Universidade do Minho
Escola de Ciências da Saúde

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# Characterization of the Antitumoral Activity of Portuguese Propolis

Master thesis
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Work performed under the guidance of:
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# Abstract

Over the years, interest in natural products has increased because they are a promising source of new pharmaceutical agents. Propolis is a natural resinous product, collected from several plants by honeybees, which is composed by beeswax, resin and volatile compounds. Propolis has several biological properties that have been widely investigated. In the last years, a number of studies have demonstrated the antitumoral activity of many propolis samples; however, studies with Portuguese propolis are scarce, especially regarding this activity. Taking that into account, we aimed to characterize the chemical composition and the antitumoral activity of a Portuguese propolis sample that was harvested in 2010 in the district of Guarda.

The chemical profiling of an ethanol extract of Pereiro propolis (P10.EE) was done by HPLC-MS and it allowed confirming that the composition of the studied sample is generally similar to other poplar propolis type. Relatively to the antitumoral activity, we first observed that P10.EE affects the cell viability of different tumor cell lines: breast, prostate and brain, being the MDA-MB-231 (breast) and DU145 (prostate) two of the most sensitive ones after 48 hours of treatment ($IC_{50}$ = 0.015 and 0.007 mg/ml respectively). The effect of P10.EE on cancer cell proliferation, cell cycle, cell death, migration, metabolism and angiogenesis was assessed on these two sensitive cell lines. A drug combinatory study with paclitaxel was also performed. Cell proliferation and migration of both cell lines decreased significantly after 24 and 48 hours, with alterations in the cell cycle and increase in cell death in both cell lines. We also observed a significant increase of glucose consumption and lactate production, which is explained, in the MDA-MB-231 cells, by the increased expression of hypoxia inducible factor-1$\alpha$ (HIF-1$\alpha$), pyruvate dehydrogenase kinase (PDK), glucose transporter 1 (GLUT1), lactate dehydrogenase (LDH) and carbonic anhydrase (CAIX). Regarding the antiangiogenic activity, P10.EE induced a decrease in total biomass and proliferation of HBMEC cells and appears to affect the natural occurring neovascularization from existing vessels in chicken chorioallantoic membrane (CAM). The drug combinatory study allowed to uncover the conditions of treatment and concentrations of P10.EE that potentiate the effect of paclitaxel in MDA-MB-231 and DU145 cell viability. In conclusion, apart from the increase in glycolytic metabolism, P10.EE appears to be a good candidate for cancer drug development since it decreases important characteristics that dictate tumorigenesis, such as cell proliferation, migration and angiogenesis and also increases cell death.

# Resumo

Ao longo dos anos, o interesse em produtos naturais tem aumentado uma vez que são uma fonte promissora de novos fármacos. O própolis é um produto natural resinoso, obtido pelas abelhas a partir das plantas, que é composto por cera das abelhas, resinas e compostos voláteis, e possui diversas propriedades biológicas que têm sido investigadas. Nos últimos anos, vários estudos têm demonstrado a atividade antitumoral de diferentes amostras de propolis, no entanto, estudos com amostras Portuguesas são escassos, sobretudo em relação a esta atividade. Assim, este estudo teve como objetivo caraterizar a composição química e a atividade antitumoral de uma amostra de própolis Português, obtida em 2010 no distrito de Guarda.

A caraterização química de um extrato etanólico do própolis do Pereiro (P10.EE) realizada por HPLC-MS permitiu confirmar que, em geral a nossa amostra é semelhante a amostras de propolis denominadas "*poplar propolis type*". Quanto à atividade antitumoral, observou-se pela primeira vez que P10.EE afeta a viabilidade celular de diferentes linhas tumorais de mama, próstata e cérebro, sendo que as linhas MDA-MB-231 (mama) e DU145 (próstata) são das mais sensíveis após 48 horas de tratamento ($IC_{50}$ = 0,015 e 0,007 mg/ml, respetivamente). Neste estudo, foi avaliado o efeito do P10.EE sobre a proliferação, ciclo celular, morte, migração, metabolismo e angiogénese nestas duas linhas celulares. Foi também realizado um estudo de combinação de fármacos. Após 24 e 48 horas, P10.EE diminuiu a proliferação e a migração em ambas as linhas celulares, provocou alterações no ciclo celular e aumento da morte. Quanto ao metabolismo, foi observado um aumento significativo do consumo de glucose e produção de lactato. Isto é explicado na linha celular MDA-MB-231 com o aumento da expressão do "*hypoxia inducible factor-1*$\alpha$" (HIF-1$\alpha$), piruvato desidrogenase quinase (PDK), transportador de glucose 1 (GLUT1), lactato desidrogenase (LDH) e anidrase carbónica IX (CAIX). Observamos ainda que P10.EE induz uma diminuição da viabilidade e proliferação da linha endotelial HBMEC e parece afetar a neovascularização natural que ocorre a partir de vasos existentes na "*chicken chorioallantoic membrane*" (CAM). A combinação de fármacos permitiu revelar as condições de tratamento e as concentrações de paclitaxel e P10.EE que potenciam a redução da viabilidade em ambas as linhas celulares. Em conclusão, apesar do aumento do metabolismo glicolítico, P10.EE parece ser um bom candidato para o desenvolvimento de agentes anticancerígenos, visto que diminui caraterísticas importantes que determinam a tumorigénese, como sejam a proliferação celular, migração e angiogénese e aumento da morte celular.

