria (inoculum) were prepared in LB medium at a ratio water/volume of 2:5 and incubation (Infors HT Multitron Standard Incubator Shaker) was performed at 37 °C and 200 rpm. Bacterial growth was monitored by optical density at 600 nm (OD_{600}) (Thermo Scientific Genesys 20).

3.6.1.2. Yeast Culture Media and Growth Conditions

Yeast cell cultures were prepared in liquid YPD medium (Difco™ YPD Broth BD) which is composed by 1% (w/v) of yeast extract, 2% (w/v) of peptone and 2% (w/v) of dextrose/glucose. The solid medium YPDA, used to maintain the cultures, was made by adding 2% (w/v) of agar to the recipe.

Yeast overnight cultures (inoculum) were prepared in YPD liquid medium at a ratio water/volume of 1:5 and incubation were performed at 30 °C and 200 rpm. Culture growth was monitored by optical density at 600 nm (OD_{600}).

3.6.2. Agar Dilution Method

The antimicrobial activity of propolis from Gerês was evaluated by calculating the minimum inhibitory concentration (MIC) values of G18.EE and G20.miEEs using an adaptation of the agar dilution method (Sforcin *et al.*, 2000; Stepanović *et al.*, 2003). Briefly, yeast and bacterial strains were grown on YPD and LB media (see 3.6.1), respectively. Overnight cultures were diluted with fresh medium to an OD_{600} of 0.1 and incubated until OD_{600} reached 0.4 to 0.6, corresponding to the mid-exponential phase of growth. A volume of 100 µl of each suspension was then serially diluted from 10^{-1} to 10^{-4} and 5 µl-drops of each dilution were transferred to YPDA or LBA plates containing G18.EE and G20.miEEs at concentrations of 10, 50, 100, 200, 500, 750, 1000, 1500, and 2000 $\mu g ml^{-1}$, or with an equal volume of absolute ethanol, used as control. Plates with only LBA and YPDA were also used as control. Plates were incubated at 30 °C (Heraeus Incubator) for 48 h, for yeasts, and at 37 °C (Incucell MMM MedCenter Incubator) for 24 h, in the case of bacteria, being observed for the presence or absence of growth and photographed using the VWR GenoSmart Gel Documentation System. MIC values were expressed as the lowest concentrations where no growth was verified.

3.7. *In vitro* Evaluation of Propolis Antitumoral Activity in Melanoma

3.7.1. Sample Preparation

G18.EE and its fractions, particularly *n*-Butanol, *n*-Hexane, and ethyl acetate, were dissolved in dimethyl sulphoxide (DMSO) (Honeywell) to obtain stock solutions of 162.1 mg/ml, 172.2 mg/ml, 66 mg/ml, and 234.2 mg/ml, respectively. These stock solutions were used to prepare the working solutions at desired concentrations.

The sample final concentrations used in the subsequent experiments were obtained by diluting the stock solutions in a 0.5% Fetal Bovine Serum (FBS) medium (PAN-Biotech™) (complete medium). DMSO concentrations never exceeded 0.25% in well.

3.7.2. Cell Lines and Culture Conditions

The *in vitro* assays were performed using two distinct human melanoma cell lines: A375 and WM9 **(Figure 12)**, which were cultured in Dulbecco´s Modified Eagle´s Medium (DMEM; PAN-BIOTEC™) supplemented with 10% FBS. Cell lines were incubated at 37 °C in a humidified environment containing 5% CO_2.

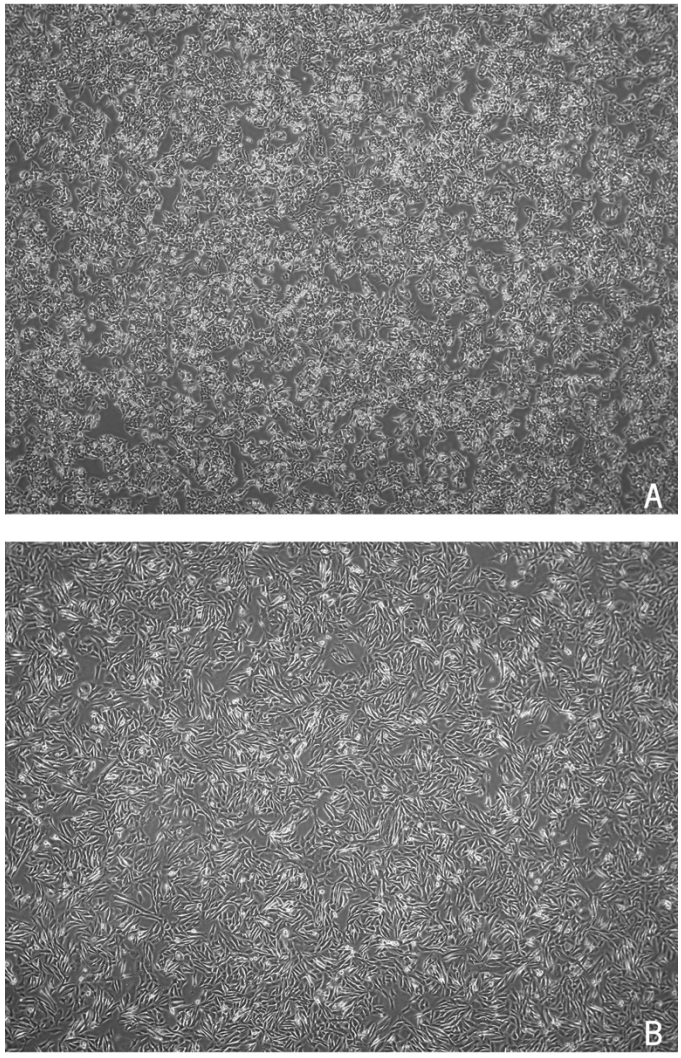

Figure 12 - Microscopy pictures of A375 (A) and WM9 (B) cell lines. Selected cell lines images were taken with 100x magnification (Olympus CKX41 Inverted Phase Contrast Microscope).

The *BRAF*^{V600E} mutant cell lines A375 and WM9 were established from malignant melanoma. A375 Cell line was obtained from Dra. Marta Viana-Pereira (University of Minho, Braga, Portugal) and WM9 cell line from Dr. Josane F. Sousa (University of São Paulo, Ribeirão Preto, Brazil) (Sousa *et al.*, 2010).

3.7.3. Cell Viability Assay

Cell susceptibility of melanoma cell lines to propolis extract and its fractions was analyzed using Sulforhodamine B assay (SRB, TOX-6, Sigma-Aldrich). The SRB assay, one of the most widely used techniques for cytotoxicity screening, was established by Skehan *et al.* (1990) and some years later improved by Vichai & Kirtikara (2006). The assay is based on the ability of SRB, a bright

pink aminoxanthene dye, to bind to protein basic amino acid residues of cells that are attached to culture plates with trichloroacetic acid (TCA), under mildly acidic conditions. SRB can also be isolated from cells and solubilized for measurement under mild basic conditions. This dye's binding is stoichiometric, implying a proportional relationship between SRB extracted from stained cells and cell mass (Orellana & Kasinski, 2016), which indirectly indicates the degree of cytotoxicity caused by the tested samples.

A375 and WM9 cell lines were plated into 96-well plates, at a concentration of $25x10^4$ cells/ml in 200 µl and allowed to adhere overnight at 37 °C and 5% CO_2 (humidified atmosphere). On the following day, plates were subjected to serum-starvation (culture medium without FBS) for 2 h. This deprivation period was performed for cell cycle synchronization. The effect of G18.EE and its fractions *n*-butanol, *n*-hexane, and EtOAc on cell number (total biomass) was determined upon treatments for 72 h (5 to 60 µg/ml), using DMEM supplemented with 0.5% of FBS. DMSO (control) and treatments were carried out at a final concentration of 0.1% DMSO. Triplicate wells were plated for each individual dose. After reaching the specific time point, the medium was discarded, cells were fixed using 100 µl of cold 10% trichloroacetic acid (TCA)(Sigma-Aldrich; CAS Number:76-03-9) for 1 h at 4 °C. The cells were washed four times with de-ionized water and dried at 37°C for 1h30. Then, 50 µl of SRB solution (0.4% SRB in 0.1% acetic acid) were added and incubated at RT for 30 min. After staining, washing was accomplished using 1% acetic acid (to eliminate unbound dye) and dried for 30 min at 37 °C, until no liquid was evident. The dye was solubilized by adding 100 µl of 10 mM Tris base to each well and plates were incubated for 10 min at RT. Absorbance was measured at 490 nm (Thermo Scientific Varioskan Flash). The 15% inhibition concentration (IC_{15}), the 25% inhibition concentration (IC_{25}), and the half-maximal inhibitory concentration (IC_{50}) values were calculated using GraphPad Software Version 8.0. Three independent experiments were carried out, each one conducted in triplicate.

After determining the IC_{15} and IC_{25} values, another SRB test was conducted to evaluate if the concentrations of G18.EE and its *n*-Butanol and EtOAc fractions were cytotoxic for the A375 and WM9 cells after 24 h, 48 h, and 72 h of treatment (5 to 13 g/ml) **(Table 9)**. The methodology adopted is the same as the one described above, except for the treatment time. As previously, DMSO was used as control. Three independent assays were carried out, each one performed in triplicate.

Table 9 – Specific intermediate values of IC_{15} and IC_{25} concentrations of G18.EE and two of its fractions: *n*-Butanol and EtOAc to test against A375 and WM9 melanoma cell lines. The IC values were calculated using SRB assay data, after 72 h of treatment, and Graph Pad Software version 8.0.

	IC_{15} (µg/ml)	IC_{25} (µg/ml)
G18.EE	10	13
G18.EE - EtOAc	8	10
G18.EE -*n*-Butanol	5	7

3.7.4. Protein Extraction and Quantification

To evaluate protein levels through western blot, protein extraction is mandatory. For that, melanoma cell lines were plated in six-well plates at a concentration of $20x10^4$ cells/well in 2 ml of complete medium and allowed to adhere for two days in a humidified atmosphere, at 37 °C and 5 % of CO_2. The culture medium was removed, cells were subjected to a period of 2 h of serum starvation followed by treatment with 100 µg/ml, as well as IC_{15} and IC_{25} concentrations of propolis ethanol extract (G18.EE) (10 and 13 µg/ml respectively), *n*-butanol (5 and 7 µg/ml), and EtOAc (8 and 10 µg/ml) fractions, and with DMSO (control), for further 2 h. DMSO (control) and treatments were carried out at a final concentration of 0.1% DMSO. Then, cells were washed with cold phosphate-buffered saline (PBS) 1x and protein was obtained by scraping the cells after adding lysis buffer (50 mM Tris pH 7.6 – 8, 150 mM NaCl, 5 mM EDTA, 1 mM NaOVa, 10 mM NaF, 10 mM NaPyrophosphatase, 1 % NP-40 and 1:7 of Protease cocktail inhibitors (Roche®)). Lysed cells were collected, kept on ice for half an hour, and centrifuged at 13000 rpm, 4 °C for 15 min. Protein was collected in the final supernatant.

Protein quantification was performed with Bradford reagent (Sigma-Aldrich). The Bradford method is based on the ability of Coomassie Brilliant Blue G-250 dye to bind to proteins, which results in a maximum dye absorption wavelength shift from 465 nm to 590 nm, which can be monitored (Kruger, 1994). For protein quantification, 2 µl of protein extracts were added to wells of a 96-well plate, jointly with 98 µl of PBS 1x and 200 µl of Bradford's reagent. Absorbances were measured at 590 nm after a 5 min incubation period. Protein was quantified using an established calibration curve for bovine serum albumin (BSA) (Sigma Aldrich; CAS Number: 9048-46-8) concentration absorbances.

3.7.5. Western Blot

Western blotting is a common approach for identifying individual proteins in a complex biological mixture and determining their molecular weight. Separation of denatured proteins by size, followed by transfer to a membrane, and selective immunodetection of an immobilized antigen are all part of the gel electrophoresis technique (Silva-Carvalho *et al.*, 2014; Hnasko & Hnasko, 2015).

Protein samples were prepared through the addition of 40 µg of protein to 10 µl of 2x Laemmli Sample Buffer (Bio-Rad Laboratories, Inc.) previously mixed with β-mercaptoethanol (Bio-Rad®). The molecular weight marker (Plus, Grisp) and the samples were loaded onto 10% and 12% acrylamide gels **(Figure 13)** and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V. Protein was transferred into nitrocellulose membranes (Amersham Biosciences®) using transfer buffer (10x Tris/Glycine Buffer (Bio-Rad®)) and 20% methanol (Honeywell) during 30 min in the Trans-Blot Turbo® transfer system (Bio-Rad®) (25 V, 1 A). To prevent unspecific binding, membranes were blocked with TBS-0.1% Tween containing 5% of BSA for 1 hour at RT and incubated overnight with primary antibodies at 4 °C **(Table 10)**. The following day, membranes were washed in TBS-0.1% tween three times, 5 min each, and then incubated with a secondary antibody coupled to horseradish peroxidase (1:2500, Cell Signalling) **(Table 10)**. After this final incubation, membranes were washed 2x5 min and 1x15 min with the same solution previously used. Loading was assessed by Tubulin protein levels at 1:2000 dilution. Signals of the bound antibodies were detected by chemiluminescence (WesternBright ECL HRP substrate, Advansta) using Sapphire Biomolecular Imager (Azure Biosystems).

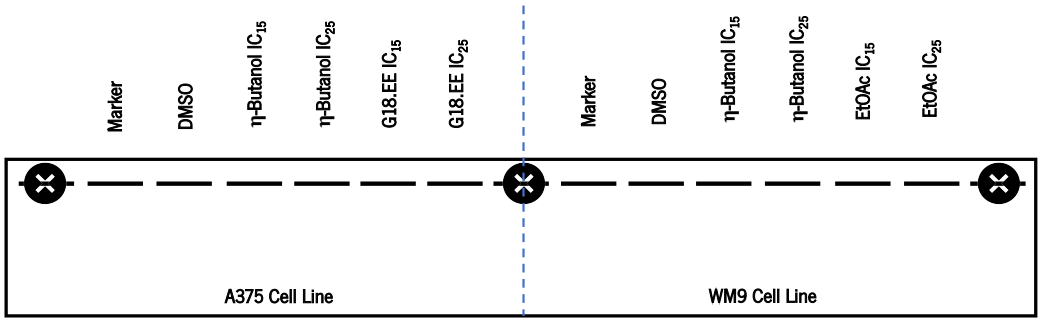

Figure 13 - Schematic representation of the order of protein addition to the electrophoresis gel. Protein was extracted from cells without treatment (Control with DMSO) and cells treated with IC_{15} and IC_{25} concentrations of G18.EE and of its *n*-Butanol and EtOAc Fractions.

Table 10 - Antibodies used in the Western blot. Specification of the dilution of antibodies, animal source, and supplier.

Protein Target	Dilution	Source	Brand
Bax	1:2000	Rabbit	Cell Signaling
Bcl-2	1:1000	Mouse	Santa Cruz Biotechnology
Bcl-xL	1:1000	Mouse	Cell Signaling
Caspase 3_{total}	1:1000	Rabbit	Cell Signaling
Caspase 9_{total}	1:1000	Mouse	Cell Signaling
Catalase	1:1000	Rabbit	Abcam
CD147	1:500	Mouse	Santa Cruz Biotechnology
LDHA	1:1000	Mouse	Cell Signaling
MCT1	1:500	Mouse	Santa Cruz Biotechnology
MCT4	1:500	Mouse	Santa Cruz Biotechnology
p-AMPK	1:500	Rabbit	Cell Signaling
AMPK	1:500	Rabbit	Cell Signaling
PFK-L	1:250	Rabbit	Abcam
p53	1:1000	Rabbit	Cell Signaling
phospho p53	1:1000	Rabbit	Cell Signaling
Tubulin	1:2000	Rabbit	Santa Cruz Biotechnology
Anti-mouse	1:2500	———	Santa Cruz Biotechnology
Anti-rabbit	1:2500	———	Santa Cruz Biotechnology

3.7.6. Metabolism Assay: Extracellular Lactate Measurement

The quantitative lactate determination method is a colorimetric assay based on the oxidation of lactate to pyruvate by lactate oxidase, forming hydrogen peroxide. In the presence of 4-aminophenazone, 4-chlorophenol, and peroxidase, a red quinone forms, which is a colorimetric and fluorometric product, proportional to the lactate concentration present in the medium. A calibration curve based on a range of lactate solutions with different concentrations was performed (Silva-Carvalho, 2013).

The measurement of extracellular lactate was carried out as previously described by Morais-Santos *et al.* (2015). A375 and WM9 cells were plated into 48-well plates at a concentration of $25x10^4$ cells/ml in 300 µl and allowed to attach overnight to the bottom of the plate in a complete medium. On the following day, the medium was changed to DMEM supplemented with 0.5% of

FBS containing specific treatments: IC_{15} and IC_{25} concentrations of G18.EE, *n*-butanol, and EtOAc **(Table 9)**. DMSO was used as control. After 24, 48, and 72 h of incubation, 20 µl of each well media were removed and added to a new 96-well plate to store at -20 °C. From this plate, 2 µl of each well were removed and placed into a new 96-well plate for extracellular lactate quantification, followed by the addition of 100 µl of lactate colorimetric commercial kit (Spinreact) and 10 min incubation at RT. Absorbance was measured at 490 nm (Thermo Scientific Varioskan Flash). Results are expressed as total µg/total biomass. Total biomass was assessed through SRB assay (see section 3.7.3).

3.7.7. Cell Migration/Wound Healing Assay

The migratory capacity of melanoma cell lines was assessed by the Scratch Wound Healing Assay, which is based on the creation of a "wound gap" in a cell monolayer and subsequently monitoring of the gap "healing" by cell migration. This assay is a simple and inexpensive method that mimics cell migration during wound healing *in vitro* (Rodriguez *et al.*, 2005), thus allowing to estimate the migratory capacity of melanoma cells after the desired treatments. As previously described by Martinho *et al.* (2012), A375 and WM9 melanoma cell lines were seeded onto a 12-well-plate, at a density of $9x10^5$ and $5x10^5$ cells/well respectively and allowed to adhere overnight in a humidified atmosphere at 37 °C and 5% CO_2. The following day, the cell monolayer (nearly 95% of confluence) was "wounded" through scraping with a 200 µl pipette tip and washed once with PBS 1x. Next, cells were covered with DMEM supplemented with 0.5% of FBS containing IC_{15} and IC_{25} concentrations **(Table 9)** of G18.EE and its *n*-Butanol and EtOAc fractions. DMSO was used as a control.