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# List of Abbreviations

ALD – aldolase

Ang – angiopoietin

BrdU - 5-bromo-2´-deoxyuridine

CA - caffeic acid

CAIX – carbonic anhydrases IX

CAM - chick chorioallantoic membrane

CAPE - caffeic acid phenethyl ester

CDK – cyclin dependente kinase

ECM – extracellular matrix

EEP - ethanolic extract of propolis

ENO1 – enolase 1

ERK1/2 - extracellular signal-regulated kinase 1/2

ESI - MS - electrospray ionization-mass spectrometry

ESI - $MS^n$ - electrospray ionization-tandem mass spectrometry

GC - MS - gas chromatography-mass spectrometry

GLUT - glucose transporter

HE - hexane extract

HIF-1 $\alpha$ - Hypoxia-inducible factor 1-alpha

HK - hexokinase

HPLC - high-performed liquid chromatography

LC-MS - liquid chromatography-mass spectrometry

LDH - lactate dehydrogenase

ME - methanolic extract

MMP - matrix metalloproteinases

MS/MS - tandem mass spectrometry

MCTs - monocarboxylate transporters

NFAT - Nuclear factor of activated T-cells

NF-κB - factor nuclear kappa B

NHE1 - $Na^+$ /$H^+$ exchanger 1

NMR - nuclear magnetic resonance

OXPHOS - oxidative phosphorylation

P10.EE - ethanolic extract of Pereiro propolis

PDK1 - pyruvate dehydrogenase kinase-1

PFK - phosphofructokinase

PGK1 - phosphoglycerate kinase 1

PHD2 - prolyl hydroxylase domain protein 2

PHS - propolis hydroalcoholic solution

PKM2 - pyruvate kinase M2

PPP - pentose phosphate pathway

Rb – retinoblastoma

RNS - nitrogen species

ROS - reactive oxygen

TGF - tumor growth factor

TNF - tumor necrose factor

VEGF - vascular endothelial growth factor

VHL - von Hippel-Lindau protein complex

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# Chapter 1: Introduction

## 1.1. Propolis: a Natural Product that can be used as Source of New Drugs

Over the years, natural products have been a rich and a promising source for discovery of new pharmaceutical agents that are important in medicine [1]. In fact, more than half of the currently available drugs are natural compounds or related [2]. Actually, approximately 60% of the world's population relies almost entirely on plants for medication [3]. This area of research is continually growing and is of enormous interest because the different structural range of natural compounds can provide lead compounds for therapeutic improvement by molecular modification [4]. In science, herbs and other natural plant products have become interesting sources of investigation, but modified plant products by animals have been largely ignored and wasted [5]. However, over the years, because natural products are a promising source of new pharmaceutical agents, there has been an increased interest in a modified plant product, more specifically one produced by bees, propolis [6].

Propolis, a complex mixture of compounds that is also called bee glue, is a natural resinous product collected from several plants, buds and exudates by honeybees (*Apis mellifera*), that is obtained by mixing beeswax and salivary enzymes with the collected resin [7-11]. In terms of characteristics, it is a lipophilic material that is hard and breakable when cold but soft, flexible, and very sticky when warm; it possesses a pleasant aromatic smell and varies in terms of coloration, including brown, green and red among others, depending upon the plant types used as source [5, 7]. In terms of chemical composition, propolis is generally composed by 50% resin, 30% wax, 10% essential oils, 5% pollen and 5% other substances which include minerals and organic compounds like phenolic acids (cinnamic and caffeic acid) or their esters, flavonoids (flavones, flavanones, flavonols, dihydroflavonols chalcones), terpenes, aromatic aldehydes and alcohols, fatty acids, stilbenes and β-steroids [8, 9]. Nevertheless, it is known that the chemical composition of propolis is difficult to standardize because it depends on the vegetation, climate, season and environmental conditions of the site of collection, since bees select different plants as source of propolis in different habitats [6, 8-10].

Etymologically, the Greek word propolis means "a substance in defense of the hive" (*pro*-, for or in defense, and *polis*, the city) [11]. Bees are flying insects that exist for more than 125 million years, and they are one of the species that can explore virtually all habitats on Earth. This

is only possible due to the evolutionary success that they have suffered, since they are able to produce specific products, such as honey, propolis, royal jelly, beeswax and bee venom that are used to meet certain needs [10]. Defense is definitely one of the most important factors in a species life. Regarding that, it is known that most bees can efficiently defend their hives when they are attacked by predators [12]. For that, they apply a thin layer of propolis on the internal walls of their hive, which make the entrance of the hive easier to defend against predators. Also, propolis is used against microorganisms, to repair the block holes and cracks in the hives and to prevent the decomposition of intruders that are killed by bees after an invasion of the hive and are too heavy to be thrown off (e.g., small snakes) [7, 9, 11, 13].

## 1.2. Use of Propolis in Ancient and Current Times

Propolis is not a new discovery. In fact, since bee's domestication that Man explores the bees' products to his own benefit and propolis, one of the most important chemical weapons of bees, is no exception, having been employed extensively by Humans since ancient times [6, 14-17].

It is stated that propolis use dates back to ancient times, at least to 300 BC, where it was used in folk medicine and other activities in many parts of the world [15]. Propolis was very well known to the Egyptians, in particular by the priests who had controlled medicine and chemistry and knew very well its anti-putrefactive properties. Normally, this natural product was used to perform the mummification of corpses. Also, the Greek and the Roman physicians acknowledged the potential of propolis by employing it in wound treatment, as an antiseptic and cicatrizing agent, and as mouth disinfectant. Populations of the new world, like Incas, also used propolis as an anti-pyretic agent. Between the seventeenth and twentieth century this natural product became very popular in Europe. In 1969 in the former Union of Soviet Socialist Republics (USSR) the use of propolis was accepted in human and veterinary medicine, with several applications including the treatment of tuberculosis, where the regression of lung problems and recovery of appetite was observed. Also, it was accepted to cure some diseases in folk Georgian medicine. During World War II (1939-1945) doctors used propolis to treat wounds [14-16], but only in 1985, in Japan, propolis was considered as a promising possibility in pharmacology. Before that, propolis was considered a product without market value, especially because its production affected honey production. Nowadays in Japan, propolis is widely imported from Brazil and it is an important product in alternative medicine [13]. Curiously, in the seventeenth century, The

Italian Antonio Stradivari, considered the most significant and greatest crafter of string instruments, like violins, used propolis as an ingredient in the varnish of his instruments [15].

Propolis is one of the few natural products that maintained popularity for a long period of time, although it is not considered a therapeutic agent in conventional medicine. Actually, it is widely used as a component in pharmaceutical and cosmetic products such as anti-acne creams, facial and body creams, ointments and lotions, and toothpaste solutions for oral hygiene [14, 15]. Furthermore, it has been used in some foods and beverages, or simply as a food supplement or healthy drinks. This name was attributed to the drinks because it is thought propolis improves human health and prevents diseases such as heart disease, diabetes and others [18]. Interestingly, such as in the seventeenth century, propolis is currently used in rosin for stringed instruments and in the repair of accordions [15].

## 1.3. Origin and Composition of Propolis

Useful knowledge of propolis has been obtained, for the last 30 years, through intense studies of its chemical and pharmacological activity. In 1960s, it was thought that, despite its complexity, the chemical composition of propolis was more or less constant; nevertheless, in the following years, analysis of a large number of samples of different geographic origins revealed that the chemical composition of propolis is highly variable [10]. Despite being a product modified by animals, propolis comprises many compounds of plant origin that are the responsible for the various biological activities. Generally, the main constituents of propolis are beeswax, which is secreted by the bees, and resin and volatiles, which are substances obtained from plants [9, 10, 13]. In fact, for propolis manufacturing, bees take the material from plant secretions as well as from wounds in plants or by cutting fragments of vegetable tissues [9, 13]. As already said, the chemical composition of propolis is difficult to standardize because it depends on factors such as the vegetation, climate, season and environmental conditions of the site of collection [6, 8-10].

In Europe, North America and temperate zones of Asia, the main source of resin relies in buds from *Populus* species, more specifically *Populus nigra* [7, 13, 19] (Table 1). In other regions where these plants are non-native, alternative plants are sources of propolis resin. For example, the source of resin in Venezuela is *Clusia minor* [20], in Cuba is *Clusia rosea* [21] and in Brazil is *Baccharis*, more predominantly *Baccharis dracunculifolia* [13]. In propolis from Taiwan, Okinawa and Solomon Islands, the main source of resin is *Macaranga tanariu*. In Canary Island the botanical origin of propolis is unknown [22]. Although less frequently, different genus of plants

such as *Pinus, Acacia, Quercus, Betula, Ulmus, Salix* are also used as sources of propolis resin [23]. Marcucci [7] and Bankova *et al* [11] registered more than 300 known substances in propolis and reports from the last years showed the presence of compounds never mentioned before in several samples of propolis [16, 23, 24]. In a study of propolis standardization, Bankova *et. al.* classified the different types of propolis as poplar propolis, green propolis, red propolis, "Pacific" propolis and "Canarian" propolis (Table 1) [9].

Samples of poplar propolis (e.g. from Europe, North America, New Zealand and temperate

**Table 1 –** Propolis types according to their plant origin and chemical composition

| Propolis type | Geographic origin | Plant source | Main biologic active substances | References |
|---|---|---|---|---|
| **Poplar propolis** | Europe, North America, New Zealand and temperate zones of Asia | Buds from *Populus* spp, more specifically *Populus nigra* | Pinocembrin, pinobanksin, chrysin, galangin, caffeic acid, ferulic acid, cinnamic acid and their esters | [11, 22, 23] |
| **Green propolis** | Brazil | *Baccharis* spp predominantly *Baccharis dracunculifolia* | Prenylated phenylpropanoids, prenylated *p*-coumaric acids, acetophenones, diterpenic acids, caffeoyl quinic acids, kaempferide and isosakuranetin | [9, 11, 24, 25] |
| **Red propolis** | Cuba, Venezuela | *Clusia* spp, more specifically *Clusia rosea* and *Clusia minor* | Polyisoprenylated benzophenones, more specifically nemoronose, xanthochymol and guttiferone E | [9, 20, 21, 26] |
| **"Pacific" propolis** | Okinawa, Taiwan, Indonesia | *Macaranga tanariu* | C-prenylflavonones, alk(en)ylresorcinols, cycloartane-type triterpenes, 27-hydroxyisomangiferolic acid | [27, 28, 29] |
| **"Canarian" propolis** | Canary Islands | Unknown | Furofuran lignans | [30] |

zones of Asia) have a similar chemical composition. Mainly, they are composed by flavones, flavanones, the flavonoids pinocembrin, pinobanksin, chrysin and galangin, the phenolic acids caffeic acid, ferulic acid and cinnamic acid, and their esters [11, 22, 23]. In the last few years, propolis from tropic regions, like Brazil, Cuba and Venezuela has become a subject of interest. Prenylated phenylpropanoids, prenylated *p*-coumaric acids, acetophenones, diterpenic acids, caffeoyl quinic acids were shown to be very common and abundant in propolis from Brazil, mainly from the southeastern region [9, 11, 24]. The flavonoids kaempferide and isosakuranetin were also found in Brazilian propolis samples [25]. Cuban propolis has a peculiar enrichment in polyisoprenylated benzophenones, more specifically nemoronose, which turns this type of sample chemically distinct from both European and Brazilian propolis. It is also composed in a minor

proportion by a mixture of xanthochymol and guttiferone E [9, 21]. Venezuela propolis is also composed by polyisoprenylated benzophenones in addition to the other constituents that are usual in samples of tropical regions [9, 20, 26]. The chemical composition of samples of "Pacific" propolis (e.g. Okinawa, Taiwan and Indonesia) was also analyzed. Okinawa propolis is composed by a new family of compounds, the C-prenylflavonones, more specifically isonymphaeol-B. Also, three already known compounds, nymphaeol-A, nymphaeol-B and nymphaeol-C, were isolated in these samples [27]. Taiwanese propolis is composed by eight prenylated flavanones, among then nymphaeol-A, nymphaeol-B and nymphaeol-C [28]. In another sample of Pacific region, the Indonesian propolis, an inseparable mixture of four alk(en)ylresorcinols (5-pentadecylresorcinol, 5-(8'Z,11'Z-heptadecadienyl)-resorcinol, 5-(11'Z-heptadecenyl)-resorcinol and 5-heptadecylresorcinol along with four prenylflavonones, propolin D, propolin C, propolin F, propolin G, and three cycloartane-type triterpenes, mangiferolic acid, isomangiferolic acid and 27-hydroxyisomangiferolic acid were identified [29]. Different phenolic profiles were found in propolis from Canary Islands in which furofuran lignans are the main compounds. Four furofuran lignans were isolated and characterized as sesamin, aschantin, sesartenin and yangambin. It also contained sugars and sugar alcohols [30].

Portuguese propolis, although little studied, appears to be composed by phenolic acids and flavonoids, similar to the ones found in samples of European origin, but it also contains several other new compounds that had never been referred in the literature. These compounds are new methylated, esterified and hydroxylated derivatives of common poplar flavonoids and pinocembrin/pinobanksin derivatives containing a phenylpropanoic acid derivative moiety in their structure. Also, it contains a *p*-coumaric ester derivative dimer [23, 24].

In addition, propolis contains some minerals such as magnesium, calcium, iodine, potassium, sodium, copper, zinc, manganese and iron, some vitamins like B1, B2, B6, C and E, a number of fatty acids and also some enzymes like succinic dehydrogenase, glucose-6-phosphatase, adenosine triphosphatase and acid phosphatase [16].

In recent years, different analytical approaches have been used for phenolic characterization of propolis [19, 23, 31]. The need for sample preparation depends strongly on the sample type and the techniques used for analysis. First, to analyze and characterize propolis is essential to perform the fractioning of the samples, which is difficult due to its chemical complexity. For that, different solvents that solubilize and extract different compounds are commonly used. Ethanol and methanol, at different concentrations, and water are the most common substances for propolis

extracts preparation. Ethanol extraction is probably the most used type of propolis fractionating in different studies and this procedure allows obtaining enriched samples in phenolic acids and flavonoids [6, 7]. After propolis fractioning, the compounds in the extracts can be analyzed by a large number of analytical methods. Nowadays, spectrophotometry [31], capillary electrophoresis [32], thin-layer chromatography [33], gas chromatography-mass spectrometry (GC-MS) [34], high-performance liquid chromatography (HPLC) [35, 36], liquid chromatography-mass spectrometry (LC-MS) [37-39], electrospray ionization-mass spectrometry (ESI-MS) and electrospray ionization-tandem mass spectrometry (ESI-$MS^n$) [36, 40, 41], and nuclear magnetic resonance (NMR) [42] are used in this type of studies. Among all the referred methods, the most often used is LC-MS, a powerful chemistry technique for analysis of natural products. LC-MS combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry, which has a high sensitivity and allows the discovery of new constituents, difficult to obtain using conventional methods. It is also possible to characterize unknown compounds in propolis samples of different geographic origin, without reference to standards, using tandem mass spectrometry (MS/MS) [43].

## 1.4. Biological Properties and Therapeutic Activity of Propolis

Propolis maintained popularity for a long period of time; however it is not considered a therapeutic agent in conventional medicine because the standardization of chemical composition and biological activity is lacking. Standardization of these two parameters is absolutely essential for acceptance of this natural compound in the health system. For this purpose, it is necessary to characterize different types of propolis, according to its vegetal origin and corresponding chemical profile. Any type of study about the bioactivity of different extracts of propolis must begin with chemical profiling of the extracts because it is essential to have detailed and reliable comparative data of each type of biological activity, combined with chemical data. For that, as already mentioned, a great number of techniques can be used. Knowing the plant origin and the major and minor constituents of propolis allows extrapolating the possible activity and mechanism of action of propolis under study and this provides substantial clues for the development of new drug candidates [6, 9].