To evaluate the percentage of wound coverage, specific scratching sites of the wound were analyzed and photographed at 0 h (immediately after scratching and medium addition), 3, 6, 9, 12, 24, 48 and 72 h or until complete closure of the wound. Image analyses were performed using beWound 1.7.1 version software (besurg) (Morais-Santos *et al.*, 2015) to address the width of the wound. Results were calibrated considering the width on day 0. This migration assay was performed in triplicate, with three independent assays.

3.7.8. Three-dimensional (3D) Cell Culture Methods (Spheroids)

Three-dimensional cultures are increasing in popularity due to their ability to reflect the tumor microenvironment (TME), which is an advantage when compared to two-dimensional (2D) cell cultures. This 3D models mimic the mass of solid tumors in a better way (Celeiro *et al.*, 2020).

Tumor spheroids of A375 melanoma cells were obtained by agarose 1.5% coating method in 48-well plates. Cells were plated onto 48-well plates, at a density of 2500 cells/well and allowed to grow and form spheroids over a four-day period.

3.7.8.1. Assessment of Tumor Spheroid Growth

Melanoma cell lines were assessed for tumor spheroid growth as described by Pires Celeiro (2018). On day 4 of tumor spheroid formation, spheroids were treated with 0.5% FBS DMEM containing the specific treatments: G18.EE, *n*-butanol, and EtOAc IC_{15} and IC_{25} concentrations **(Table 9)** (final volume of 300 µl). Photographs were taken on days 4 (corresponding to day 0, when the therapy began), 7, 10, 12, and 14. The area of the tumor spheroid was calculated using ImageJ software, and the results were normalized for the spheroid area acquired on day 4 (considered 0%). Three independent assays were carried out.

3.7.8.2. Tumor Spheroid Migration Assay

Tumour spheroid migration was determined according to Pires Celeiro (2018). On day 4th, these 3D structures were transferred to a 96-well plate. The 96-well plate was previously coated with 50 µl of gelatine 0.1% (v/v). Spheroids were treated with 0.5% FBS DMEM containing the specific treatment: G18.EE, *n*-butanol, and EtOAc at IC_{15} and IC_{25} concentrations **(Table 9)** (final volume of 200 µl). Migration was assessed at 0, 24, 48, and 72 hours. ImageJ software was used to analyze the taken photographs. The spheroid area acquired on day 0 was used to calibrate the results, which were set to 0%. Three independent assays were performed.

3.7.9. ROS Production and Mitochondrial Membrane Potential

The influence of G18.EE and its two fractions, *n*-butanol and EtOAc, on ROS production and mitochondrial activity was evaluated as previously described Miranda-Gonçalves *et al.* (2017). A375 and WM9 cell lines were plated at a density of $80x10^4$ cells/well and allowed to adhere overnight in a complete medium. The medium was discarded and DMEM supplemented with 0.5% FBS was added. For both assays, cells were treated with DMSO (control), 100 µg/ml and IC_{15} and IC_{25} concentrations of propolis extract and its fractions **(Table 9)**. DMSO (control) and treatments

were carried out at a final concentration of 0.1% DMSO. After 24 h, adherent cells were incubated with molecular probes for a period of 4 h at 37 °C in the dark. 10 mM of Dihydroethidium (DHE, Molecular Probes) was used to assess ROS production. For mitochondrial polarization and mitochondrial biomass, 50 nM of Mitotracker Red and 30 nM of Mitotracker Green (Molecular Probes) were employed, respectively. Cells and respective supernatants were then collected into cytometry tubes and centrifuged for 5 min at 900 rpm and 4 °C. Cells were washed with PBS 1x and centrifuged again under the same conditions. Lastly, PBS 1x was removed and 300 µl of Fluorescence-activated Cell Sorting (FACs) was added to each flow cytometer tube. Analysis of ROS production and mitochondrial activity was performed by Flow Cytometry (BD LSR II). Assays were carried out in duplicate, in three independent experiments.

3.8. Statistical Analyses

Results were expressed as mean ± standard deviation (SD) and statistically analyzed using the GraphPad Prism 8 software. Comparisons between different conditions were performed using the Two-way ANOVA test (Cell Viability Assay, Wound-Healing Assay, Tumor Spheroids Migration Assay, Tumor Spheroid Growth Assay, and Extracellular Lactate Measurement) and One-way ANOVA test (Metabolic Markers Expression Levels, DHE, ROS and Mitochondrial Membrane potential). The threshold used for statistical significance was $p<0.05$.

Chapter 4 - Results

Samples of Portuguese propolis from Gerês (G) are usually obtained by mixing propolis from three different locations - Bugalho (b), Toutelo (t), and Felgueiras (f) (Freitas *et al.*, 2018). This mixture has shown consistent chemical and biological profiles over time, being this constancy an unique aspect of Gerês propolis , that has renewed the interest in this research (Freitas *et al.*, 2019). In 2017, a fire destroyed the apiary located in Bugalho and, as result, propolis from Gerês started to be harvested from a different combination of three apiaries (Roca (r), Toutelo (t) and Felgueiras (f)). However, G20 was only collected from Toutelo and Felgueiras. Thus, we decided to study $G20_t$ and $G20_f$ alone as well as the mixture of both propolis samples $G20_{tf}$ to understand whether the biological and chemical profiles of these propolis samples remain similar to the previous years.

4.1. G18.EE exhibits higher antibacterial activity than G20.miEEs

Ethanol microextraction was performed to obtain the dried ethanol micro-extracts $G20_t$.miEE, $G20_f$.miEE, and $G20_{tf}$.miEE, with extraction yields of 57.8%, 59.3%, and 58.1%, respectively, and antimicrobial activity of these G20.miEEswas evaluated using an adaptation of the agar dilution method. For each microorganism tested, MIC values were calculated by observing the lowest concentration at which no growth occurred **(Table 11)**.

Table 11 - MIC values (µg/ml) of G20.miEEs against the panel of susceptibility indicator strains.

		MIC (µg/ml)		
	Strains	**$G20_t$.miEE**	**$G20_f$.miEE**	**$G20_{tf}$.miEE**
Gram-positive bacteria	*Bacillus cereus*	50	100	100
	Bacillus megaterium	50	100	100
	Bacillus subtilis	50	100	100
	Methicillin-sensitive *Staphylococcus aureus* (MSSA)	1000	750	750
	Methicillin-resistant *Staphylococcus aureus* (MRSA)	>2000	>2000	>2000
	Propionibacterium acnes	>2000	>2000	>2000
Gram-negative bacteria	*Escherichia coli*	>2000	>2000	>2000
Yeast	*Candida albicans*	>2000	2000	>2000
	Saccharomyces cerevisiae	>2000	2000	>2000

In general, bacteria are more vulnerable to the activity of the G20.miEEs when compared to yeast. Gram-positive bacteria are more susceptible to the micro-extracts action than Gram-negative ones, with the genus *Bacillus* being the most affected (MIC = 50 or 100 µg/ml). No activity or similar MIC values (MIC=2000 µg/ml) were obtained against MRSA, the methicillin-resistant *Staphylococcus aureus* strain; the Gram-positive bacteria *P. acnes*; the Gram-negative *E. coli*, and the yeast *C. albicans* and *S. cerevisiae*. Despite having a consistent pattern, G20.miEEs reveal differences in MIC values: $G20_t$.miEE is the most active micro-extract against *Bacillus* bacteria, whereas $G20_f$.miEE is the most promising extract against yeast-sensitive indicator strains. *S. aureus* is more susceptible to the action of $G20_f$.miEE and $G20_{tf}$.miEE, the extract resulting from a mixture of both propolis samples.

Ethanol extracts of propolis from Gerês such as G18.EE have been showing strong antibacterial properties, particularly against Gram-positive bacteria (Freitas *et al.*, 2022). Considering this, we tested the antimicrobial activity of G18.EE **(Table 12)**, an extract prepared in 2018 (Freitas *et al.*, 2022) and preserved at -20 °C until 2021, to compare its activity with that of G20.miEEs and select the best G20.miEE for use in the subsequent experiments.

Table 12 - MIC values (µg/ml) of G18.EE against the panel of susceptibility indicator strains.

		MIC (µg/ml)
	Strains	**G18.EE**
Gram-positive bacteria	*Bacillus cereus*	50
	Bacillus megaterium	50
	Bacillus subtilis	50
	Methicillin-sensitive *Staphylococcus aureus* (MSSA)	500
	Methicillin-resistant *Staphylococcus aureus* (MRSA)	>2000
	Propionibacterium acnes	500
Gram-negative bacteria	*Escherichia coli*	>2000
Yeast	*Candida albicans*	>2000
	Saccharomyces cerevisiae	>2000

Gram-negative bacteria and yeast are less sensitive to G18.EE than Gram-positive bacteria, according to the observed sensitivity pattern of indicator strains, which is identical to that reported for G20.miEEs and generally known for propolis antibacterial activity. The genus *Bacillus* (MIC = 50 µg/ml), Methicillin-sensitive *Staphylococcus aureus* (MSSA) (MIC=500 µg/ml) and *P. acnes* (MIC=500 µg/ml) are the most susceptible microorganisms to G18 ethanol extract action.

Based on the specific values of MIC against each bacterium or yeast tested, we can infer that G18.EE has stronger antimicrobial activity than any of the dried ethanol micro-extracts $G20_t$.miEE, $G20_f$.miEE, and $G20_{tf}$.miEE **(Table 13)**. G18.EE showed the lowest MIC values against *Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Staphylococcus aureus* (MSSA), and *Propionibacterium acnes*.

Table 13 - Comparison between G20.miEEs and G18.EE MIC values (µg/ml) against the indicator strains. The best MIC values are highlighted in red and bold.

	MIC (µg/ml)			
	$G20_t$miEE	**$G20_f$.miEE**	**$G20_{tf}$.miEE**	**G18.EE**
Bacillus cereus	**50**	100	100	**50**
Bacillus megaterium	**50**	100	100	**50**
Bacillus subtilis	**50**	100	100	**50**
Methicillin-sensitive *Staphylococcus aureus* (MSSA)	1000	750	750	**500**
Methicillin-resistant *Staphylococcus aureus* (MRSA)	>2000	>2000	>2000	>2000
Propionibacterium acnes	>2000	>2000	>2000	**500**
Escherichia coli	>2000	>2000	>2000	>2000
Candida albicans	>2000	**2000**	>2000	>2000
Saccharomyces cerevisiae	>2000	**2000**	>2000	>2000

4.2. G18.EE displays stronger radical scavenging activity than G20.miEEs

To evaluate the free-radical scavenging activity of the ethanol extract of propolis collected from Gerês in 2018 (G18.EE) and the G20 ethanol micro-extracts ($G20_t$.miEE, $G20_f$.miEE, and $G20_{tf}$.miEE), we employed a methodology based on the reduction of a stable free radical, the DPPH•. The value of EC_{50} (concentration that generates half of the maximal response) determined for the G18.EE was 10.90 ± 0.34 μg/ml **(Table 14)** lower than the EC_{50} values obtained for the G20 micro-extracts - 20.68 ± 1.98 μg/ml for $G20_t$.miEE; 18.37 ± 0.67 μg/ml for $G20_f$.miEE; and 21.49 ± 1.15 μg/ml for $G20_{tf}$.miEE. According to Sheng *et al.* (2007) a natural substance can be recognized as a possible natural antioxidant if it exhibits DPPH• scavenging activity. As a result, we can assume that G18.EE and G20.miEEs might be potential natural antioxidants, with the first being significantly stronger. Gallic acid, used as a conventional standard for DPPH assay, displayed anEC_{50} value of 1.21 ± 0.08 μg/ml.

The 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay was also used to assess and confirm the higher antioxidant activity of G18.EE. ABTS assay uses the absorbance of the ABTS• colored radical cation to measure a compound's antioxidant capacity. Trolox was used as a standard and the EC_{50} value for G18.EE was 9.83 ± 0.21 μg/ml **(Table 14)**, in line with the previous results of the DPPH assay.

Table 14 - DPPH• and ABTS• scavenging activities of G18.EE and G20.miEEs. Results are expressed in EC_{50} (μg/ml), as mean ± standard deviation (SD). Gallic acid and Trolox were used as standards for the DPPH and ABTS assays, respectively.

	DPPH•	ABTS (Absolute Ethanol)
	EC_{50} (μg/ml)	EC_{50} (μg/ml)
G18.EE	10.90 ± 0.34	9.83 ± 0.21
$G20_t$.miEE	20.68 ± 1.98	-----*
$G20_f$.miEE	18.37 ± 0.67	-----*
$G20_{tf}$.miEE	21.49 ± 1.15	-----*
Gallic Acid	1.21 ± 0.08	-----
Trolox	-----	3.46 ± 0.22

"-----" = value not applicable ; "-----*" = value not determined for lack of sample

We decided to pursue and focus our work with the G18 ethanol extract since its significant antibacterial activity has been validated and preserved after 3 years of storage at -20 °C, and it outperforms the antibacterial potential of G20 micro-extracts **(Table 13)**. In fact, none of the three micro-extracts of G20 exhibit the exact antimicrobial profile of G18.EE. Other major factors that influenced this decision included the higher antioxidant potential of G18.EE compared to G20 micro-extracts **(Table 14)**, and meanwhile, the confirmation and report of G18.EE promising antitumoral effectiveness against Renal Cell Carcinoma (RCC) by Freitas *et al.* (2022).

4.3. G18 fulfills propolis quality requirements

The sample of propolis used in this research, from the Gerês region, was collected in 2018 and stored at 4 °C until 2021. G18 was quite aromatic and had a dark-brownish coloration **(Figure 14)**, both typical characteristics of this type of propolis. A few impurities visible to the human eye were identified, such as threads from scraping nets, fragments of wood, and small parts of bees and other insects.

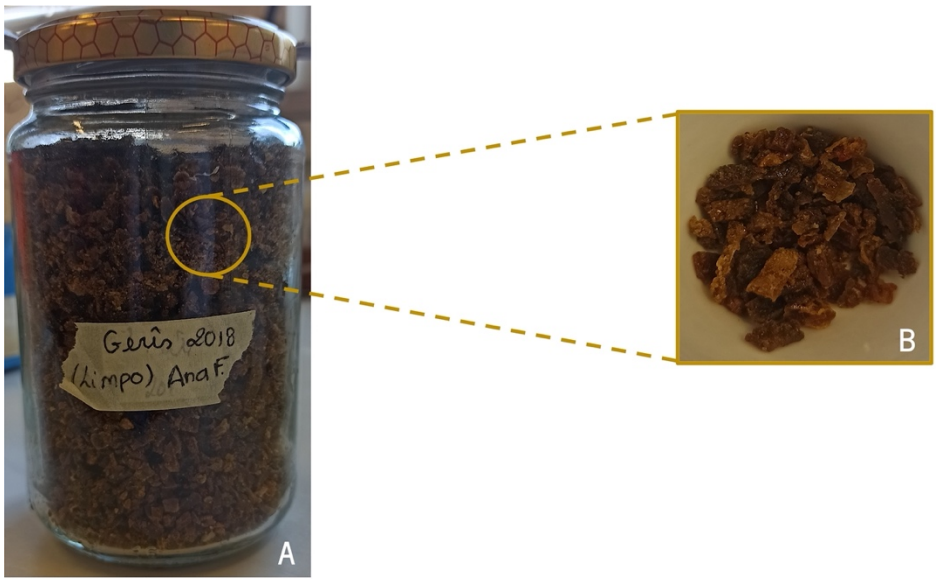

Figure 14 - Propolis sample from Gerês harvested in 2018. Crude sample before **(A)** and after **(B)** fragmentation into small pieces and removal of visible impurities.

In addition to the presence or absence of mechanical contaminants, parameters such as water, ash, wax, and balsamic contents must be considered when evaluating propolis quality (Lopes *et al.*, 2017). These analyses were performed in this work as such data was unavailable and the values obtained for the 3-years stored G18 crude sample are within the maximum and

minimum limits established in the literature by the Technical Regulation of Propolis Identity and Quality (TRPIQ) (2000) (Pereira *et al.*, 2020), Bogdanov (2011), and Falcão (2013), for the referred parameters **(Table 15)**.

Table 15 - Quality requirements determined for the crude sample of G18 and comparison with values established by the TRPIQ (2000), Bogdanov (2011) for European propolis, and with those proposed by Falcão (2013) as a reference for Portuguese propolis types I and II. Results obtained for the G18 sample are expressed as mean ± SD. Three independent assays were carried out in triplicate.

	G18	TRPIQ Brazilian Propolis	European Propolis	Portuguese Propolis	
				Type I	Type II
Dry residue free of volatile substances (% m/m)	5.25 ± 0.30	≤ 8	Non-specified	≤ 5	≤ 5
Ash Content (% m/m)	0.73 ± 0.06	≤ 5	Non-specified	≤ 2	≤ 4
Beeswax Content (% m/m)	2.18 ± 0.19	≤ 25	≤ 25	≤ 25	≤ 31
Balsamic Content (% m/m)	56.67 ± 13.12	Non-specified	≥ 45	≥ 65	≥ 45

The amount of water present in the G18 is 5.25 ± 0.30 % is quite closer to the TRPIQ and Falcão (2013) prescribed limits **(Table 15)**. Ash, when present in large amounts, reduces the bioactive compounds in propolis (Pereira *et al.*, 2020), but G18 showed a content of 0.73 ± 0.06 %, which is significantly lower than the established limits reported in the literature **(Table 15)**. Beeswax content, like the ash content, might affect propolis commercial value because a high quantity of wax leads to a reduction in bioactive compounds (Pereira *et al.*, 2020). G18 presented 2.18 ± 0.19 % of wax, a value that is well below the maximum threshold considered acceptable for European and Portuguese type I propolis **(Table 15)**, which includes propolis from northern regions of Portugal like G18. Lastly, the balsamic content of G18 was 56.67 ± 13.12 %, being, in general, considerably higher than the minimum limits **(Table 15)**. This is one of the most important propolis quality indicators since it refers to the ethanol-soluble fraction, which contains the bioactive

phenolic compounds as well as other product components such as esters, ketones, and alcohols (Burdock, 1998; Bankova *et al.*, 2016; Bogdanov, 2016).