In the last decades, several studies have demonstrated the biological and pharmacological actions of different propolis samples, such as antimicrobial, antibacterial and antifungal [6, 7, 44-47], antiviral [7, 47, 48], anti-inflammatory [49], antioxidant [50-52], immunomodulatory [6, 53,

54] and antitumor activities [8, 16, 52, 54], among others [6, 7], revealing the interest of researchers in this bee product and its potential for the development of new drugs as well. Table 2 briefly shows the compounds responsible for the biological activity of different propolis types.

**Table 2 -** Compounds responsible for the biological activities of different propolis types.

| Propolis type | Antioxidant activity | Anti-inflammatory activity | Immunomodulatory activity | Antimicrobial activity | Antitumoral activity |
|---|---|---|---|---|---|
| **Popolar propolis** | Flavonoids, phenolic acids and their esters [50-52, 65] | Flavanones, flavones [68, 69], phenolic acids and their esters (like CAPE) [49, 66, 67, 69, 70] | Phenolic acids and their esters (Caffeic acid phenethyl ester) [53, 80] and flavonoids [75] | Flavanones, flavones, phenolic acids and their esters [46, 48, 81, 82, 86, 95, 101], terpenes [88] | Flavonoids, phenolic acids and their esters [8, 52, 102, 103], like caffeic acid phenethyl ester [16, 109-115, 120, 132] |
| **Green propolis (Baccharis type)** | Prenylated *p*-coumaric acids, flavonoids [10] | Flavanones, flavones [68, 66, 71] | Flavonoids, prenylated *p*-coumaric acids [78] and other phenolic acids and their esters [53, 72, 74, 76, 79] | Prenylated phenylpropanoids, flavonoids and phenolic acids and their esters (artepilin C) [84, 85, 89, 100] | Flavones, flavavones, phenolic acids and their esters (*p*-coumaric and cinnamic acid), cinnamic acid derivatives (artepilin C, drupanin, baccharin) [16, 104-108, 117-119, 128, 130, 135] |
| **Red propolis** | Flavonoids [64], polyisoprenylated benzophenone [21] | Not reviewed | Not reviewed | Phenolic compounds, flavonoids [98] (2',4'-dihydroxychalcone [45]), polyisoprenylated benzophenones [94] | Prenylated benzophenones [10] |
| **"Pacific" and "Canarian" propolis** | Prenylated flavavones [10] | Not reviewed | Flavonoids [77] | Phenolic acids and their esters, flavonoids, di- and tri-terpenes, lignanes [47] | Prenylflavanones [16] |

### 1.4.1. Antioxidant Activity

Oxidative stress is generated when the equilibrium between reactive oxygen and nitrogen species (ROS/RNS) production and the antioxidant defense is disrupted. Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen that are produced through leakage of these species from the mitochondrial electron transport chain. Generation of ROS also occurs by the action of different exogenous agents like UV, toxins, drugs, among others. These species induce damage in biomolecules, such as carbohydrates, proteins, lipids, and nucleic acids, which may alter the cell and lead to its death [55-57]. Oxidative stress is responsible for the occurrence of a wide variety of human diseases, such as neurodegenerative [58] or cardiovascular diseases [59, 60], cancer [55, 61], diabetes [6] and atherosclerosis [62].

In the last years, several studies have been performed to evaluate the antioxidant capacity of natural products, using different assays like the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-

scavenging activity. Propolis is composed by polyphenols, known to have antioxidant activity [57, 63]. Other propolis compounds, like flavonoids [10, 64], among others, also exhibit antioxidant activity. Valente *et al* [52] demonstrated that Portuguese propolis protects human erythrocytes from damage caused by free radicals by decreasing lipid peroxidation. In other words, this suggests that Portuguese propolis is a powerful antioxidant agent that can be used against oxidative stress, thus maintaining the structural and functional integrity of the cells. Also, Moreira [50] and Miguel *et al* [51], using different techniques, obtained results that indicate that Portuguese propolis is an important source of total phenols, flavones and flavonols and it shows antioxidant properties that could be beneficial for human health. Cuesta-Rubio *et al* [21] demonstrated, that nemorosone, the most abundant polyisoprenylated benzophenone present in Cuban propolis, exhibits antioxidant capacity. However, when this compound suffers methylation, a process that facilitates the separation of the compound from the propolis sample, the antioxidant property is abolished. Also, a study using two ethanolic extracts of Slovenian propolis (PEE70 and PEE96) showed that this type of propolis is rich in bioactive compounds that have antioxidant activity. Mavri *et al* [65] showed that, despite the sample PEE96 presented a slight enrichment in three specific compounds, caffeic acid, ferulic acid, and luteolin, PEE70 had slightly higher amounts of phenolic compounds (e.g. quercetin, galangin, chrysin, among others), and displayed a stronger reducing power and ability to scavenge free radicals and metal ions.

### 1.4.2. Anti-Inflammatory Activity

Inflammation is an event that normally occurs in response to the constant exposure to environmental and endogenous stimuli as well as to accidental damage and can be divided into acute and chronic inflammation [49]. Several studies have associated different types of propolis and its various constituents with anti-inflammatory activity [49, 66-71]. Recently, the role of the flavonoids quercetin, flavonols and flavones in modulating inflammatory cell function was studied [68]. All of these compounds are present in different samples of propolis from various geographic origins.

The release and oxygenation of arachidonic acid is a critical event in inflammation. Mirzoeva *et al* [66] demonstrated that propolis components such as caffeic acid phenethyl ester (CAPE), caffeic acid (CA), quercetin and naringenin, among others, inhibit the production of eicosanoids. In fact, these components significantly suppressed the lipoxygenase pathway of arachidonic acid

metabolism, being CAPE the most potent modulator. Also, CAPE appears to be effective in inhibiting cyclooxygenase activity [67].

According to Naito *et al* [71], topical application of propolis is effective in inhibiting carrageenan-induced rat hind paw edema. Propolis appears to inhibit the chemotaxis of human polymorphonuclear leukocytes (PMNs), which also contributes to its anti-inflammatory affects. Hu *et al* [49] showed the anti-inflammatory effects of two different extracts (ethanol and water) of a Chinese sample of propolis in imprinting control region (ICR) mice and Wistar rats. In this study, both samples inhibit the activation and differentiation of mononuclear macrophages, and probably were also involved in the decrease of prostaglandin-E2 (PGE 2) and nitric oxide (NO) levels. Márquez *et al* [70] showed that CAPE had inhibitory effects on transcription factors NF-κB and NFAT and, as a consequence, it inhibited IL-2 gene transcription, IL-2 receptor expression, and proliferation of human T cells. This provides new information into the molecular mechanisms involved in the anti-inflammatory and immunomodulatory activities of this natural compound.

### 1.4.3. Immunomodulatory Activity

In the treatment of different diseases, natural substances have been considered as alternative adjuvant therapies due to its immunomodulatory effects. In the case of propolis, the information about this type of activity was scarce until the 1990s; nevertheless, published work in the last years has provided information about the influence of different propolis samples on the immune system [53, 72-80].

In a study using Brazilian green propolis, it was seen that the administration for 3 days of an ethanolic extract of propolis to male BALB/c mice modulated the activation of the initial steps of the immune response by up-regulating TLR-2 and TLR-4 expression and pro-inflammatory cytokines (IL-1 and IL-6) production by macrophages and spleen cells [72]. In another study using chronically stressed mice, propolis exerted an immunomodulatory action by up-regulating TLR-2 and TLR-4 expression, which contributes to the recognition of microorganisms, favoring the initial steps of the immune response during stress [79]. Orsi *et al* [74] performed a study which evaluated the effect of Brazilian green propolis on macrophage activation by hydrogen peroxide ($H_2O_2$) and NO metabolite determination. Depending on concentration, propolis increased $H_2O_2$ generation and decreased the NO generation, which favors the killing of microorganisms. In addition, using stressed mice, it was seen that propolis treatment also potentiated $H_2O_2$

generation and inhibited NO production by peritoneal macrophages [76]. According to Wang *et al* [80], CAPE can be useful in the treatment of asthma and other allergic diseases because it shows to be capable of inhibiting cytokines and chemokines production by human monocyte-derived dendritic cells (MoDCs), which might be related to the NF-κB signaling pathway. Propolis hydroalcoholic solution from Indonesia, more specifically from Lawang, was used to assess its immunomodulatory activity. Syamsudin *et al* [77] concludes that PHS increases the IgG generation and the macrophage phagocytosis activity and capacity in male BALB/c mice.

The immunomodulatory action of propolis does not occur only at the level of macrophages. In fact, some studies show that this action has also an effect on lymphocyte proliferation [73, 75]. Sá-Nunes *et al* [75] showed inhibitory effects of Brazilian green propolis on splenocyte proliferation, effect that was attributed to flavonoids, and enhancement effects on IFN-γ production by spleen cells. It was also described [73] that under certain conditions, cinnamic acid, a usual component of propolis, increases lymphocyte proliferation and release of the cytokine interleukin-1 (IL-1) and interleukin-2 (IL-2), which contributes to increased host-defense response.

### 1.4.4. Antimicrobial Activity

Antimicrobial activity, one of the most studied biological properties of propolis, is well documented against different virus, bacteria and fungi. This biological activity has been largely investigated in the last years due to the need of new treatments against infectious diseases, especially with the increase of pathogens which have developed resistance to current antibiotics.

#### 1.4.4.1. Antiviral Activity

Propolis comprises a complex of chemicals which play a role in antiviral protection. Several *in vitro* studies have shown the effect of propolis in different viruses, including herpes simplex types 1 and 2, adenovirus type 2, influenza virus or human immunodeficiency virus (HIV), among others [7, 48, 81-86].

Schnitzler*et al* [86] analyzed the antiviral effect of aqueous and ethanol extracts of propolis as well as of the different constituents caffeic acid, *p*-coumaric acid, benzoic acid, galangin, pinocembrin and chrysin, against herpes simplex virus type 1 (HSV-1) in cell culture. Both extracts exhibited high anti-HSV-1 activity when cells were treated prior to viral infection, being

galangin and chrysin the main bioactive compounds. Ethanol extracts of Brazilian propolis were also used to assess anti-HSV-1 activity. The three extracts used were effective against cutaneous HSV-1 infection in mice and some were effective in reducing virus titers in the skin and brain. Besides the anti-viral activity, these propolis extracts appear to exhibit immunological activity against intra-dermal HSV-1 infection in mice, especially by increasing IFN-γ production [84]. Amoros *et al* [48] showed the activity of the major flavonoids of propolis, more specifically flavonols and flavones, being flavonols more active than flavones against HSV-1. Additionally, they analyzed the effect of propolis on several DNA and RNA viruses (HSV-1, HSV-2, adenovirus type 2, vesicular stomatitis virus (VSV) and poliovirus type 2). The propagation of poliovirus was also inhibited. At a concentration of 30 μg/ml, propolis reduced the titer of herpes simplex virus; however, vesicular stomatitis virus and adenovirus were less susceptible. In addition, propolis appears to exert a virucidal action on the enveloped viruses HSV and VSV [82]. Serkedjieva. *et al* [83] performed an *in vitro* study where the antiviral activity of constituents of propolis, such as esters of substituted cinnamic acids was assessed. One of them, isopentyl ferulate, inhibited significantly the infectious activity of influenza virus A/Hong Kong (H3N2) *in vitro*. Also, Shimizu *et al* [85] demonstrated that Brazilian propolis possess anti-influenza virus activity and relieve influenza symptoms in mice.

Over recent years therapeutic benefits of propolis have been described in the treatment of HIV. Gekker *et al* [81] assessed the anti-HIV-1 activity of propolis in $CD4^+$ lymphocytes and microglial cell cultures, observing that propolis inhibited viral expression in a concentration-dependent manner. The possible mechanism of propolis antiviral property was suggested to involve inhibition of viral entry.

#### 1.4.4.2. Antibacterial Activity

The *in vitro* activity of propolis against several bacterial strains has been reported in some studies [7, 44-47, 87]. The effect of ethanolic extract of propolis on the physiology of microorganisms was investigated using different strains of bacteria (*Bacillus subtilis, Escherichia coli and Rhodobacter sphaeroides*). Ethanolic extract of propolis was effective against Gram-positive and some Gram-negative bacteria. It was found that propolis and some of its components, like cinnamic acid and flavonoids, affect the ion permeability of the inner bacterial membrane causing the dissipation of the membrane potential and inhibiting bacterial motility

[87]. Also, five terpenes isolated from Cretan propolis show antimicrobial activity against some Gram-positive and Gram-negative bacteria [88]. The efficacy of other EEP against *Staphylococcus aureus* cultivated in complex media or milk was evaluated in another study. EEP (0–0.5 mg/ml) decreased *S. aureus* growth in a complex media and 1 mg/ml was bactericidal against cell suspensions. In milk, the bactericidal dose of propolis was greater [89].

It has been suggested that the combination of propolis with other antibiotics would allow dose reduction of selected antibiotics, thus potentiating their effect. The antibacterial activity of EEP in some clinically isolated Gram-positive strains, as well as the synergetic effect with some antibiotics was assessed by Scazzocchio e*t al* [44]. The results of this study indicated a significant EEP antimicrobial activity towards all tested strains. Additionally, EEP drastically increased the effect of ampicillin, gentamycin and streptomycin and moderated the action of chloramphenicol, ceftriaxone and vancomycin. No effect was observed when propolis was used simultaneously with erythromycin. Furthermore, Orsi *et al* [90] showed the synergetic effect between ethanolic extracts of propolis from Brazil and Bulgaria and the antibiotics amoxicillin, ampicillin and cefalexin against *Salmonella typhi*. Both samples showed an antibacterial action, being the Bulgarian propolis more efficient, and they have a similar synergetic effect.