To summarize, since all the quality metrics assessed for G18 are within acceptable limits for high-quality propolis, it is reasonable to assume that it is a good quality sample with potential applications. This finding is extremely significant to us since these values were obtained 4 years after sample collection and storage and we evaluated propolis as an antitumoral agent against *BRAF*-mutated melanoma cells.

4.4. Antitumoral activity of G18.EE and its fractions on melanoma cells

The ethanol extract of propolis from Gerês (G18.EE) was fractionated into *n*-hexane, ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH) fractions (Freitas *et al.*, 2022). To evaluate G18.EE and its fractions antitumoral potential in *BRAF*-mutated melanoma cells we first established the half-maximal inhibitory concentration (IC_{50}), the 25% inhibition concentration (IC_{25}), and the 15% inhibition concentration (IC_{15}) for each component and cell line used.

4.4.1. G18.EE and its fractions decrease melanoma cell viability in a dose-dependent manner

The cytotoxic effect of G18.EE and its fractions was evaluated in A375 and WM9 human *BRAF*-mutated melanoma cell lines through the SRB assay. In **Figure 15** it is possible to observe a decrease in melanoma cells biomass after a 72-h treatment with G18.EE and its fractions in a dose-dependent manner. However, melanoma cells viability is affected differently by the various fractions tested: A375 cells **(Figure 15A)** appear to be more sensitive to G18.EE, *n*-BuOH, and EtOAc fractions, whereas WM9 cells **(Figure 15B)** are more sensitive to *n*-BuOH and EtOAc. The *n*-hexane fraction was the least active and *n*-BuOH the most active fraction against both melanoma cell lines tested.

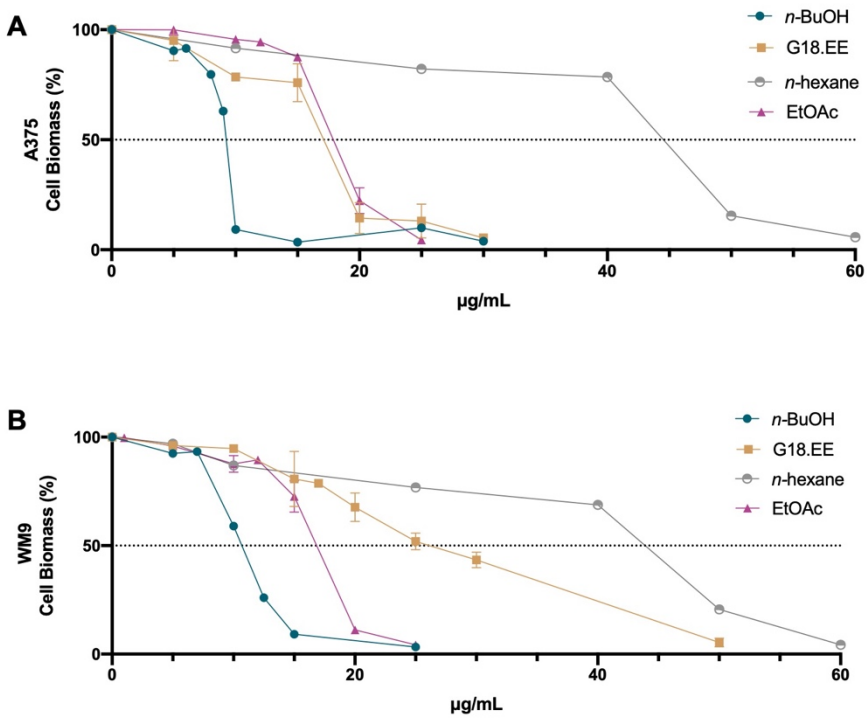

Figure 15 - Effect of G18.EE and its fractions on total cell biomass of melanoma cells. A375 **(A)** and WM9 **(B)** cell lines were treated with a range of concentrations (5 to 60 µg/ml) of propolis extract (G18.EE) and its fractions (*n*-hexane, EtOAc, and *n*-BuOH) for 72 h to determine the IC_{50} concentrations. Cell biomass was measured by the Sulphorhodamine B (SRB) assay. At each time point, data was normalized for total biomass. Results represent the mean ± SD of three independent experiments carried out in triplicate.

The IC_{50}, IC_{25}, and IC_{15} values **(Table 16)** were calculated through the curve obtained in **Figure 15**. For subsequent studies, we chose the two treatments with the lowest IC_{50} value for each cell line: *n*-BuOH and G18.EE for A375 cells; *n*-BuOH and EtOAc for WM9 cells. As previously mentioned, *n*-hexane was the fraction with the lowest toxicity for both cell lines.

Table 16 - IC_{50}, IC_{25}, and IC_{15} values of the Portuguese propolis ethanol extract under study (G18.EE) and respective fractions (*n*-hexane, EtOAc, and *n*-BuOH) against melanoma cell lines. A375 and WM9 cells were treated for 72 h with 5 to 60 µg/ml of each fraction. Results are expressed as mean ± SD.

	IC_{50} (µg/ml)		IC_{25} (µg/ml)		IC_{15} (µg/ml)	
	A375	WM9	A375	WM9	A375	WM9
G18.EE	16.98 ± 0.93	25.03 ± 1.34	10.85 ± 0.12	15.32 ± 0.14	8.88 ± 0.12	12.05 ± 0.14
n-hexane	45.71 ± 1.69	39.54 ± 0.17	24.79 ± 0.09	20.49 ± 0.04	19.13 ± 0.11	15.48 ± 0.04
EtOAc	17.12 ± 0.72	16.39 ± 0.46	12.8 ± 0.03	8.56 ± 0.11	10.9 ± 0.09	6.69 ± 0.15
n-BuOH	8.14 ± 0.03	11.22 ± 1.66	6.16 ± 0.10	8.08 ± 0.22	4.57± 0.09	6.01 0.11

4.4.2. Melanoma cell viability for IC_{15} and IC_{25} concentrations of G18.EE and its fractions

Instead of using the IC_{15} and IC_{25} concentrations of each fraction for each cell line **(Table 16)**, we selected an intermediate concentration of each fraction **(Table 17)** for both cell lines for the following assays. For example, the IC_{25} values of *n*-butanol for A375 and WM9 cells were 6.16 µg/ml and 8.08 µg/ml, respectively, therefore we chose 7 µg/ml as the IC_{25} value for *n*-BuOH. The cytotoxicity of these IC_{15} and IC_{25} concentrations was assessed by SRB assay over time **(Figure 16)**.

Table 17 - IC_{15} and IC_{25} intermediate values selected for G18.EE and η-BuOH and EtOAc fractions against melanoma cells.

	IC_{15} (µg/ml)	IC_{25} (µg/ml)
G18.EE	10	13
EtOAc	8	10
***n*-BuOH**	5	7

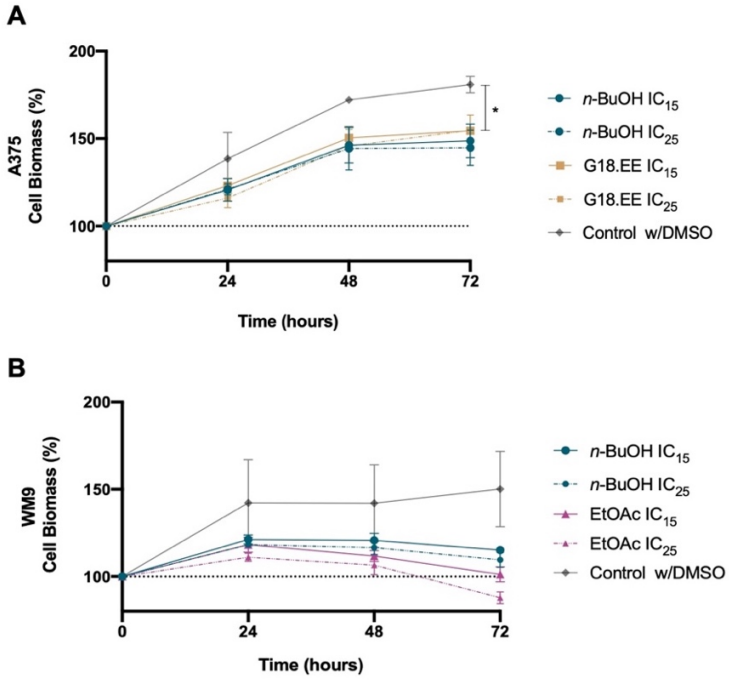

Figure 16 - Effect of the two selected concentrations of propolis fractions on cell biomass (A375 and WM9 cells). Cell biomass was measured at 24, 48, and 72 h by SRB assay after treatment with IC_{15} and IC_{25} concentrations of **(A)** *n*-BuOH (5 and 7 µg/ml) and G18.EE (10 and 13 µg/ml) for the A375 cell line and of **(B)** *n*-BuOH (5 and 7 µg/ml) and EtOAc (8 and 10 µg/ml) for the WM9 cell line. Results are expressed as mean ± SD. Three independent experiments were carried out in triplicate.* $p<0.05$.

G18.EE, *n*-BuOH, and EtOAc, even at lower doses, have an impact on melanoma cell viability at the initial time points **(Figure 16)**. Statistical analyses verified if these lower doses affected melanoma cell biomass over time and independently. There was no statistically significant association between the effects of treatment on cell biomass over time, in the A375 cell line (p=0.5901; **Appendix 1**). Simple main effects analysis showed however that time and treatment independently have a significant influence on cell biomass (p<0.0001; p=0.0205; respectively; **Appendix 1**). Respective multiple comparisons to the control demonstrated that G18.EE IC_{15} has a significant effect on A375 cell biomass at 72 h (p=0.0406; **Figure 16A**). Regarding the WM9 cell line, a statistically significant association was verified between the effects of treatment and time (p=0.0074). Time has an independent statistically significant influence (p=0.0013) whereas treatment did not significantly affect cell biomass (p=0.1252).

4.4.3. G18.EE and its fractions decrease the migratory capacity of *BRAF*-mutated melanoma cells

Cell migration is an energy-intensive mesenchymal feature associated with the invasion-metastasis cascade (Zanotelli *et al.*, 2021). Indeed, abnormal cell migration regulation is crucial to the progression of cancer cell invasion and metastasis into surrounding tissues, generating secondary tumors (Yamaguchi & Condeelis, 2007).

Melanoma can rapidly disseminate to a variety of organs, including the liver, bone, lung, skin, muscle, and brain (Alqathama, 2020). Thus, the effect of IC_{15} and IC_{25} intermediate values **(Table 17)** of G18.EE and of G18.EE-fractions in the migratory capacity of A375 and WM9 human *BRAF*-mutated melanoma cell lines was evaluated through the wound-healing assay (Martinho *et al.*, 2012) (2D Cell Culture Model) over time **(Figures 17** and **18)**. G18.EE and its selected fractions have an impact on melanoma cell migratory capacity. Time and treatment individually had a substantial impact on the migratory capacity of the A375 (p<0.0001; p<0.0001; respectively; **Appendix 2**) and WM9 (p<0.0001; p<0.0001; respectively; **Appendix 2**) melanoma cell line. In addition, a statistically significant association between treatment and time was confirmed (p<0.0001 for both A375 and WM9 cell lines; **Appendix 2**).

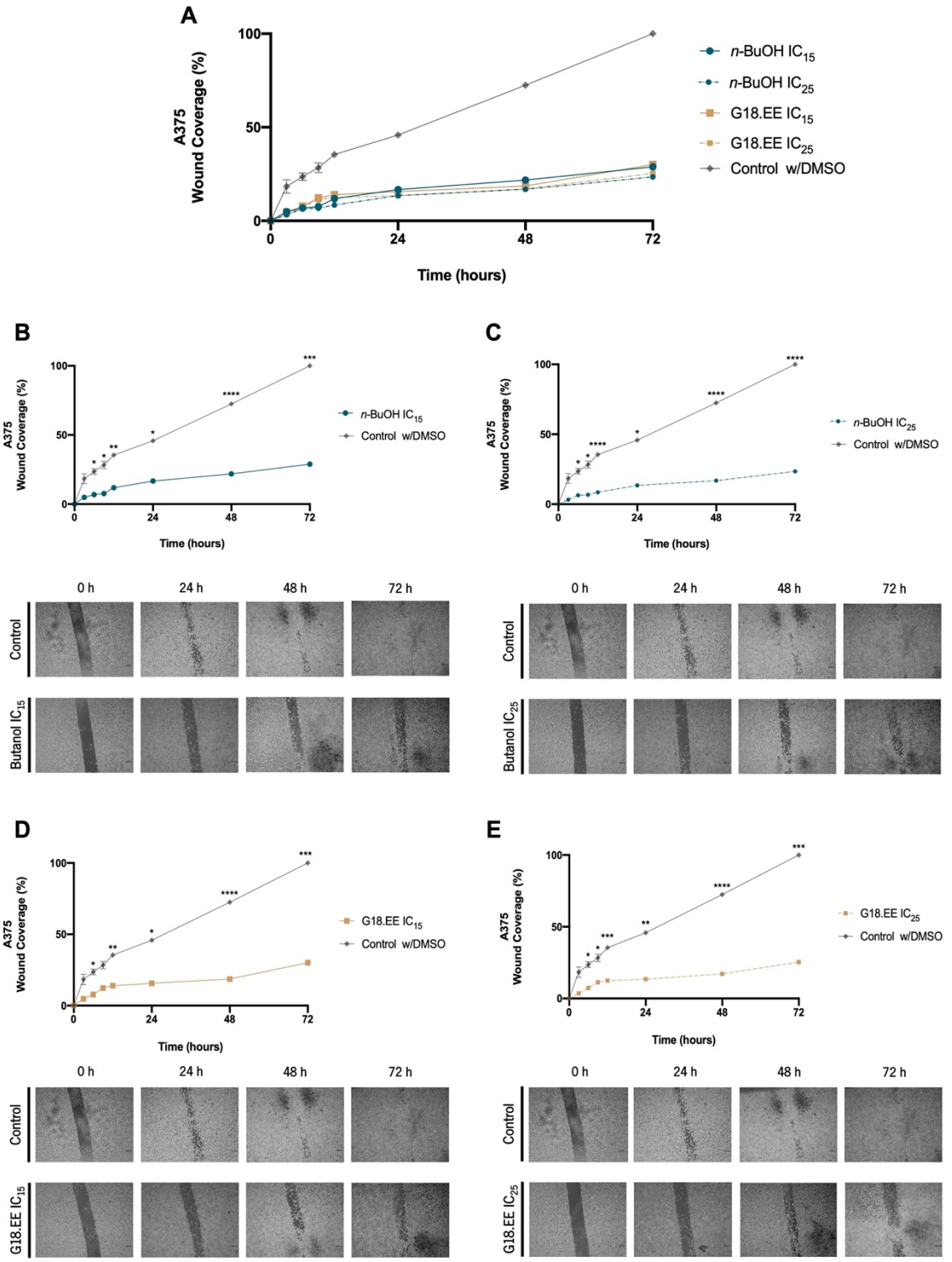

Figure 17 - Effect of the G18.EE and its selected fractions on the A375 melanoma cell line migration. Wound healing assay of A375 cells treated with DMSO and **(A)** G18.EE and its selected fractions, specifically **(B)** (5 µg/ml) *n*-BuOH IC_{15}; **(C)** (7 µg/ml) *n*-BuOH IC_{25}; **(D)** (10 µg/ml) G18.EE IC_{15} and **(E)** (13 µg/ml) G18.EE IC_{25}. Cell migration normalized to time 0 was evaluated through a 72 h period. Results are expressed as mean ± SD. These results are relative to three independent assays carried out in triplicate. Statistical analyses were performed using the Two-Way ANOVA test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (Images were taken at x40 magnification).

Multiple comparisons test demonstrated that the migratory capacity of A375 cells was significantly lower with G18.EE and *n*-butanol throughout time, when compared to the control condition **(Figure 17)**. Specifically, at the time points 6, 9, 12, 24, 48, and 72 h statistically significant differences were obtained after treatment with *n*-BuOH IC_{15} (p=0.0324; p=0.0337; p=0.0016; p=0.0055; p=0.0001 and p=0.0003; respectively; **Figure 17B**); *n*-BuOH IC_{25} (p=0.0304; p=0.0309; p<0.0001; p=0.0012; p=0.0001 and p=0.0001; respectively; **Figure 17C**) and G18.EE IC_{25} (p=0.0337; p=0.0491; p=0.0002; p=0.0066; p=0.0001 and p=0.0001; respectively; **Figure 17E**). Relatively to the G18.EE IC_{15} treatment, non-significant differences were observed at 9 h (p=0.0550; **Figure 17D**), contrarily to what happened at the time points 6, 12, 24, 48 and 72 h (p=0.0337; p=0.0017; p=0.0064; p=0.0001 and p=0.0001; respectively; **Figure 17D**).

Regarding the WM9 cell line, respective multiple comparisons to the control demonstrated that *n*-butanol and EtOAc significantly decreased cells' migration capability **(Figure 18)**. In fact, we observed statistically significant differences at the time points 12, 24, and 48h after treatment with *n*-BuOH IC_{15} (p=0.0121; p=0.0124; p=0.0002; respectively; **Figure 18B**), *n*-BuOH IC_{25} (p<0.0001; p=0.0135; p=0.0001; respectively; **Figure 18C**), EtOAc IC_{15} (p=0.0334; p=0.0183; p<0.0001; respectively; **Figure 18D**), and EtOAc IC_{25} (p=0.0371; p=0.0103; p=0.0005; respectively; **Figure 18E**). Regarding the WM9 cells treated with *n*-BuOH IC_{25}, significant differences were also obtained at 9 h (p=0.0387; **Figure 18C**). Overall, the latest time points, namely 48 and 72 h, exhibited a greater statistical significance.