Diverse studies show that, as the composition of propolis varies from region to region, the antibacterial activity also displays some variations [47]. According to Seidel *et al* [91], susceptibility of different Gram-positive bacteria to EEP varies with the place of propolis collection. It was seen that the antibacterial effect is greater for samples from a wet-tropical rain forest-type climate. Orsi *et al* [92] performed a study where macrophages, which play an important role in the early phase of Salmonella infection, were pre-stimulated with Brazilian or Bulgarian propolis and then they were challenged with *Salmonella typhimurium*. Depending on the concentrations, both samples increased the bactericidal activity of macrophages; however Brazilian propolis appears to be more efficient than the Bulgarian one. Another study against *Salmonella typhi* was performed in order to investigate a possible synergism between Brazilian and Bulgarian propolis and antibiotic drugs acting on DNA (ciprofloxacin and norfloxacin) and on the metabolism (cotrimoxazole). Both samples had antibacterial activity, but no synergistic effects with the three tested antibiotics was detected [93]. In other studies it is possible to see the influence of the geographical origin of propolis in its antibacterial properties. Samples of Northern Argentine propolis, Cuban propolis and samples of propolis harvested from different areas of Portugal,

which have different composition, exhibit antibacterial activity against different strains of bacteria [45, 46, 94, 95].

#### 1.4.4.3. Antifungal Activity

As the antibacterial and antiviral activities, also the antifungal activity is influenced by the chemical variation of propolis [47]. Recently, several *in vitro* studies have shown the effect of propolis, from different geographic origin, in different fungi [96-101].

The antifungal activity of Brazilian propolis against some *Candida* strains (20 strains of *Candida albicans*, 20 strains of *Candida tropicalis*, 20 strains of *Candida krusei* and 15 strains of *Candida guilliermondii*) was studied by Ota *et al* [96]. All strains used were shown to be sensitive to the alcoholic solution of propolis, being *C. albicans* the most sensitive and *C. guilliermondii* the most resistant. Paracoccidioidomycosis, the most important systemic mycosis in Latin America, is a human systemic mycosis caused through inhalation of airborne conidia or mycelial fragments of the fungus *Paracoccidioides brasiliensis*. Murad *et al* [97] used a sample of Brazilian and Bulgarian propolis to assess the antifungal activity of both samples against *P. brasiliensis*. After stimulating the peritoneal macrophages from BALB/c mice with both samples of propolis, they challenged them with the fungi. Independently from geographic origin, the results of this work suggest an increase in the fungicidal activity of macrophages, which could be through the liberation of fungicidal substances, such as oxygen and nitrogen metabolites, as well as inducing production of some cytokines by the macrophages. Additionally, the *in vitro* antifungal activity of Brazilian green and red propolis was tested against different species of *Trichophyton*, the species of fungi that cause dermatophytosis. Both samples exhibit antifungal activity, being red propolis ethanolic extract more efficient than the green one [99]. A recent study has shown that the use of propolis microparticles, which are obtained from the EEP, gives some advantages in the treatment of vulvovaginal candidiasis [100].

There are many other studies that confirm the influence of the chemical variability of propolis composition derived from different geographic origin in the antifungal activity. Quiroga [98] and Bonvehí *et al* [101] demonstrated the antifungal activity of propolis from the northwest of Argentina and propolis from northern Spain against different fungus, respectively. However, Quiroga focused his study for the environment and the development of agrochemicals with reduced economic costs, possibly containing propolis extracts and its isolated compounds, such as pinocembrin and galangin, as active principles.

### 1.4.5. Antitumoral Activity

Recognition of the hallmarks of cancer affects the search and development of new methods and therapeutic agents with a sufficiently large therapeutic window to kill tumor cells while sparing normal cells. In the last years, the natural product propolis has been the target of a growing interest by a huge number of researchers since it contains a variety of phytochemical compounds that may act through multiple pathways and reduce the development and other malignant characteristics of cancer cells. In fact, over 60% of anticancer compounds are either natural products or substances derived from natural products [4].

Recently, several *in vitro* studies have shown a cytotoxic action of propolis from different geographic origin and of some of its isolated compounds on various tumor cells. Also, *in vivo* studies shows their potential in the development of new antitumor agents, since propolis administration to mammals (e.g. rats) does not lead to side-effects [4]. Briefly, this natural product can block specific oncogene signaling pathways, which in turn leads to a decrease in cell proliferation and growth and can also act by decreasing the cancer stem cell population, increasing apoptosis, exerting antiangiogenic effects and modulating the tumor microenvironment [54, 102, 103].

Cancer cells present uncontrolled growth and proliferation, which is a result of the abnormal function of various genes. Some researchers showed the effect of different types of propolis and its constituents on cancer cell growth, proliferation and apoptosis. In this case, the effect of propolis is also influenced by its chemical variation. The hexane extract (HE) of *Trigona laeviceps* propolis from Thailand contains compounds with antiproliferative activity against five tested cancer cell lines (Chago, KATO-III, SW620, BT474 and Hep-G2) but not against the normal cell lines used (HS27 fibroblast and CH-liver) [5]. Also, three prenylated flavanones isolated from Taiwanese propolis showed more potent cytotoxicity against PC-3 cell line than 5-flurouracil (5-FU) [28]. Akao *et al* [104] demonstrated the cell growth inhibitory effect of two ingredients of propolis (drupanin and baccharin) in human cancer cell lines. Both compounds promoted morphological changes and nucleosomal DNA fragmentation, in other words promoted apoptosis. However their effects were less potent compared to a known antitumor compound from Brazilian propolis, artepillin C. Other studies showed the antitumor activity of Brazilian propolis [105-108]. Extracts of green propolis together with artepillin C, when administered to mice with tumors associated with neurofibromatosis, blocked PAK1 signaling, which is required for tumor growth. As a result, the tumor was suppressed almost completely [106]. Human pancreatic cancer cells

such as PANC-1 are known to exhibit tolerance to nutrition starvation. A methanolic extract (ME) of Brazilian red propolis (10 μg/ml) was found to kill 100% PANC-1 cells in the nutrient-deprived condition, being (6aR,11aR)-3,8-dihydroxy-9-methoxypterocarpan (DMPC) the most potent compound isolated from this sample of propolis [107]. Furthermore, the ethanolic extracts of Brazilian propolis regulate the protein expression of cyclin D1, B1 and cyclin dependent kinase (CDK) as well as p21 in human prostate cancer cells, which significantly affect proliferation [108].