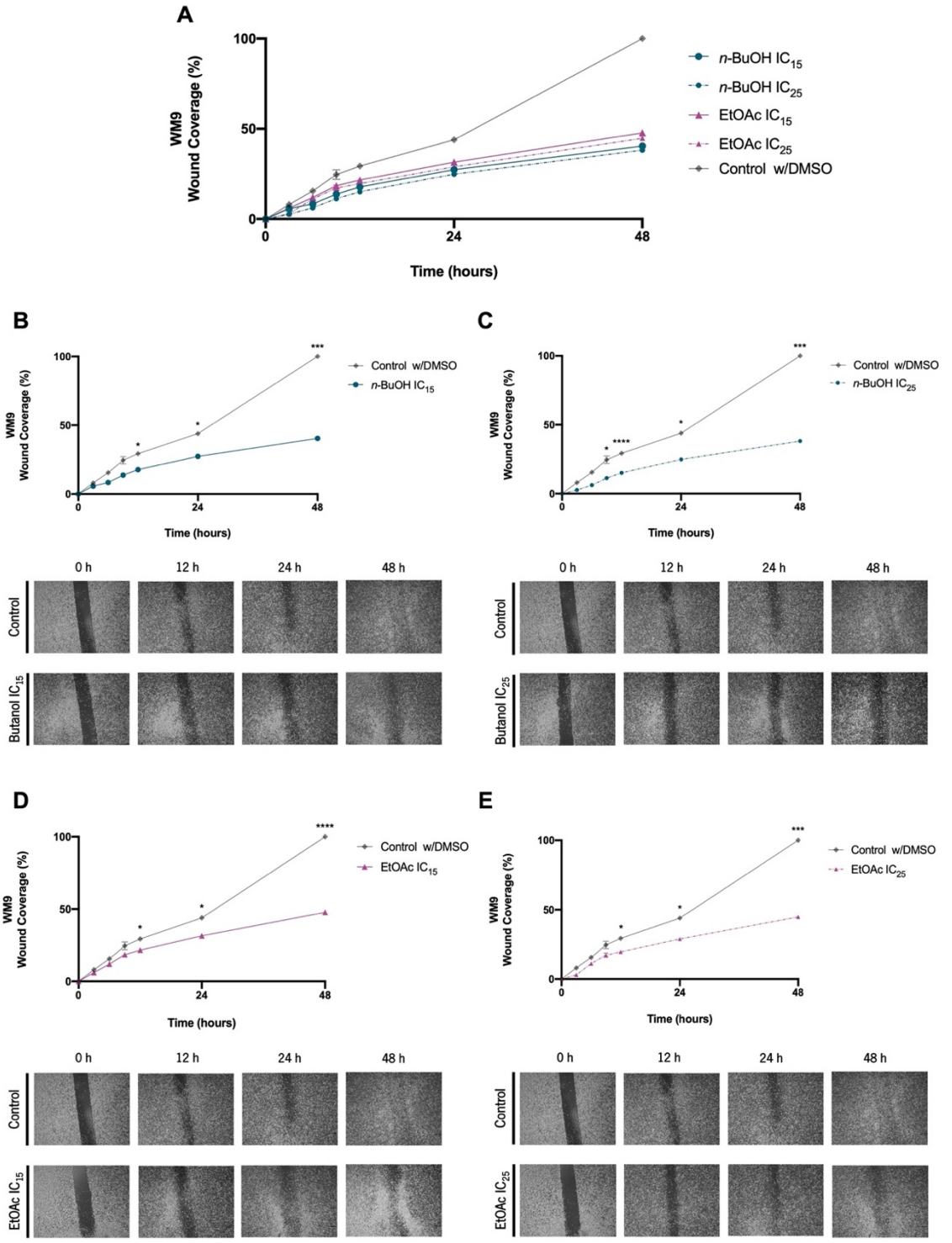

Figure 18 - Effect of the G18.EE selected fractions - *n*-butanol and EtOAc - on the WM9 melanoma cell lines migration. Wound healing assay of WM9 cells treated with DMSO and **(A)** G18.EE selected fractions, specifically **(B)** (5 µg/ml) *n*-BuOH IC_{15}; **(C)** (7 µg/ml) *n*-BuOH IC_{25}; **(D)** (8 µg/ml) EtOAc IC_{15}, and **(E)** (10 µg/ml) EtOAc IC_{25}. Cell migration normalized to time 0 was evaluated through a 72-h period. Results are expressed as mean ± SD. These results are relative to three independent assays carried out in triplicate. Statistical analyses were performed using the Two-Way ANOVA test. *p<0.05, **p<0.01; ***p<0.001, ****p<0.0001 (Images were taken at x40 magnification).

Thus, by comparing all the A375 and WM9 cell line results (**Figures 17A** and **18A**; respectively), we may deduce that *n*-butanol treatment had the highest impact on cell migration, resulting in the lowest percentage of wound coverage in the final time point.

4.4.4. Three-dimensional (3D) Cell Culture Model

Three-dimensional (3D) cell culture models may be an intermediary step between the two-dimensional (2D) *in vitro* assays and the *in vivo* experiments. Since solid tumors grow as a 3D conformation and tumor cells are exposed to suboptimum conditions, such as cell-cell interactions, hypoxia, or low nutritional levels, 3D culture systems can better mimic *in vivo* growth conditions and allow researchers to investigate the broader aspects of tumor biology (Vinci *et al.*, 2012; Ishiguro *et al.*, 2017). In standard 2D cell cultures, the complexity and heterogeneity of tumors *in vivo* may be lost because of the cellular adaptability that is required for survival *in vitro* (Ishiguro *et al.*, 2017).

The formation of A375 and WM9 spheroids was attempted by employing two distinct methodologies: the agarose 1.5% coating (Pires Celeiro, 2018) and the hanging drop (Foty, 2011), which were carried out in 48-well and Petri dishes, respectively. However, spheroids obtained through the hanging drop approach were loose and difficult to transfer **(Figure 19A)**. Compact and tight spheroids are crucial aspects for collecting this cell culture tumor model and measuring growth and migration over time. As a result, we proceeded with the agarose 1.5% coating technique and, after procedure optimization, three assays were performed to optimize the number of cells per spheroid. However, WM9 spheroids disintegrated over time with this methodology, and we were unable to conduct 3D cell culture studies with this cell line **(Figure 19B)**. So, only the A375 cell line was used for size optimization and evaluation of tumor spheroid growth and migration capacity. The cells concentration chosen for the further 3D cell culture assays was 2500 cells/ml because spheroids were tight, compact, and have a perfect size for growth and migration measurements (diameter fits within the size range of 300 to 500 μM, at day 4) **(Figure 19C)** (Vinci *et al.*, 2012; Pires Celeiro, 2018).

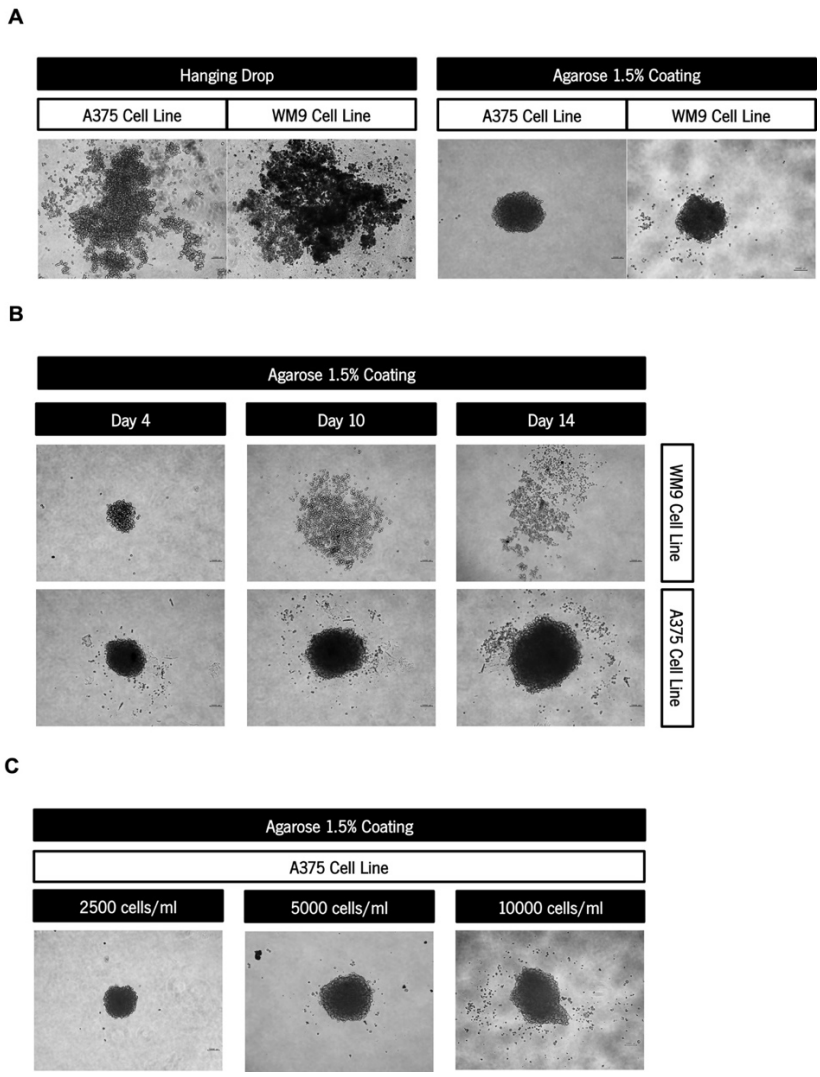

Figure 19 – A375 and WM9 spheroids for 3D cell culture assays: procedure and size optimization. (A) The development of A375 and WM9 tumor spheroids was evaluated using two different approaches: hanging drop and agarose 1.5% coating (procedure optimization); **(B)** Tumor spheroids of A375 and WM9 cell lines throughout time. WM9 spheroids seem to be looser after day 4 and disintegrated after day 10; **(C)** Cell concentration optimization to obtain A375 spheroids. Three concentrations were evaluated, with the 2500 cells/ml being the optimum concentration. Three independent assays were carried out not only for the procedure but also for the size optimization.

4.4.4.1. G18.EE and its *n*-butanol fraction decrease the tumor spheroid migratory capacity

The migratory capacity of A375 tumor spheroids was evaluated in gelatin-coated flat-bottomed 96 well plates. Tumor spheroids were transferred to the gelatin-coated plates on day 4. Following the transference, images were taken for each well (40x magnification), corresponding to day 0. Migration was followed over time after the addition of G18.EE and of *n*-butanol fraction dosages **(Table 17)**, and the respective zones of migration for days 1, 2, and 3 were calibrated to day 0 **(Figure 20A)**.

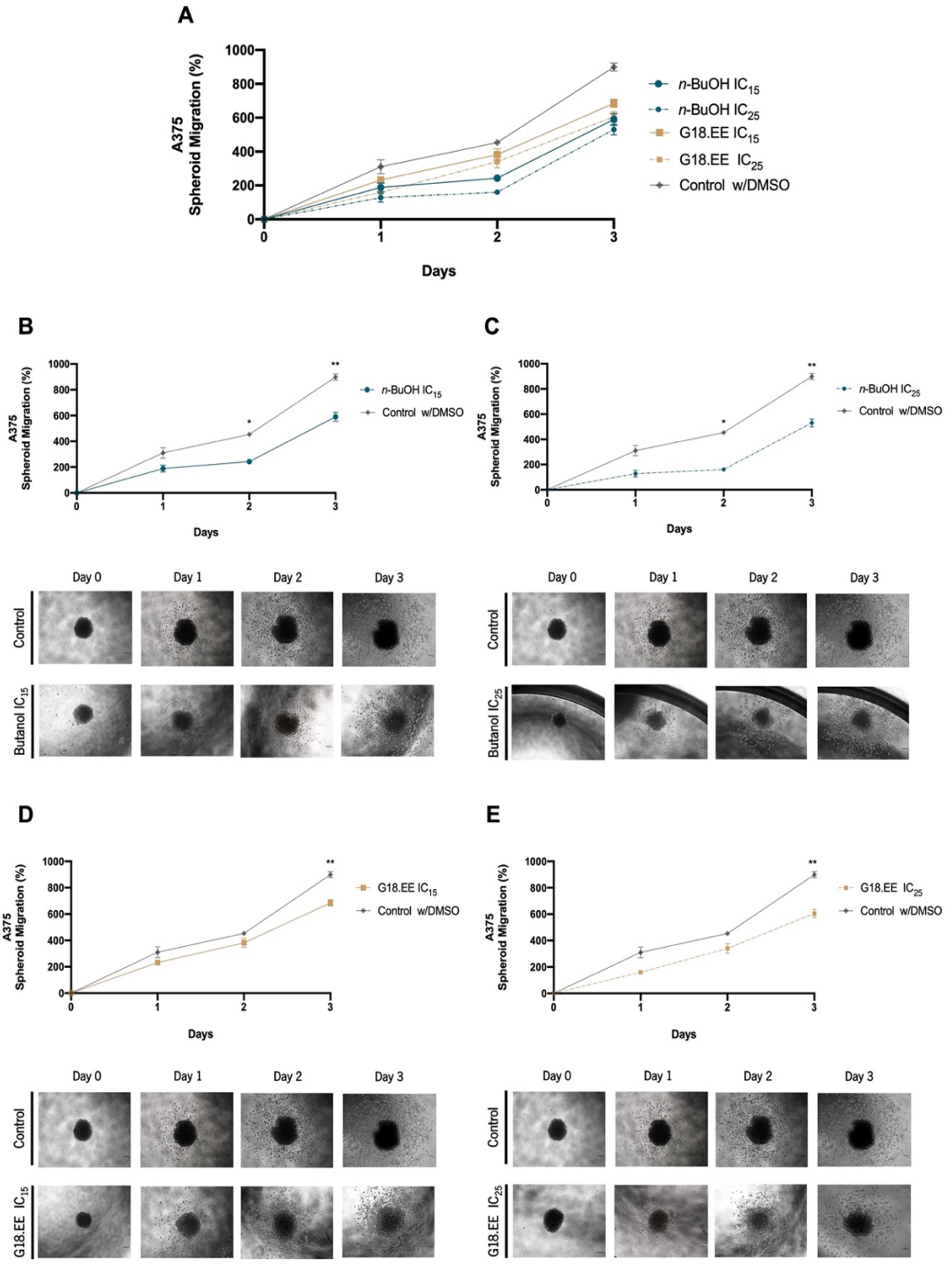

Figure 20 - Effect of the G18.EE and its *n*-butanol fraction on the A375 tumor spheroid migratory capacity. Tumor spheroid migration assay of A375 spheroids treated with **(A)** DMSO (control) and different concentrations of G18.EE and its selected fraction, namely **(B)** (5 µg/ml) *n*-BuOH IC_{15}; **(C)** (7 µg/ml) *n*-BuOH IC_{25}; **(D)** (10 µg/ml) G18.EE IC_{15}; and **(E)** (13 µg/ml) G18.EE IC_{25}. Spheroid migration normalized to day 0 was assessed by the measurement of the area through a 3-day period. Results are expressed as mean ± SD. These results are relative to three independent assays performed in triplicate. Statistical analyses were performed using the Two-Way ANOVA test. *p<0.05, **p<0.01 (Images were taken at x40 magnification).

Both G18.EE and *n*-BuOH affect the migratory potential of A375 tumor spheroids, even at low dosages, resulting in a reduction in 3D migration **(Figure 20)**. Statistical analyses revealed that both time and treatment had a significant impact on the A375 spheroid migration ($p<0.0001$; $p<0.0001$; respectively; **Appendix 3**), as well as a statistically significant interaction between these two factors ($p<0.0001$; **Appendix 3**).

According to the multiple comparisons test, the migratory capacity of A375 spheroids was significantly reduced on days 2 and 3, after treatment with *n*-BuOH IC_{15} ($p=0.0200$; $p=0.0081$; respectively; **Figure 20B**) and *n*-BuOH IC_{25} ($p=0.0101$; $p=0.0025$; respectively; **Figure 20C**). Both G18.EE IC_{15} and IC_{25} treatments showed statistically significant changes only on day 3 when compared to the control condition ($p=0.0085$; $p=0.0058$; **Figure 20D,E**; respectively). These findings support prior 2D migratory capability data, indicating that the ethanol extract of propolis from Gerês as well as its selected fractions are potential cell migration inhibitors, being the *n*-BuOH fraction the strongest one.

4.4.4.2. G18.EE and its *n*-butanol fraction decrease tumor spheroid growth

The examination of tumor spheroids growth (TSG) along time is the *in vitro* assay that comes closest to replicating the growth capacity of a tumor *in situ*. As previously described, A375 tumor spheroids were grown for four days until their diameters fell within the size range of 300 to 500 μM. Then, on day 4, G18.EE and its *n*-BuOH dosages **(Table 17)** were added to the corresponding wells. The development of the spheroids was tracked for 14 days **(Figure 21)**, being images acquired (x40 magnification) on days 4, 7, 10, 12, and 14. The medium was renewed at each time point.

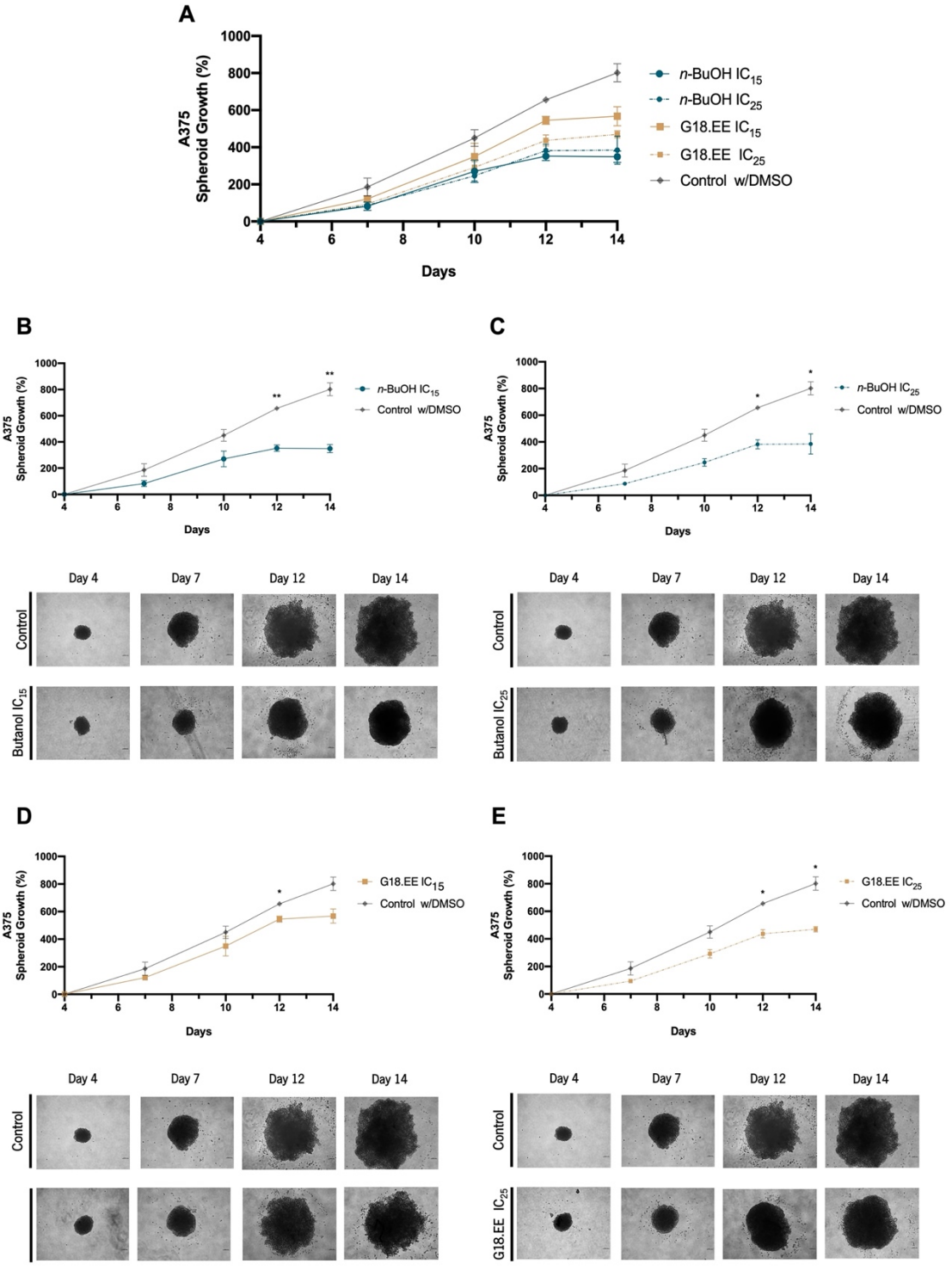

Figure 21 - Effect of the G18.EE and its *n*-butanol fraction on the A375 tumor spheroid growth. Tumor spheroid growth assay of A375 spheroids treated with **(A)** DMSO (control) and different concentrations of G18.EE and its selected fraction, namely **(B)** (5 µg/ml) *n*-BuOH IC_{15}; **(C)** (7 µg/ml) *n*-BuOH IC_{25}; **(D)** (10 µg/ml) G18.EE IC_{15}; and **(E)** (13 µg/ml) G18.EE IC_{25}. Spheroid growth was normalized to day 4 and assessed by area measurement (ImageJ) through a 10-day period. Results are expressed as mean ± SD. These results are relative to three independent assays performed in triplicate. Statistical analyses were performed using the Two-Way ANOVA test. *p<0.05, **p<0.01 (Images were taken at x40 magnification).

According to the results of the A375 tumor spheroid growth assay, G18.EE and the *n*-butanol fraction decrease spheroids' growth capacity over time **(Figure 21A)**. Statistical analysis revealed a significant interaction between time and treatment (p<0.0001; **Appendix 4**) and both had a significant effect on the A375 spheroids' growth (p=0.0001; p<0.0001; respectively).

The multiple comparisons test showed that A375 spheroid growth was significantly reduced on days 12 and 14, with *n*-BuOH IC_{15} (p=0.0027; p=0.0077; respectively; **Figure 21B**), *n*-BuOH IC_{25} (p=0.0154; p=0.0367; respectively; **Figure 21C**), and G18.EE IC_{25} *n*-BuOH IC_{25} (p=0.0193; p=0.0293; respectively; **Figure 21E**) treatments. A375 spheroids treated with G18.EE IC_{15} were significantly smaller when compared to the control condition, only on day 12 (p=0.0430; **Figure 21D**). Overall, the latest time points were the ones with statistically significant lower tumor spheroids area compared to the DMSO condition.

Comparing all the A375 spheroids growth results (**Figures 21A**), we may conclude that G18.EE and its chosen fraction are potential cell growth inhibitors, with *n*-butanol being the most effective and impactful treatment tested.

4.4.5. G18.EE and its selected fractions affect the glycolytic metabolism of melanoma cell lines

Cancer metabolic reprogramming, an emerging hallmark of cancer metabolism, is required for tumorigenesis. Cancer cells, in fact, have a remarkable ability to regulate their metabolism to promote tumor development, growth, and survival. In 1927, Otto Warburg discovered that cancer cells have a high rate of aerobic glycolysis, resulting in the conversion of up to 85% of incoming glucose to lactate - the "Warburg Effect." (Vander Heiden *et al.*, 2009; Dang *et al.*, 2011). Lactate promotes carcinogenic processes, such as angiogenesis, metastasis, tumor resistance, and immunosuppression, playing a key role in cancer progression. High rates of lactate are usually linked with higher tumor aggressiveness, so therapy is expected to decrease the extracellular levels of lactate (Pérez-Tomás & Pérez-Guillén, 2020).

The impact of Portuguese propolis from Gerês on cancer metabolism remains unknown. Therefore, in this study, the effect of chosen dosages **(Table 17)** of G18.EE and its selected fractions on the extracellular levels of lactate, which represents the lactate exported by the cells, were assessed in A375 and WM9 melanoma cell lines after 24, 48, and 72 h of treatment **(Figure 22)**.

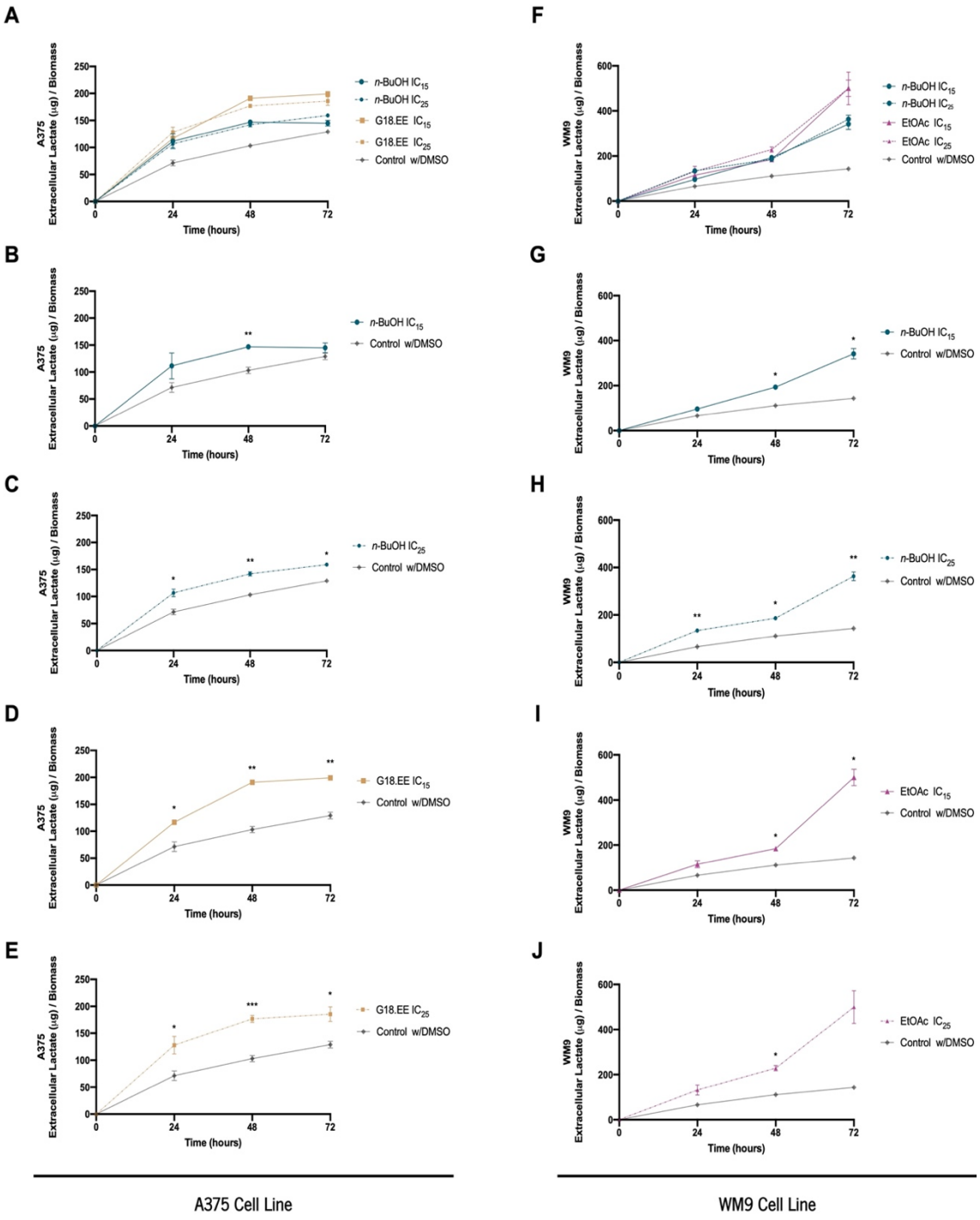

Figure 22 - Effect of the G18.EE and its selected fractions on A375 and WM9 extracellular levels of lactate. Extracellular lactate was measured after treatment with different concentrations of **(A)** G18.EE and its *n*-BuOH fraction in the A375 cell line, namely **(B)** (5 µg/ml) *n*-BuOH IC_{15}; **(C)** (7 µg/ml) *n*-BuOH IC_{25}; **(D)** (10 µg/ml) G18.EE IC_{15}; and **(E)** (13 µg/ml) G18.EE IC_{25}; and with distinct dosages of *n*-BuOH and EtOAc fractions in the **(F)** WM9 cell line, specifically (**G)** (5 µg/ml) *n*-BuOH IC_{15}; **(H)** (7 µg/ml) *n*-BuOH IC_{25}; **(I)** (8 µg/ml) EtOAc IC_{15}; and **(J)** (10 µg/ml) EtOAc IC_{25}. DMSO was used as a control. Lactate extracellular levels were measured throughout a 72-hour period. Results are expressed as mean ± SD. These results are relative to three independent assays carried out in triplicate. Statistical analyses were performed using the Two-Way ANOVA test. *p<0.05, **p<0.01, ***p<0.001.

G18.EE and its selected fractions (*n*-butanol and EtOAc) influence the extracellular levels of lactate over time in A375 **(Figure 22A)** and WM9 cells **(Figure 22F)**. Statistical analyses revealed that time and treatment, as independent parameters, had a noteworthy impact on the extracellular lactate levels of both the A375 ($p<0.0001$; $p<0.0001$; respectively; **Appendix 5**) and the WM9 cell lines ($p<0.0001$; $p<0.0001$; respectively; **Appendix 5**). Furthermore, for both melanoma cell lines tested, a statistically significant association between time and treatment was established ($p<0.0001$ for A375 cells; $p=0.0002$ for WM9 cells; **Appendix 5**).

The A375 cells multiple comparisons test revealed that the extracellular levels of lactate in this melanoma cell line were significantly higher after treatment with G18.EE and *n*-butanol throughout time, when compared to the control **(Figure 22)**. Specifically, at the time points 24, 48, and 72 h statistically significant differences were obtained after treatment with *n*-BuOH IC_{25} ($p=0.0459$; $p=0.0044$; $p=0.0117$; respectively; **Figure 22C**), G18.EE IC_{15} ($p=0.0132$; $p=0.0025$; $p=0.0014$; respectively; **Figure 22D**), and G18.EE IC_{25} ($p=0.0304$; $p=0.0004$; $p=0.0221$; respectively; **Figure 22E**). A375 extracellular lactate levels were significantly higher than the control condition only 48 hours after treatment with *n*-BuOH IC_{15} ($p=0.0038$; **Figure 22B**). Regarding the WM9 cell line, respective multiple comparisons to the control demonstrated that *n*-butanol and EtOAc significantly increased the extracellular lactate levels **(Figure 22).** In fact, we observed statistically significant differences at the time points 48 and 72 h after treatment with *n*-BuOH IC_{15} ($p=0.0147$; $p=0.0250$; respectively; **Figure 22G**), *n*-BuOH IC_{25} ($p=0.0121$; $p=0.0099$; respectively; **Figure 22H**), and EtOAc IC_{15} ($p=0.0286$; $p=0.0205$; respectively; **Figure 22I**). Regarding the WM9 cells treated with *n*-BuOH IC_{25}, significant differences were also observed at 24 h ($p=0.0033$; **Figure 22H**). Lastly, treatment with EtOAc IC_{25} only originates a significant difference at 48h ($p=0.0196$; **Figure 22J**).

By comparing all the A375 and WM9 cell line results (**Figure 22A,F**; respectively), we may infer that *n*-butanol was the treatment that resulted in the lowest increase of the extracellular lactate levels compared to the control condition.

4.4.6. G18.EE and its selected fractions affect the expression of metabolic markers in melanoma cell lines

The impact of Portuguese propolis from Gerês on the expression of metabolic proteins is unknown. After determining the effects of G18.EE and its selected fractions (*n*-butanol and EtOAc) on the extracellular levels of lactate, western blot analysis was performed to assess the effect of these dosages **(Table 17)** on the expression levels of several proteins that are important to the glycolytic phenotype in the *BRAF*-mutated melanoma cell lines A375 and WM9 **(Figure 23)**. Proteins involved in ROS (Catalase) and tumor metabolism (LDHA, MCT1, MCT4, PFKL, CD147, AMPK) were evaluated in this study **(Figure 23A)**.

The Western Blot results reveal that Gerês propolis treatments affect the metabolism of A375 and WM9 *BRAF*-mutated melanoma cell lines changing the expression levels of metabolic proteins **(Figure 23A,B)**. Both cell lines tested seem to display higher levels of LDHA, although not significant, which are established indicators of lactate metabolism **(Figures 23C,D)**. Also, MCT-1, a membrane protein implicated in lactate transport, appears to be enhanced in WM9 cells after exposure to *n*-BuOH and EtOAc dosages. These findings support the previous results of the extracellular lactate levels quantification assay. The expression levels of CD147, the MCTs chaperone, and MCT-4 were unaffected. In addition, propolis therapies appear to increase the expression of the metabolic marker AMPK (activated form), whose major role is to operate as an energy sensor for cell metabolism regulation and as a metabolic gatekeeper to inhibit cancer cell development **(Figures 23C,D)**. Furthermore, although not statistically significant, western blot quantifications revealed a decrease in catalase and PFKL protein expression levels in the different propolis treatment conditions tested **(Figures 23C,D)**.

Propolis extract and its fractions significantly affected the expression levels of Catalase in the A375 cell line (p=0.0285; **Appendix 6**), as well as the AMPK levels in the WM9 cell line (p=0.0103; **Appendix 6**) **(Figure 23C,D)**. More specifically, the levels of catalase were significantly decreased with *n*-BuOH IC_{15} (p=0.0428; **Figure 23C**) and G18.EE IC_{25} (p=0.0102; **Figure 23C**) in A375 cells compared to the control condition. In the WM9 cell line, AMPK was significantly higher with EtOAc IC_{15} treatment (p=0.0467; **Figure 23D**).

Overall, we may deduce that G18.EE and its chosen fractions (*n*-butanol and EtOAc) interfere with the tumor metabolism of melanoma cells, increasing ROS and lactate secretion rates, as well as modulating glucose metabolism.

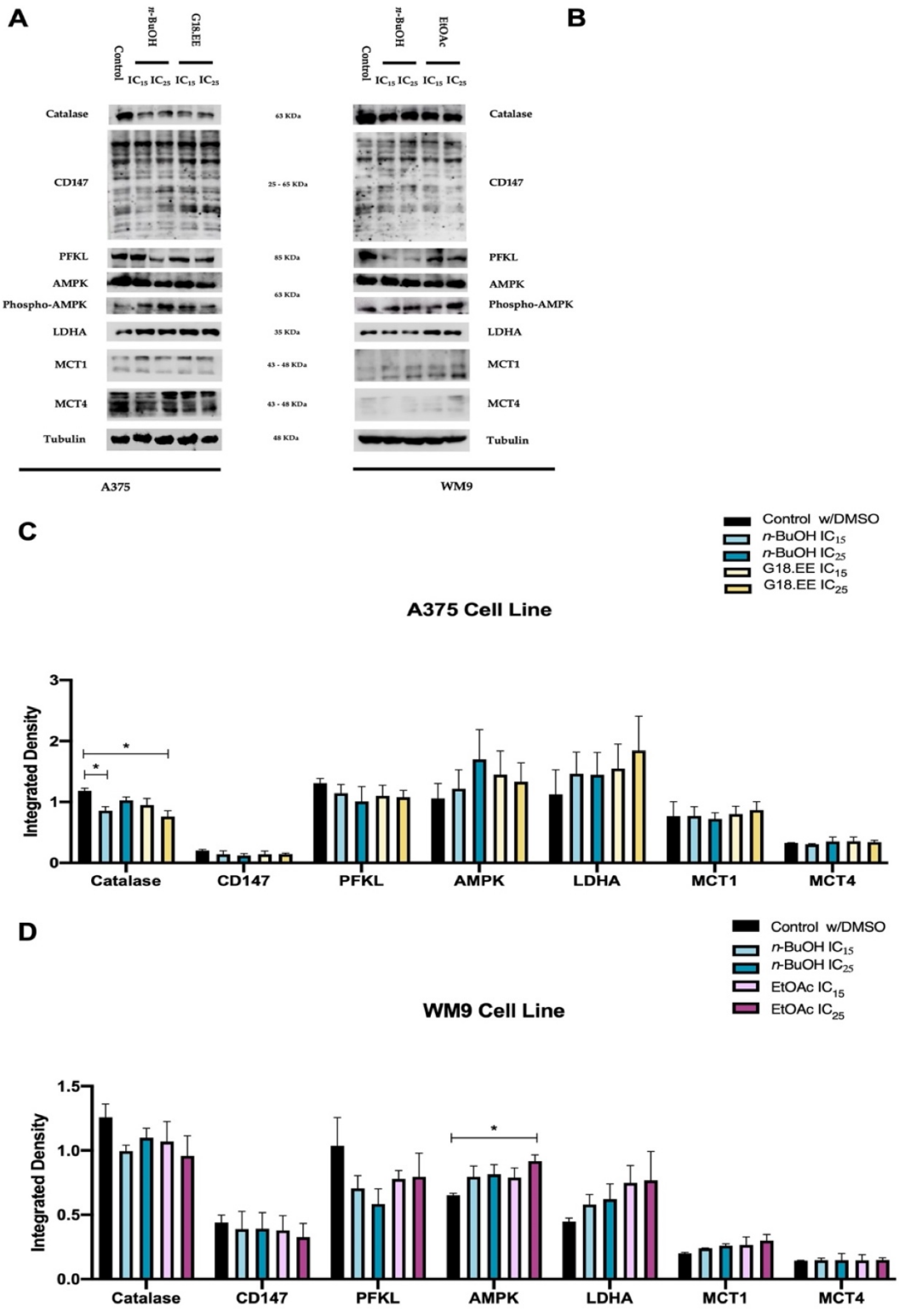

Figure 23 - Effect of G18.EE and its selected fractions on the expression levels of metabolic markers in melanoma cells. Metabolic markers were evaluated in the different conditions by Western Blot and quantified in **(A,C)** A375 and **(B,D)** WM9 cell lines. A375 and WM9 cells treated with DMSO were used as controls. Except for phospho-AMPK, which was normalized for total AMPK, the remaining proteins were normalized to tubulin. Results are expressed as mean ± SD and are relative to three independent experiments carried out in triplicate. *p<0.05.