It is known that propolis containing CAPE are different from those with artepillin C. However, with CAPE it is possible to obtain a similar inhibitory effect that is obtained by artepillin C. In fact, some articles analyzed the effect of CAPE in different cancer cell lines and many of its effects have been shown to be mediated through inhibition of NF-κB [54, 109]. It was reported that CAPE can inhibit the proliferation of the colorectal cell line SW480 by decreasing the β-catenin, c-myc and cyclin D1 protein expression [110]. Chuu *et al* [111, 112] observed that CAPE suppressed the proliferation of LNCaP, DU-145, and PC-3 human prostate cancer cells in a dose dependent manner and also inhibited the tumor growth of LNCaP xenografts in nude mice. It was suggested that it acted thought inhibition of p70S6K (an intermediary of the PI3K/AKT pathway responsible for the protein synthesis) and some Akt signaling networks. An *in vivo* study showed that CAPE reduces the growth of C6 glioma cells in mice by inhibiting cell proliferation. Histochemical and immuno-histochemistry analysis revealed a significant reduction in the number of mitotic cells and proliferating cell nuclear antigen (PCNA)-positive cells in C6 glioma [113]. Wu *et al* [114] demonstrated that CAPE inhibits *in vitro* and *in vivo* MCF-7 and MDA-MB-231 tumor growth without much effect on normal mammary cells by reducing the expression of growth and transcription factors, including NF-κB. It also induces cell cycle arrest and apoptosis. As previously said, propolis can also act by decreasing the cancer stem cell population. Recently, Omene *et al* [115] showed the effect of CAPE in breast cancer steam cells. Using the putative breast cancer stem cells with the characteristic CD44 (+)/CD24 (−/low) phenotype and the capability to generate mammospheres from single cells, they found that CAPE caused a dose-dependent inhibition of cancer stem cells self-renewal, progenitor formation, and clonal growth.

Recently, some *in vitro* studies showed different sensitivities of tumor cells to propolis extracts in the context of apoptosis. For example, the study of the apoptotic effect of different extracts of Turkish propolis in the human breast cell line MCF-7 showed that two of the seven extracts of propolis (0.125 mg/ml) induced apoptosis in association with increased number of

TUNEL positive cells, and also an increase in the expression of caspases [116]. In order to evaluate the antitumoral effect of propolis, a more complex study was performed in two cell lines (HL-60 and HT-116) using a sample of Iraqian propolis [17]. The results showed that this sample of propolis inhibits the proliferation of HL-60 cells and leads to down-regulation of Bcl-2 and activation of Bax, thus inducing apoptosis. In HCT-116 cells, propolis inhibits colony formation potential and some necrotic changes were observed, like increase in size, swelling of cytoplasm, loss of membrane integrity, cell rupture and release of cellular contents. An *in vivo* study with HCT-116 tumor-bearing mice showed that, at non-toxic doses, Iraquian propolis decreases mitotic cells and increases p53 and Ki-67 expression [17].

Szliszka *et al* [117, 118] have performed many studies to analyze the antitumoral effect of Brazilian green propolis and its constituents in prostate cancer cells (LNCaP and DU145). It has been proved that these cells are resistant to TRAIL-induced apoptosis. However ethanolic extract of Brazilian propolis and its constituents artepillin C, quercetin, kaempferol and *p*-coumaric acid, sensitize these cells to TRAIL-induced death. Additionally, Brazilian EEP enhanced the expression of TRAIL-R2 and decrease the activity of NF-κB in LNCaP cells [118], and co-treatment with TRAIL and artepillin C induced the significant activation of caspase-8 and caspase-3, as well as a significant disruption of the mitochondrial membrane potential [119].

The cancer inhibitory effects of CAPE have been confirmed in a variety of culture cell lines and many studies have been conducted to understand the pathways involved in the apoptotic effect of this propolis constituent. CAPE induces cell cycle arrest, apoptosis and reduces expression of NF-κB in MDA-MB-231 and MCF-7 human breast cancer [114]. In MCF-7 cells, Fas activation plays a role in NF-κB inhibition-induced apoptosis [120]. In addition, Kamiya. *et al* [121] revealed that an ethanol extract of Brazilian red propolis and CAPE reduced MCF-7 cell viability through induction of mitochondrial dysfunction, caspase-3 activity and DNA fragmentation. Also, an increase expression in CHOP (CCAAT/enhancer-binding protein homologous protein) was seen, which only occurs in the presence of endoplasmic reticulum stress. So, MCF-7 cell apoptosis occurs, at least in part, by ER stress-related signaling.

In PC3 prostate cancer cell lines, CAPE induced apoptosis in a dose-dependent manner that was associated with the loss of apoptosis expression of the inhibitors of apoptosis cIAP-1, cIAP-2 and XIAP [122]. CAPE (6 μg/ml) also induces apoptosis in human leukemic HL-60 cells by activation of caspase-3, down-regulation of Bcl-2, and up-regulation of Bax [123].

It has been known that cancer microenvironment is very important for carcinogenesis and it consists of stromal, endothelial, immune and cancer cells. Intervening in the symbiosis of cancer microenvironments can be achieved by using natural products, like propolis and their constituents. It was demonstrated by Lee *et al* [124] that CAPE (12.5 μM) could effectively suppress the adhesion and invasion potential of human hepatocellular carcinoma cells (SK-Hep1) by inhibiting completely the expression of MMP-2 and MMP-9 and significantly the NF-κB. Taking into consideration all the results, it was postulated that CAPE has anti-metastatic potential. Additionally, CAPE has the same effect in HT1080 human fibrosarcoma cells [125]. Another study that demonstrates that cancers and their tumor microenvironment can be countered by natural products has been performed recently by Hattori *et al* [126]. They examined components which are derived from Brazilian green propolis - bacharin, beturetol, kaempferide, isosakuranetin and drupanin - and found that some components significantly inhibited the expression of the HIF-1α protein and HIF-1α downstream target genes such as glucose transporter 1, hexokinase 2, and vascular endothelial growth factor A (VEGF-A). They also exhibit anti-angiogenic effects (300 ng/CAM) in the chick chorioallantoic membrane (CAM) assay.

Angiogenesis plays a limiting role in tumor growth due to the requirement of oxygen and nutrients to sustain rapid uncontrolled proliferation and metastization. Both tumor and stromal cells can secrete pro-angiogenic factors that stimulate the formation and maintenance of new vessels, such as vascular endothelial growth factor (VEGF) [127]. Taking that into account, it was interesting to find if propolis has antiangiogenic effects. In an *in vitro* tube formation assay the EE of Brazilian propolis could significantly reduce the number of newly formed vessels and suppress the proliferation of Human umbilical vein endothelial cells (HUVECs). Furthermore, the authors demonstrated that the propolis compound responsible for such action was artepillin C (3.15-50 μg/ml) only [128]. Subsequently, the authors investigated the molecular mechanism that leads to the inhibition of angiogenesis. For that, they analyzed two major survival signals, the extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt, after treating the cells. The results showed that the anti-angiogenic effect was mainly mediated through inducing apoptosis in tube-forming endothelial cells through the inactivation of the survival signal ERK1/2 [129]. In an *in vivo* study, Dornela *et al* [130] observed that water extract of propolis inhibits angiogenesis in N-butyl-(-4-hydroxybutyl) nitrosamine (BBN)-induced rat bladder cancer.