4.4.7. G18.EE and its fractions (*n*-BuOH and EtOAc) promote mitochondrial ROS production in melanoma cells

ROS generation was assessed to verify if the selected therapies **(Table 17)** - *n*-BuOH and G18.EE for A375, and *n*-BuOH and EtOAc for WM9 - had an antioxidant effect on A375 and WM9 *BRAF*-mutated melanoma cell lines **(Figure 24)**, as expected based on the DPPH• and ABTS• scavenging activity results **(Table 14)**. In the DPPH and ABTS assays, a stock solution with 150 µg/mL of propolis was used. Thus, in an attempt to compare with these assays, 100 µg/mL of each fraction was the dose used in the ROS assays. However, results show that all the treatments significantly increased ROS production in melanoma cell lines in a dose-dependent manner **(Figure 24A,B)** ($p<0.0001$; $p=0.0157$, respectively; **Appendix 7**). Dunnett's test for multiple comparisons demonstrates that 100 µg/mL of *n*-BuOH and G18.EE were significantly different than the control condition (DHE) ($p<0.0001$; $p<0.0001$; **Figure 24A**). Regarding WM9 cells, the same multiple comparison tests revealed that 100 µg/mL of *n*-BuOH and EtOAc significantly increased ROS levels compared to the control ($p=0.0072$; $p=0.0047$; **Figure 24B**).

Mitochondria are the primary generator of intracellular ROS (Snezhkina *et al.*, 2020). Thus, the activity of this membrane-bound cell organelle was measured to understand if the greater levels of ROS observed **(Figure 24A,B)** are explained by a higher mitochondrial activity **(Figure 24C,D)**. Mitochondrial activity was assessed through the ratio of mitochondrial polarization: mitochondrial mass. Although no statistically significant differences were detected between treatments, these seem to be associated with higher mitochondrial activity in both cell lines **(Appendix 8)**. In the A375 cells multiple comparisons test, mitochondrial activity was significantly higher with 100 µg/mL of G18.EE than in the control condition ($p=0.0460$; **Figure 24C**). In WM9 cells, 100 µg/mL of either *n*-BuOH or EtOAc seems to induce an increase in mitochondrial activity compared to the control. However, these results were not statistically significant **(Figure 24D)**.

A significant impact of G18.EE and its selected fractions (*n*-BuOH and EtOAc) was observed in the mitochondrial biomass of both cell lines ($p=0.0028$; $p=0.0002$, respectively; **Appendix 9**; **Figure 24F,H**). The 100 µg/mL dosage of *n*-BuOH and G18.EE in the A375 cell line significantly decreased the biomass of this organelle ($p=0.0306$ and $p=0.0253$, respectively; **Figure 24F**). In WM9 cells, 100 µg/mL of *n*-BuOH and IC_{15}, IC_{25} and 100 µg/mL of EtOAc decreased mitochondrial biomass ($p=0.0013$, $p=0.0051$, $p=0.0168$, and $p=0.0024$; **Figure 24H**).

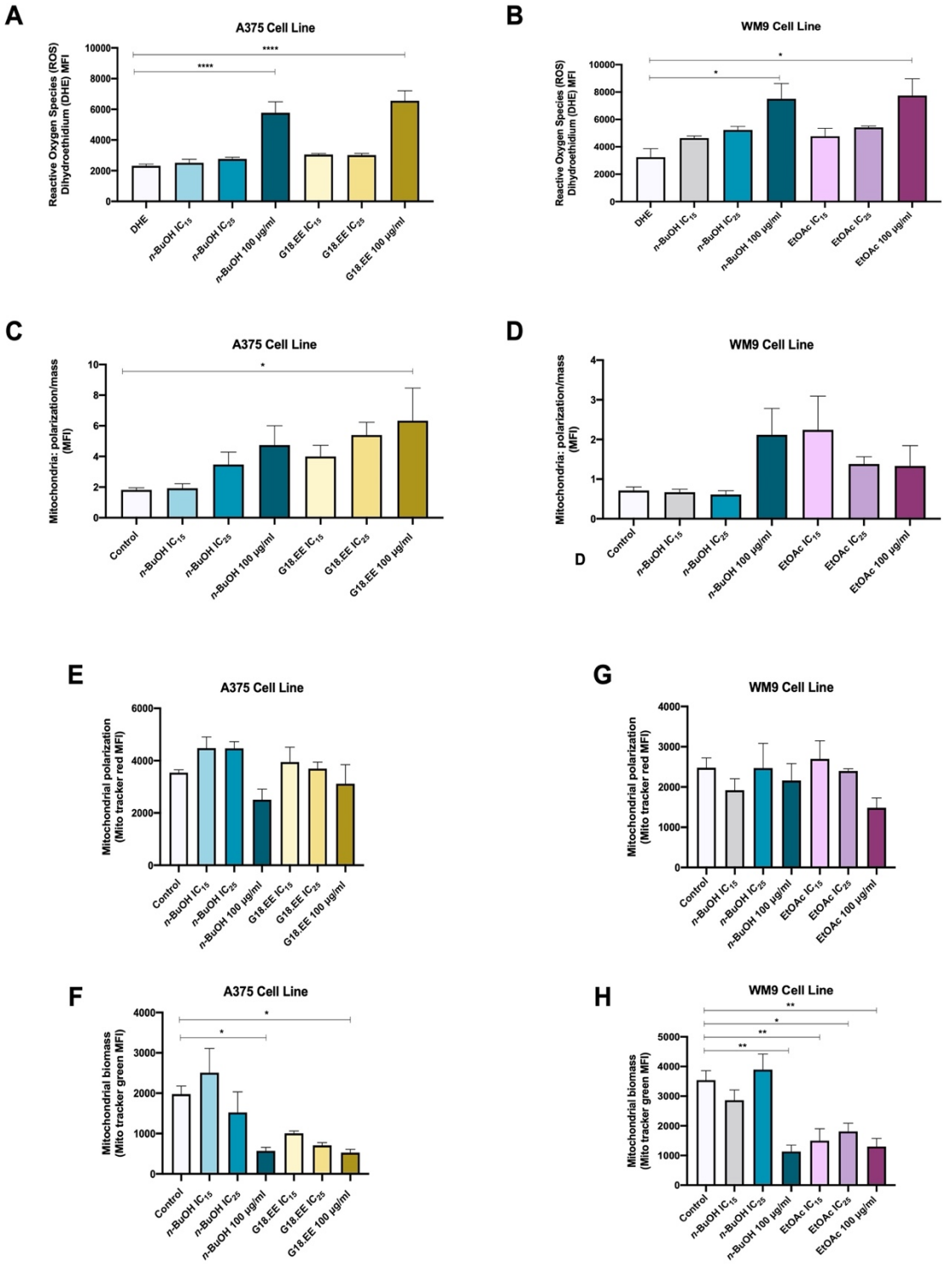

Figure 24 - Effect of the G18.EE and its selected fractions on ROS production and mitochondrial activity in melanoma cells. Treatments with G18.EE, n-BuOH, and EtOAc increase ROS levels and mitochondrial activity. Results were obtained after 24 h treatment with DMSO (control); *n*-BuOH 5 µg/ml (IC_{15}), 7 µg/ml (IC_{25}), and 100 µg/ml; and G18.EE 10 µg/ml (IC_{15}), 13 µg/mL (IC_{25}), and 100 µg/ml. ROS levels were measured in A375 **(A)** and WM9 **(B)** cell lines. Mitochondrial activity was measured in **(C)** A375 and **(D)** WM9 cell lines through the ratio of the respective **(E,G)** mitochondrial polarization by the **(F,H)** mitochondrial biomass. Results are expressed as mean ± SD. Three independent experiments were carried out in triplicate. *p<0.05, ** p<0.01, **** p<0.0001.

4.4.8. G18.EE, *n*-BuOH, and EtOAc treatments induce apoptosis in melanoma cell lines

ROS overproduction can stimulate tumor cell apoptosis (Ivanova *et al.*, 2016). Therefore, the levels of apoptotic markers were evaluated to understand if the different propolis treatments induced this cell death mechanism **(Figure 25)**. The levels of anti-apoptotic, Bcl-2, and Bcl-XL; pro-apoptotic, Bax, and p53; and apoptotic-related proteins, such as caspase 3 and caspase 9, were evaluated **(Figure 25A)**. Results suggest that Gerês propolis treatments trigger cell death by a regulated cell death mechanism. Except for Bcl-XL in WM9 cells, anti-apoptotic Bcl-2 and Bcl-XL proteins appear to be reduced in the different treatment conditions **(Figure 25B,C)**. Both cell lines tested seem to display higher levels of Bax and p53, although not significant, which are known pro-apoptotic indicators **(Figure 25B,C)**. The apoptotic proteases caspases 3 and 9 also appear to be enhanced by propolis treatments too **(Figure 25B,C)**.

Propolis extract and its fractions significantly affected caspase 9 expression levels in A375 (p=0.0201; **Appendix 11**) and WM9 cells (p=0.0006; **Appendix 11**), as well as the Bcl-XL levels in A375 (p=0.0014; **Appendix 11**) cell line **(Figure 25B,C)**. More specifically, the levels of caspase 9 were significantly increased with G18.EE IC_{25} (p=0.0063) in A375 cell line, when compared to the control; and with EtOAc IC_{15} (p=0.0046) and IC_{25} (p=0.0007) in WM9 cell line **(Figure 25B,C)**. In the A375 cell line, Bcl-XL was significantly lower with *n*-BuOH IC_{15} (p=0.0035), G18.EE IC_{15} (p=0.0087), and IC_{25} (p=0.0005) treatments **(Figure 25B)**.

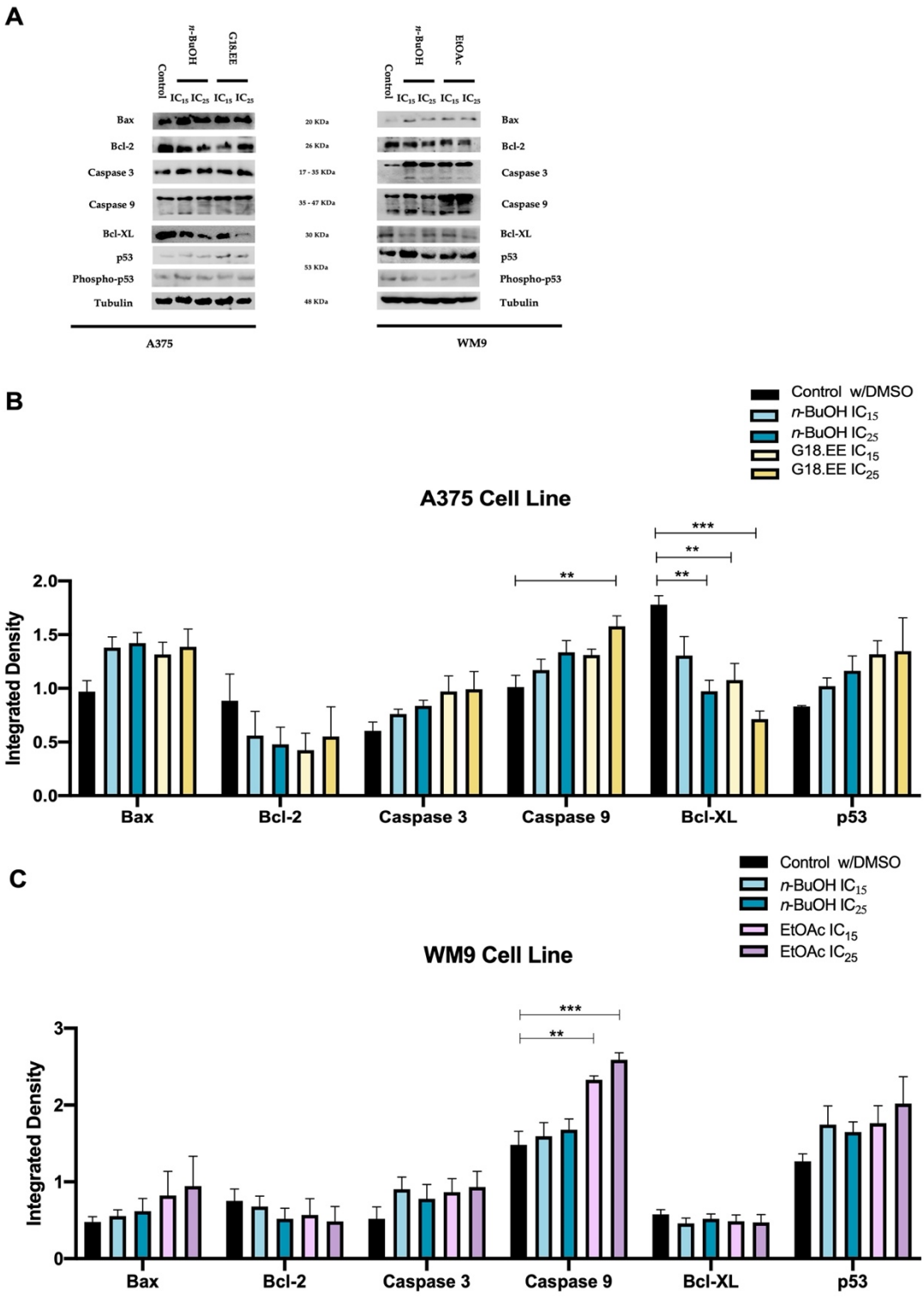

Figure 25 - Effect of the G18.EE and its selected fractions on the expression levels of apoptotic markers in melanoma cell lines. Pro-apoptotic mechanisms are activated by G18.EE, *n*-BuOH, and EtOAc in melanoma cells. Apoptotic markers were evaluated in the different conditions by **(A)** Western blot and quantified in A375 **(B)** and WM9 **(C)** cell lines. A375 and WM9 cells treated with DMSO were used as controls. Except for phospho-p53, which was normalized for total p53, the remaining proteins were normalized to tubulin. Results are expressed as mean ± SD. Results are from three independent experiments carried out in triplicate. ** $p<0.01$, *** $p<0.001$.

Chapter 5 - Discussion

Propolis is an animal-modified plant product, specifically by bees, that has been extensively used since ancient times (Ghisalberti, 1979; Silva-Carvalho *et al.*, 2014) The chemical composition of this resinous product is very complex and widely variable, with over 500 distinct compounds identified (Marcucci, 1995; Bankova *et al.*, 2000). One of the major struggles with the use of propolis as a therapeutic agent in conventional medicine is the lack of chemical composition and biological activity standardization. Standardization of these two parameters is critical for the acceptance and application of propolis in the healthcare system (Silva-Carvalho *et al.*, 2014; Silva-Carvalho *et al.*, 2015). Despite these limitations, the interest of the scientific community in this natural compound has been expanding. This intensification of studies allowed the identification of several biological, pharmacological, and biomedical properties, including antimicrobial, antioxidant, and antitumor/anticancer (Silva-Carvalho *et al.*, 2015). Propolis antitumor bioactivity has been described in some types of cancer, including skin cancer. However, there is a scarcity of data linking propolis and melanoma. Besides that, nothing is known about Portuguese propolis effect in this skin cancer type.

Taking that into account, in the present study, we evaluated the antimicrobial, antioxidant, and antitumoral activity of a Portuguese propolis sample collected in an apiary located in the northern region of Portugal (Montalegre, Gerês), which has been little explored but appears to have intriguing biological potential. Aside from the scientific knowledge acquired, such investigations can contribute to the valorization of bee products, and consequently contribute significantly to the Portuguese economy.

Propolis has been described as a powerful antimicrobial agent. This bioactivity is its most well-known property, and it has been demonstrated against a panel of pathogens, including bacteria and yeast, mainly using the agar diffusion method (Bogdanov, 2016). Prior research has shown that propolis ethanol extracts are more effective against Gram-positive bacteria than Gram-negative bacteria (Bankova *et al.*, 2000; Sforcin *et al.*, 2000; Vardar-Ünlü *et al.*, 2008). This work reveals that G18.EE and G20.miEEs exhibit lower MIC values when tested against Gram-positive bacterial strains **(Table 13)**, which is consistent with previous studies reporting higher propolis activity against such bacterial type. This variation in propolis action could be explained by structural differences in the cell wall of Gram-positive and Gram-negative bacteria since this last one presents an inner membrane and a very impermeable outer lipid membrane (Silici & Kutluca, 2005). According to Zhang *et al.* (2022), Chinese red propolis extract is exceedingly active against MRSA (MIC=50 µg/ml), even more than against *S. aureus* (MIC=100 µg/ml). Pamplona-Zomenhan *et al.*

(2011) also demonstrated that Brazilian propolis has remarkable antibacterial efficacy against the same referred strains (MIC=1.420 μg/ml). However, at the concentrations tested, none of the G.EEs covered in this study was active against the Gram-positive MRSA (MIC>2000 μg/ml) **(Table 13)**. Using data acquired in 2018 (harvest year of the G18 sample), Freitas *et al.* (2022) reported a similar MIC value to the one obtained in this work for the genus *Bacillus* (MIC= 50 g/ml) and the species MRSA and *E. coli* (both with MIC>2000 μg/ml). The result reported for *Staphylococcus aureus* (MIC= 200 μg/ml) was, however, slightly lower than the MIC achieved in our study (MIC= 500 μg/ml). These findings suggest that the Gerês propolis sample (2018) has maintained its antibacterial efficacy after three years of storage at -20 °C, with only a relatively slight loss of action against some specific microorganisms. On the other hand, comparing the G18.EE and the G20.miEEs MIC values **(Table 13)**, it is feasible to deduce that changes in the composition of 2020 Gerês propolis samples resulted in a significant reduction of its antibacterial capacity. As a result, G18.EE is an attractive type of propolis for antibacterial applications, preferential against Gram-positive bacteria.

Regarding the antifungal activity of Portuguese propolis, the results of this study reveal that G18.EE and the three G20.miEEs were unable to inhibit the growth of *S. cerevisiae* and *C. albicans* (MIC≥2000 μg/ml) **(Table 13)**. The MIC values reported by Peixoto *et al.* (2021) for five propolis samples collected in consecutive years, namely G11.EE, G12.EE, G13.EE, G14.EE and G15.EE, are consistent with these results. In contrast, a study carried out by Bonvehí & Gutiérrez (2012) revealed that the growth of *S. cerevisiae* was inhibited by a Spanish propolis ethanol extract, with MIC values ranging from 500 to 1500 μg/ml. Similar conclusions were obtained in studies using French propolis samples against *C. albicans*, with a MIC value of 31.25 μg/ml (Velikova *et al.*, 2000). As an outcome, G18.EE and G20.miEEs are ineffective against the tested yeast strains.

Once elucidated the antimicrobial potential of the propolis ethanol extracts under study, we shifted our attention to another essential biological property of propolis: the antioxidant activity. Considering that DPPH• is a stable nitrogen-centered free radical, substances that can react with it, leading to a change of color from purple to yellow, are called antioxidants and therefore antiradical agents (Brand-Williams *et al.*, 1995). In the case of ABTS• scavenging assay, the compounds have antioxidant capacity if a reduction of ABTS• was verified by decolorization (Ilyasov *et al.*, 2020). Previous studies classified Portuguese propolis as a natural product with antioxidant activity (Moreira *et al.*, 2008). Falcão *et al.* (2013) verified that propolis samples from the north and coast of Portugal, as well as from Azores, have the best DPPH• scavenging effects,

with EC_{50} values ranging from 10 to 30 µg/ml, when compared to propolis from other zones of Portugal. In particular, the sample collected in Montalegre (north of Portugal) exhibited a scavenging activity effect with an EC_{50} value of 10 µg/ml. According to Freitas *et al.* (2022), G18.EE has an EC_{50} value of 12.40 ± 0.43 µg/ml. This value was calculated in 2018, the sample's harvest year. In this study, the EC_{50} values calculated for G18.EE were 10.90 ± 0.34 (DPPH assay) and 9.83 ± 0.21 µg/ml (ABTS assay) **(Table 14)**, indicating that the antioxidant capacity of Gerês (2018) Portuguese propolis was preserved over the years. This discovery is significant because it provides value to samples that were stored and undrained - often designated as leftovers - due to their limited quantity. Comparing the EC_{50} values of G18.EE (10.90 ± 0.34 µg/ml) and G20.miEEs (ranging from 18.37 to 21.49 µg/ml) from the DPPH assay **(Table 14)**, it is reasonable to conclude that G18.EE has a higher antioxidant capacity than the G20 micro-extracts, which can be explained by the adjustment in the propolis mixture composition in the year 2020 due to the apiary problem. Despite this fact, according to the data published by Da Cruz *et al.* (2021), the EC_{50} of G18.EE and G20.miEEs are much lower than other values published for worldwide samples, implying that they had stronger DPPH• scavenging activity (Da Cruz *et al.*, 2022). As an outcome, Portuguese propolis from Gerês is an attractive type of propolis for antioxidant applications.

As reviewed earlier, the determination of propolis quality parameters is essential for its commercialization and application in a variety of fields, including industry and health (Woisky & Salatino, 1998; Bankova *et al.*, 2016;). So, before conducting the antitumoral assays, the quality criteria of the propolis ethanol extract selected for the study (G18.EE) was assessed. Our results show that the values acquired for each parameter, namely the amount of water (5.25 ± 0.30 µg/ml), ash (0.73 ± 0.06 µg/ml), beeswax (2.18 ± 0.19 µg/ml), and balsamic content (56.67 ± 13.12 µg/ml) **(Table 15)**, are consistent with a propolis sample of high quality (Bogdanov, 2011; Falcão, 2013). This outcome is important taking into consideration that we want to evaluate propolis potential in melanoma.

Skin cancer is the third most prevalent human malignancy (Zheng *et al.*, 2018). The number of new cases reported has risen at an exponential and alarming rate over the years (Ishiguro *et al.*, 2017). Melanoma is the most aggressive, severe, and invasive type of skin cancer, with the highest mutation rate (Bandarchi *et al.*, 2010; Prieto-Granada *et al.*, 2015) *BRAF* mutated melanomas are the most common and are associated with poorer prognosis and overall survival (Ny *et al.*, 2020) These mutated melanomas are highly aggressive being more predisposed to metastasize (Ascierto *et al.*, 2012; Lo & Fisher, 2014). Despite all the advances and research in

melanoma treatment, finding effective therapies remains a challenge. Acquired resistance and the adverse effects to the standard therapies used in the clinics are the root causes of the current therapeutic problem (Gastaldello *et al.*, 2021). Due to the drawbacks of standard treatments, natural products, such as propolis, have the potential to be added to the therapeutic arsenal of cancer (Prieto-Granada *et al.*, 2015; Gastaldello *et al.*, 2021).

The increasing number of studies testing propolis as an antitumoral agent against several tumor types has allowed researchers to identify and distinguish the effects of this natural product in cancer cells. Propolis, in fact, inhibits specific oncogenic pathways, resulting in decreased cell proliferation and growth capacities, increased apoptotic marker levels, antiangiogenic effects, and tumor microenvironment modulation (Araújo *et al.*, 2011; Sawicka *et al.*, 2012; Chan *et al.*, 2013). Thus, we analyzed the effect of G18.EE and its isolated fractions on proliferation, migration, growth, apoptosis, metabolism, ROS production, and mitochondrial potential on the chosen *BRAF*-mutated melanoma cell lines.

Regarding the antiproliferation capacity of propolis, our results reveal that G18.EE and its fractions are able to reduce A375 and WM9 cell viability throughout the time. These findings corroborate prior research that identified other propolis samples as cytotoxic agents in melanoma, such as Moroccan (Cisilotto *et al.*, 2018) and Algerian (Guo *et al.*, 2013), among others. In addition, antiproliferative effects were also detected in melanoma (Me45 and B16-BL6 cell lines) after treatment with the Netherlands and Polish propolis (Banskota *et al.*, 2002; Kubina *et al.*, 2015). Comparing all the fractions evaluated in our study, it is possible to infer that *n*-hexane was the least active fraction **(Figure 15** and **Table 16)** whereas *n*-BuOH was the most cytotoxic one for both melanoma cell lines. Knowing that propolis is a complex mixture of multiple plant compounds and that *n*-hexane is the solvent with lower polarity (Relative polarity=0.009) (Valença et al., 2013), the proportion of polyphenols and flavonoids, two significant bioactive components of propolis, will be lower in this fraction than in the others (*n*-BuOH and EtOAc). This might explain why n-hexane has a poor antiproliferative effect on *BRAF*-mutated melanoma cells at low dosages. In addition, CAPE is a propolis component that presents important anticancer and chemoprotective activities (Farooqui & Farooqui, 2010; Silva-Carvalho *et al.*, 2014). Oliveira *et al.* (2022) used a UPLC-DAD-ESI/MSn and identified high amounts of CAPE in the *n*-BuOH fraction and a lower total area value in the *n*-hexane fraction. These results support our findings from the cell viability assay, suggesting that CAPE can be a bioactive compound against melanoma cells.

Since *n*-BuOH/G18.EE and *n*-BuOH/EtOAc were the most active fractions for A375 and WM9 cells, respectively, we tested the *in vitro* cytotoxic effect of intermediary concentrations between the IC_{15} and IC_{25} of these treatments **(Figure 16** and **Table 17)**. The different fractions treatments, even at lower doses, seem to affect melanoma cell viability at early time points **(Figure 16)**. Taking this into consideration, further anticancer activity experiments to evaluate other important cancer hallmarks for melanoma progression were conducted using the IC_{15} and IC_{25} intermediary concentrations **(Table 17)**.

3D cell culture models are the most accurate *in vitro* models to reproduce the *in vivo* TME, and they can be considered a crucial intermediary step between the 2D cell culture assays and the *in vivo* experiments (Vinci *et al.*, 2012; Ishiguro *et al.*, 2017). Migratory capacity was assessed through 2D and 3D cell cultures. The wound-healing assay (2D cell culture) reveals that G18.EE and its selected fractions have an influence on melanoma cell migratory capacity over time **(Figure 17** and **Figure 18)**, resulting in a lower percentage of cells migrating. The tumor spheroid growth experiment (3D cell culture) confirms these previous results, demonstrating that even at low doses, Portuguese propolis from Gerês (2018) reduces the migratory capability of A375 tumor spheroids, resulting in a reduction in 3D migration **(Figure 20)**. In both assays, the *n*-butanol fraction of G18.EE was the treatment with the strongest influence on the migratory rate of melanoma cells. The migration results of this work are consistent with those of Cisilotto *et al.* (2018), who reported that Brazilian propolis significantly reduced the migration rate (40-80 % of reduction) of SK-MEL-28 melanoma cell lines. Inhibition of migration using propolis therapy was also verified in other tumor types, such as pancreatic cancer (Chinese propolis) (Silva-Carvalho *et al.*, 2014; Tao *et al.*, 2021), breast cancer (Portuguese propolis from Beira Alta) (Silva-Carvalho *et al.*, 2014), glioblastoma (Polish propolis) (Borawska *et al.*, 2016), colorectal cancer (Cuban Propolis) (Frión-Herrera *et al.*, 2020), among others.

The effect of G18.EE and its selected fractions on tumor spheroids' growth over time was also investigated. Studies establishing a link between propolis, melanoma, and tumor spheroid growth are nonexistent. As a novelty for the melanoma field, we found that treatment with G18.EE and *n*-BuOH decreased the growth area of the A375 spheroids over time **(Figure 21)**. This outcome can be justified by the inhibition of some important hallmarks that are implicated in tumor growth, namely the cell proliferation capacity, which as we saw previously is decreased after treatment with propolis dosages (Hanahan & Weinberg, 2011).

The effect of a Portuguese propolis sample from Gerês on cancer cell metabolism has never been described in the literature, to the best of our knowledge. Indeed, only two studies have been published regarding the influence of Portuguese propolis samples on the glucose uptake of breast and prostate cancer cells (Silva-Carvalho *et al.*, 2014), as well as human colorectal cancer cells (Valença *et al.*, 2013). According to Silva-Carvalho *et al.* (2014), the ethanol extract of a propolis sample from Beira Alta (Center region of Portugal) promoted a significant increase in glucose consumption and lactate production in the prostate (DU145) and breast (MDA-MB-231) cell lines. Valença *et al.* (2013) observed the opposite behavior in colorectal cancer cells (HCT-15), reporting a decrease in glycolytic metabolism after treatment with propolis from Azores, Portugal.

Tumorigenesis requires cancer metabolic reprogramming, an emerging hallmark of cancer metabolism. As previously stated, tumor cells had a higher rate of glycolytic metabolism than normal cells. Proliferating cancer cells promote glycolysis to ensure energy production and tumor development, which is translated into an increase in glucose uptake and lactate production even in the presence of oxygen (Vander Heiden *et al.*, 2009; Dang *et al.*, 2011)

In the present study, the impact of G18.EE and its selected fractions on the lactate production in A375 and WM9 cells was evaluated. Overall, it was observed that G18.EE, *n*-BuOH, and EtOAc IC_{15} and IC_{25} intermediary concentrations **(Table 17)** generated a significant increase in the lactate extracellular levels of both cell lines **(Figure 22)**. Among the tested fractions, *n*-butanol was the treatment that resulted in the lowest increase in the lactate levels. Interestingly, these results match with the ones reported by Silva-Carvalho *et al.* (2014) and by Ueda *et al.* (2013). To further complement these results, we evaluated the expression levels of several different metabolism-related proteins that are crucial for glycolytic phenotype, namely CD147, PFKL, AMPK, LDHA, MCT-1, and MCT-4 **(Figure 23)**. According to our findings, after exposure to G18.EE and its fractions, the expression levels of LDHA and MCT-1 appear to be upregulated in both A375 and WM9 melanoma cells. Since these two proteins are involved in the generation and transport of glycolysis-derived lactate, this result confirms the predetermined induction of lactate production. Given this, it's plausible to assume that propolis antitumoral activity may be related to the induction of glycolytic metabolism. Until now, we cannot understand how these cancer cell lines metabolism changes, so, more studies are needed to unravel this outcome.

The AMPK protein is an energy sensor that is crucial for glucose and lipid metabolic regulation (Hardie & Carling, 1997). When cellular energy is depleted (AMP:ATP and ADP:ATP levels increase), AMPK is activated, which accelerates ATP-generating catabolic pathways including

glycolysis and fatty acid oxidation, while suppressing ATP-consuming anabolic pathways (Lee *et al.*, 2007). The evaluation of AMPK expression in melanoma cells treated with propolis was never performed before. Our results indicate that after treatment with G18.EE and its selected fractions, AMPK expression levels increase in A375 and WM9 cells **(Figure 23)**, which is consistent with the previously observed glycolytic phenotype. Prior studies only demonstrated the effect of caffeic acid and CAPE on the AMPK pathway in cancer cells, suggesting that these primary propolis components can activate AMPK and induce autophagy and apoptosis in cancer cells, two anti-tumor markers (Mirzaei *et al.*, 2021).

As inferred earlier by data from the DPPH and ABTS scavenging assays, propolis exhibits antioxidant capacity. However, this bioactivity of propolis has not been validated in cells. According to Cisilotto *et al.* (2018),Brazilian propolis increased ROS accumulation in the SK-MEL-28 *BRAF*-mutated melanoma cell line, demonstrating that propolis had a pro-oxidant function. Our DHE data support this tendency for increased ROS levels in propolis-treated cells. In A375 and WM9 cell lines, ROS production was enhanced in a dose-dependent manner **(Figure 24A,B)**. In order to complement these findings, the expression level of Catalase, a key enzyme in the protection of cells from ROS oxidative damage, was assessed. This protein is downregulated in *BRAF*-mutated melanoma-treated cells **(Figure 23)**, which is congruent with propolis and its fractions confirmed pro-oxidant activity. Since mitochondria are the primary source of ROS (Ivanova *et al.*, 2016), we decided to investigate propolis effects on mitochondrial activity. The results suggest that treatment with propolis extract and its fractions is associated with higher mitochondrial activity **(Figure 24C,D)** but lower mitochondrial biomass **(Figure 24F,H)**. Oxidative stress, which is characterized by an overproduction of ROS, can induce mitochondrial alterations and damage (Prasad *et al.*, 2017), which could explain the reduced biomass seen in treated A375 and WM9 cells. Based on these findings, we propose that the commonly used DPPH assay to measure propolis scavenging activity does not correctly reflect what happens in the cancer context. In fact, our research showed that relying just on scavenging assays, such as DPHH and ABTS, to claim that propolis has antioxidant capabilities can be misleading.

In *vitro* antioxidant activity of propolis from Gerês (G11, G12, G13, and G14) was observed by Freitas *et al.* (2019) through a DPPH scavenging assay (EC_{50} range from 14.41±0.56 to 25.24±2.45 µg/ml). These results were supported by cytometry data employing *Saccharomyces cerevisiae* as a biological model, which demonstrated that propolis from Gerês (concentrations tested ranging from 50 to 200 µg/ml) decreases intracellular oxidation triggered by H_2O_2, the most

prevalent ROS *in vivo* (Freitas *et al.*, 2019). Propolis has been described as having opposing activities, acting as an antioxidant or a pro-oxidant agent, depending on the investigation context, such as the biological model and type of experiment carried out (Guo *et al.*, 2013; Freitas *et al.*, 2019).

G18.EE and its fractions revealed a pro-oxidant activity in *BRAF*-mutated melanoma cells. Usually, ROS accumulation is linked to a pro-tumoral activity (Alqathama, 2020). However, an overproduction of ROS can also be associated with antitumor activity, prompting ROS-mediated apoptosis, a type of regulated cell death (Falcão *et al.*, 2013). Previous research has shown that ROS and p53 have a direct correlation (Skehan *et al.*, 1990). The tumor suppressor protein p53 is activated by high levels of ROS, which subsequently activates the pro-apoptotic protein Bax and inhibits Bcl-2, an anti-apoptotic protein. During apoptosis, caspase-9 expression is also elevated. This caspase activates the effector caspase-3, causing the cleavage of cellular proteins and cell demise by apoptosis (Skehan *et al.*, 1990). Taking this into consideration, in this work we looked at some specific apoptotic markers, namely Bax, Bcl-2, Caspase-3, Caspase-9, Bcl-XL, and p53, to see if there was a link between higher levels of ROS and activation of ROS-mediated apoptosis in melanoma cells. Caspase-9 (apoptosis-related protein) and Bcl-XL (anti-apoptotic protein) were significantly up and downregulated, respectively, in melanoma-treated cells **(Figure 25)**. These outcomes support previous findings, indicating that G18.EE and its fractions (*n*-BuOH and EtOAc) trigger apoptosis in melanoma cells.

Overall, for the first time, we provided evidence for the anticancer potential of Portuguese propolis in melanoma. Portuguese propolis from Gerês (2018) and its isolated fractions modulate important hallmarks that dictate tumorigeneses – cell proliferation, cell migration, and tumor growth – and promote cancer cell death via ROS-mediated apoptosis, making them a potential source of compounds for cancer drug development. Furthermore, the identification of *n*-BuOH as the G18.EE fraction with the highest activity against the most aggressive melanoma type - BRAF-mutated melanoma - was also accomplished. We believe that this natural product should be further explored as an important source of bioactive compounds with anticancer potential.

Chapter 6 – Conclusions and Future Perspectives

Natural products with potential use in a range of fields are becoming more and more popular. One prominent example is propolis, a natural product produced by honeybees. Due to its broad spectrum of biological properties, the scientific community and beekeepers have become more interested in the exploitation and investigation of this natural resource. According to several reports, Portuguese propolis is a powerful antibacterial and antioxidant agent. Our research supports this statement by showing that the G18.EE and G20.miEEs presented remarkable MIC, particularly against Gram-positive bacteria, and EC_{50} values. Among the examined propolis samples, the most active was G18.EE, which maintained its activity even after three years of storage at -20 °C.

Prior to conducting the *in vitro* antitumoral assays and considering the prospective future application of G18.EE in this healthcare sector, we evaluated parameters that are considered quality requirements for the application and commercialization of propolis. In general, all the quality parameters evaluated – water, ash, wax, and balsamic contents – are within acceptable limits for high-quality propolis. Thus, it is plausible to assume that G18 is a good quality sample, eventually of premium grade. To complement these current quality studies, it would be interesting and necessary to evaluate additional parameters, such as total phenolic compounds; total flavones and flavonols; total flavanones and dihydrofalvonols, and total flavonoids not only to characterize the extract but also to confirm its stability over time since G18.EE was prepared a few years ago.

Cancer has overtaken heart disease as the leading cause of death worldwide. Along with the increase in cancer cases, therapeutic resistance evolved into a serious public health problem. When compared to conventional chemotherapy, targeted therapies in *BRAF*-mutated melanoma exhibited higher and improved survival rates, however, patients still develop resistance to these treatments. As a result, natural compounds, such as propolis, offer great potential for the development of novel treatments, including cancer drugs. Considering this, this work is innovative since it was the first to evaluate the effect of Portuguese propolis in melanoma, specifically in *BRAF*-mutated melanoma - the most common, lethal, and aggressive form of skin cancer.

Our work confirmed the antitumoral potential of Portuguese propolis. Treatment of *BRAF*-mutated melanoma cell lines – A375 and WM9 - with G18.EE and its fractions leads to a significant decrease in several cancer-related biomarkers, such as proliferation, migration, and tumor growth. The *n*-BuOH fraction is the most active and effective treatment for both cell lines. In order to complete this work, it would be scientifically exciting to analyze how propolis influences melanoma clonogenicity and invasion capacity. Knowing that resistance to therapy is a major problem in

melanoma treatment, it would also be interesting to assess the impact of combining *BRAF* inhibitors, such as Vemurafenib and Dabrafenib, with the selected propolis fractions in the viability of melanoma cell lines.

Moreover, in this thesis, we also analyzed the effect of Portuguese propolis on cancer cell metabolism. Data showed that G18.EE and its fractions significantly increase lactate production, which can be explained by the higher expression levels of LDHA and MCT-1 in melanoma-treated cells. To confirm this glycolytic phenotype and also deepen our knowledge about propolis's effect on cancer cell metabolism, the next step will be to evaluate glucose consumption and the expression of other important metabolic markers in response to treatment.

The DPPH radical scavenging activity assay demonstrated that G18.EE is a powerful antioxidant agent. However, this propolis bioactivity was not confirmed in the cancer cell context. In fact, this work revealed that G18.EE and its selected fractions (*n*-BuOH and EtOAc) increased ROS accumulation, mitochondrial activity, and activated apoptotic mechanisms, indicating a pro-oxidant activity instead. Based on these findings, the DPPH assay seems insufficient to claim propolis as an antioxidant agent. In fact, propolis from Gerês appears to have opposing activities depending on the experimental context.

Propolis from different regions cannot be used as a mixture of several compounds; rather it must be used as isolated compounds, which ensure standardization and facilitate the development into a novel drug or a lead. The results of this thesis enabled the selection of *n*-BuOH as the G18.EE fraction with the highest activity against the most aggressive melanoma type – the *BRAF*-mutated melanoma. Knowing this, our future goals include isolating and identifying the bioactive compounds in this propolis fraction, followed by a screening to select which has the greatest potential for use as an antitumoral drug. Thus, we do believe that this natural product should continue to be explored.

Concluding, we provided evidence for the first time about the anticancer activity of Portuguese propolis in *BRAF*-mutated melanoma. We suggest that this effect is mediated by a pro-oxidant mechanism involving the accumulation of ROS and the activation of apoptotic pathways. However, further studies are required to confirm the proposed mechanism of action, in order to Portuguese propolis be accepted in the health system. In light of this, other assays such as a cell-cycle analysis could be important to increase our know-how about the propolis mechanism of action. Additionally, the evaluation of antitumoral mechanisms in *BRAF* wild-type melanoma cell lines would be essential.

Portuguese propolis is undoubtedly a fascinating product and quite promising for application in the most diverse areas. Therefore, it remains extremely important to promote and add value to this hive product and its biological properties, in order to contribute to the valorization of the beekeeping sector, a significant portion of the Portuguese economy.

Chapter 7 - References

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Appendix

Appendix 1 - Analysis of the effect of the selected propolis fractions' treatments on cell biomass along the time. Statistical analyses were performed with Two-way ANOVA for both cell lines (A375 and WM9). Bold p-values show statistically significant effects.

A375 Cell Line

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Time	31319	3	10440	F(2.096,20.96) = 72.40	**<0.0001**	0.879
Treatment	3404	4	850.9	F(4,10) = 4.774	**0.0205**	0.440
Time vs Treatment	1494	12	124.5	F(12,30) = 0.8633	0.5901	0.257
Residual	4326	30	144.2	-----	-----	-----

WM9 Cell Line

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Time	4413	3	1471	F(1.192,11.92) = 15.83	**0.0013**	0.613
Treatment	7092	4	1773	F(4,10) = 2.344	0.1252	0.718
Time vs Treatment	3330	12	277.5	F(12,30) = 2.986	**0.0074**	0.544
Residual	2788	30	92.94	-----	-----	-----

*vs = versus

Appendix 2 - Analysis of the effect of the selected propolis fractions' treatments on cell migratory capacity (2D Cell Culture Model) along the time. Statistical analyses were performed with Two-way ANOVA for both cell lines (A375 and WM9). Bold p-values show statistically significant effects.

A375 Cell Line

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Time	18287	7	2612	F(1.407,14.07) = 1755	**<0.0001**	0.994
Treatment	16176	4	4044	F(4,10) = 518.8	**<0.0001**	0.993
Time vs Treatment	9532	28	340.4	F(28,70) = 228.7	**<0.0001**	0.989
Residual	104.2	70	1.488	-----	-----	-----

WM9 Cell Line

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Time	30241	6	5040	F(2.096,20.96) = 2486	**<0.0001**	0.996
Treatment	3985	4	996.1	F(4,10) = 262.9	**<0.0001**	0.970
Time vs Treatment	5576	24	232.3	F(24,60) = 114.6	**<0.0001**	0.979
Residual	121.6	60	2.027	-----	-----	-----

*vs = versus

Appendix 3 - Analysis of the effect of the selected propolis fractions' treatments on tumor spheroid migratory capacity (3D Cell Culture Model) along the time. Statistical analyses were performed with Two-way ANOVA for the A375 cell line. Bold p-values show statistically significant effects.

A375 Cell Line						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Time	3457764	3	1152588	F(2.346,23.46) = 725.7	**<0.0001**	0.986
Treatment	306402	4	76600	F(4,10) = 54.37	**<0.0001**	0.865
Time vs Treatment	162012	12	13501	F(12,30) = 8.500	**<0.0001**	0.773
Residual	47650	30	1588	-----	-----	-----

*vs = versus

Appendix 4 - Analysis of the effect of the selected propolis fractions' treatments on tumor spheroid growth (3D Cell Culture Model) along the time. Statistical analyses were performed with Two-way ANOVA for the A375 cell line. Bold p-values show statistically significant effects.

A375 Cell Line						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Time	3005943	4	751486	F(2.573,25.73) = 204.6	**=0.0001**	0.953
Treatment	439554	4	109889	F(4,10) = 28.87	**<0.0001**	0.749
Time vs Treatment	245090	16	15318	F(16,40) = 4.171	**<0.0001**	0.625
Residual	146915	40	3673	-----	-----	-----

*vs = versus

Appendix 5 - Analysis of the effect of the selected propolis fractions' treatments on extracellular lactate levels along the time. Statistical analyses were performed with Two-way ANOVA for both cell lines (A375 and WM9). Bold p-values show statistically significant effects.

A375 Cell Line

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Time	19839	3	83327	F(1.432,14.32) = 987.9	**<0.0001**	0.887
Treatment	249982	4	4960	F(4,10) = 80.84	**<0.0001**	0.989
Time vs Treatment	9498	12	791.5	F(12,30) = 9.384	**<0.0001**	0.789
Residual	2530	30	84.34	-----	-----	-----

WM9 Cell Line

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Time	1087897	3	362632	F(1.155,11.55) = 345.7	**<0.0001**	0.972
Treatment	132490	4	33122	F(4,10) = 16.48	**<0.0001**	0.808
Time vs Treatment	157876	12	13156	F(12,30) = 12.54	**=0.0002**	0.834
Residual	31472	30	1049	-----	-----	-----

*vs = versus

Appendix 6 - Analysis of the effect of propolis fractions' treatments on the levels of metabolic markers. Statistical analyses were performed with One-way ANOVA for both cell lines (A375 and WM9). Bold p-values show statistically significant effects.

A375 Cell Line – Catalase						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.3128	4	0.07820	F(4,10) = 4.383	**0.0285**	0.6368
Residual	0.1784	10	0.01784	-----	-----	-----
WM9 Cell Line – Catalase						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.1620	4	0.04051	F(4,10) = 1.021	0.4421	0.2899
Residual	0.3968	10	0.03968	-----	-----	-----
A375 Cell Line – CD147						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.001612	4	0.0004029	F(4,10) = 0.07638	0.9878	0.0296
Residual	0.05275	10	0.005275	-----	-----	-----
WM9 Cell Line – CD147						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.01922	4	0.004806	F(4,10) = 0.1284	0.9686	0.0488
Residual	0.3742	10	0.03742	-----	-----	-----
A375 Cell Line – PFKL						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.1503	4	0.03756	F(4,10) = 0.4873	0.7451	0.1633
Residual	0.7703	10	0.07703	-----	-----	-----
WM9 Cell Line – PFKL						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.3302	4	0.08254	F(4,10) = 1.259	0.3480	0.3350
Residual	0.6555	10	0.06555	-----	-----	-----

Appendix 6 (continued) - Analysis of the effect of propolis fractions' treatments on the levels of metabolic markers. Statistical analyses were performed with One-way ANOVA for both cell lines (A375 and WM9). Bold p-values show statistically significant effects.

A375 Cell Line – AMPK						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.7029	4	0.1757	F(4,10) = 0.4543	0.7676	0.1538
Residual	3.868	10	0.3868	-----	-----	-----
WM9 Cell Line – AMPK						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.1082	4	0.02704	F(4,10) = 2.191	**0.0103**	0.4672
Residual	0.1234	10	0.01234	-----	-----	-----
A375 Cell Line – LDHA						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.7937	4	0.1984	F(4,10) = 0.3664	0.8273	0.1278
Residual	5.416	10	0.5416	-----	-----	-----
WM9 Cell Line – LDHA						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.2086	4	0.05205	F(4,10) = 0.9691	0.4658	0.2797
Residual	0.5371	10	0.05371	-----	-----	-----
A375 Cell Line – MCT1						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.03512	4	0.008779	F(4,10) = 0.1184	0.9728	0.045
Residual	0.7415	10	0.07415	-----	-----	-----
WM9 Cell Line – MCT1						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.01613	4	0.004034	F(4,10) = 1.052	0.4284	0.2961
Residual	0.03834	10	0.003834	-----	-----	-----

Appendix 6 (conclusion) - Analysis of the effect of propolis fractions' treatments on the levels of metabolic markers. Statistical analyses were performed with One-way ANOVA for both cell lines (A375 and WM9). Bold p-values show statistically significant effects.

A375 Cell Line – MCT4						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.004827	4	0.001207	F(4,10) = 0.1731	0.9472	0.0647
Residual	0.06972	10	0.006972	-----	-----	-----
WM9 Cell Line – MCT4						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	5,827e-005	4	1,457e-005	F(4,10) = 0.0048	>0.9999	0.0020
Residual	0.03014	10	0.003014	-----	-----	-----

Appendix 7 - Analysis of the effect of the selected propolis fractions' treatments on melanoma cells' ROS levels. Statistical analyses were performed with One-way ANOVA for both cell lines (A375 and WM9). Bold p-values show statistically significant effects.

A375 Cell Line						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	52679542	6	8779924	F(6,14) = 59.85	**<0.0001**	0.9625
Residual	2053646	14	146689	-----	-----	-----

WM9 Cell Line						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	41822810	6	6970468	F(6,14) = 3.972	**0.0157**	0.6299
Residual	24570069	14	1755005	-----	-----	-----

Appendix 8 - Analysis of the effect of the selected propolis fractions' treatments on mitochondrial activity. Statistical analyses were performed with One-way ANOVA for both cell lines (A375 and WM9).

A375 Cell Line						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	51.67	6	8.612	F(6,14) = 2.467	0.0769	0.5139
Residual	48.88	14	3.491	-----	-----	-----

WM9 Cell Line						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	8.320	6	1.387	F (6, 14) = 2.176	0.1082	0.4826
Residual	8.921	14	0.6372	-----	-----	-----

Appendix 9 - Analysis of the effect of the selected propolis fractions' treatments on mitochondrial biomass. Statistical analyses were performed with One-way ANOVA for both cell lines (A375 and WM9). Bold p-values show statistically significant effects.

A375 Cell Line

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	10580118	6	1763353	F(6,14) = 5.993	**0.0028**	0.7198
Residual	4119184	14	294227	-----	-----	-----

WM9 Cell Line

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	22875260	6	3812543	F (6, 14) = 10.33	**0.0002**	0.8158
Residual	5165729	14	368981	-----	-----	-----

Appendix 10 - Analysis of the effect of selected propolis fractions' treatments on mitochondrial polarization. Statistical analyses were performed with One-way ANOVA for both cell lines (A375 and WM9).

A375 Cell Line

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	9159783	6	1526631	F(6,14) = 2.662	0.0616	0.5329
Residual	8030032	14	573574	-----	-----	-----

WM9 Cell Line

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	3083553	6	513926	F (6, 14) = 1.248	0.3407	0.3485
Residual	5763906	14	411708	-----	-----	-----

Appendix 11 - Analysis of the effect of propolis fractions' treatments on the levels of apoptotic markers. Statistical analyses were performed with One-way ANOVA for both cell lines (A375 and WM9). Bold p-values show statistically significant effects.

A375 Cell Line – Bax protein						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.4133	4	0.1033	F(4,10) = 2.431	0.1161	0.4930
Residual	0.4250	10	0.04250	-----	-----	-----
WM9 Cell Line – Bax protein						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.4526	4	0.1131	F(4,10) = 0.6521	0.6385	0.2069
Residual	1.735	10	0.1735	-----	-----	-----
A375 Cell Line – Bcl-2 protein						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.3866	4	0.09666	F(4,10) = 0.6709	0.6269	0.2115
Residual	1.441	10	0.1441	-----	-----	-----
WM9 Cell Line – Bcl-2 protein						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.1520	4	0.03800	F(4,10) = 0.4382	0.7785	0.1491
Residual	0.8673	10	0.08673	-----	-----	-----
A375 Cell Line – Caspase 3 protein						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.3043	4	0.07606	F(4,10) = 2.131	0.1514	0.4644
Residual	0.3510	10	0.03570	-----	-----	-----
WM9 Cell Line – Caspase 3 protein						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.3399	4	0.08498	F(4,10) = 0.9050	0.4969	0.2658
Residual	0.9390	10	0.09390	-----	-----	-----

Appendix 11 (conclusion) - Analysis of the effect of propolis fractions' treatments on the levels of apoptotic markers. Statistical analyses were performed with One-way ANOVA for both cell lines (A375 and WM9). Bold p-values show statistically significant effects.

A375 Cell Line – Caspase 9 protein						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.5295	4	0.1324	F(4,10) = 4.811	**0.0201**	0.6581
Residual	0.2751	10	0.02751	-----	-----	-----
WM9 Cell Line – Caspase 9 protein						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	2.920	4	0.7299	F(4,10) = 12.98	**0.0006**	0.8385
Residual	0.5623	10	0.05623	-----	-----	-----
A375 Cell Line – Bcl-XL protein						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	1.937	4	0.4843	F(4,10) = 10.31	**0.0014**	0.8048
Residual	0.4697	10	0.04697	-----	-----	-----
WM9 Cell Line – Bcl-XL protein						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.02661	4	0.006653	F(4,10) = 0.3726	0.8231	0.1298
Residual	0.1786	10	0.01786	-----	-----	-----
A375 Cell Line – p53 protein						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.5521	4	0.1380	F(4,10) = 1.667	0.2331	0.4000
Residual	0.8281	10	0.08281	-----	-----	-----
WM9 Cell Line – p53 protein						
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F ratio	*p*-value	Partial Eta Squared
Treatment	0.8925	4	0.2231	F(4,10) = 1.426	0.2949	0.3632
Residual	1.565	10	0.1565	-----	-----	-----