The effect of CAPE on angiogenesis was also mentioned in some studies. Song *et al* [131] observed that CAPE inhibited angiogenesis using the *in vivo* assay chick embryo choriallantoic

membrane (CAM). CAPE also suppresses VEGF formation by MDA-MB-231 cells and formation of capillary-like tubes by endothelial cells [114, 132]. Extracts of propolis containing artepillin C and caffeic acid phenyl ester significantly reduced the newly formed vessels and expression of MMPs and VEGF production from various cells [133] and, in accordance with these results, Izuta *et al* [134] described that CAPE promotes inhibition of VEGF expression in HUVEC cells. In a recent study, Daleprane *et al* [135] characterized the molecular mechanism that explains the antiangiogenic activity of red propolis polyphenols on endothelial cells (EC). This study provides evidence that such polyphenols have antiangiogenic effects *in vitro* and *ex* and *in vivo*, resulting from effective inhibition of VEGF gene expression. Under hypoxic conditions, the inhibitory effects on VEGF gene expression were attributed to destabilization of HIF1α protein, which is achieved via an increase in the von Hippel-Lindau (pVHL)-dependent proteasomal degradation. This increase only occurs as a consequence of attenuated Cdc42 protein expression.

The first study on the antitumor activity of Portuguese propolis was only performed in 2010. In this study, Valente *et al* [52] showed a strong inhibition on human renal cell carcinoma (RCC) by two propolis samples from north eastern and central Portugal.

As reviewed here, propolis and its isolated compounds may be useful in different pathological conditions, especially against cancer. However, taking into account the different hallmarks of cancer, further studies on the anticancer activity of propolis must be carried out.

## 1.5. The Hallmarks of Cancer

Over the years, the information that allows us to understand the pathogenesis of cancer has been increasing. In fact, to understand cancer biology it is essential to comprehend the functional capabilities acquired by the different tumor types, via distinct mechanisms at various times during tumorigenesis [136, 137].

It is now clear that cancer cells have distinctive and complementary capabilities that allow tumor unlimited proliferation potential, self-sufficiency in growth signals, and resistance to anti-proliferative and apoptotic stimuli. In large part, these capabilities are achieved by modification and activation of some cellular programs that are typically used in the development [137].

Tumors are more than a mass of proliferating cancer cells. In fact, they are complex tissues composed by different cell types that interact between each other. This interaction contributes to the formation of the tumor microenvironment, which is very important for carcinogenesis and

contributes to the development of certain hallmark capabilities [136]. Also there are two very important *enabling characteristics*, genome instability and mutation, which are heritable molecular changes (genetic and epigenetic) that can orchestrate hallmark capabilities, and the tumor-promoting inflammation, where cells of the immune system are responsible, in different lesions, to promote tumor progression [136].

Hanahan and Weinberg [138] proposed in 2000 the six hallmarks of cancer (Figure 1) that collectively dictate tumorigenesis: insensitivity to growth suppressors, self-sufficiency in proliferative signals, limitless replicative potential, evasion to cell death, sustained angiogenesis and tissue invasion and metastasis [136, 138, 139]. Yet, an increasing body of research suggested two emerging hallmarks of cancer: reprogramming energy metabolism - cancer cells have the capability to reprogram cellular metabolism to support neoplastic proliferation in a most effective way - and evading immune destruction - cancer cells have the ability to evade immunological destruction, in particular by T and B lymphocytes, macrophages, and natural killer cells [136]. Recognition of these concepts its crucial to the development of new methods and therapeutic agents that display a sufficiently large therapeutic window to kill tumor cells while sparing normal cells [140].


**Figure 1 -** The hallmarks of cancer. This illustration encompasses the six hallmarks capabilities, the two emerging hallmarks and the two enabling characteristics [136].

### 1.5.1. Cancer: a Disease with Sustained Proliferation

Cell division is the process by which a parent cell divides into two or more daughter cells, in other words, it consists of two consecutive processes that are characterized by DNA replication and segregation of replicated chromosomes into two separate cells. There are four distinct phases in a proliferating cell population: the G1, S, G2 and M-phases. In a simple way, replication of DNA occurs in the S phase that is preceded by a phase where the cells are preparing for DNA synthesis, the G1 phase. After DNA replication, cells prepare for mitosis (M

phase) in the G2 phase. The cell cycle is controlled by numerous mechanisms that guarantee the correct cell division [141].

Tumors are diverse and heterogeneous, but all share the ability to proliferate. While normal tissues can maintain the homeostasis of cell number, tissue architecture and function by the controlled release of growth factors that regulate progression of cell division, tumor cells show deregulation of these signals by genetic alterations, resulting in an unrestrained cell proliferation [136]. Cancer cells can sustain proliferation by various ways: they can produce growth factor ligands and express the respective receptors; they can send signals to stimulate normal cells of the tumor-associated stroma to produce growth factors, which in turn are used by the tumor cells, and they can also increase the expression of growth factor receptors in the cell surface, leading to an increased response to growth factor even at low concentrations, or modify the receptor structure, facilitating its activation even in the absence of ligand. Additionally, tumor cells can have mutations in the receptor tyrosine kinases (RTKs) or G-protein signal transducers such as Ras, or mutations affecting one of the many intermediary signal transducers molecules in different intracellular pathways, like mutations in the gene of B-Raf, KRAS or c-Myc protein, resulting in constitutive activationoftheseintracellular pathways [136, 142].

In addition to stimulating proliferation by inducing pathways, tumor cells must also escape signaling that negatively regulates cell proliferation. Inactivation of tumor suppressor genes like retinoblastoma (Rb) and TP53 results in dysfunction of proteins that normally inhibit cell cycle progression [141]. The Rb protein regulates growth cycle and cell division. Therefore, tumor cells with defects in Rb acquire continuous cell proliferation. These defects can be achieved by deletion of the Rb gene or by deregulation of the CDKs that phosphorylate and functionally inactivate Rb. Also, in cancer, mutations have been observed in genes encoding CDK, cyclins, CDK-activating enzymes, CKI, CDK substrates, and checkpoint proteins [141, 142]. Furthermore, TP53 gene, that is important to arrest cell cycle in the presence of genome damage or in the lack of essential components for cell growth, is mutated in approximately 50% of human tumors [136]. All these events and others [136, 138] confer high malignancy to cancer cells.

### 1.5.2. Cancer Resistance to Apoptosis

In multicellular organisms, the life and death of cells that are in excess or are potentially dangerous must be controlled and balanced in order to maintain the tissue homeostasis. To achieve that balance, cells use an intrinsic mechanism of self-destruction called programmed cell

death or apoptosis [143]. Apoptosis was originally defined based on morphological features found in dying mammalian cells like nuclear condensation, nuclear fragmentation, membrane blebbing, cellular fragmentation into membrane-bound bodies, phagocytosis of the dying cell, and lack of an ensuing inflammatory response [144].

There are two major pathways that compose the apoptotic machinery: the extrinsic and intrinsic pathways (Figure 2) [136]. The extrinsic pathway is mediated by extracellular death receptor ligands such as tumor necrose factor (TNF) or CD95 that binds to their respective transmembrane receptors, whose ligation triggers recruitment and assembly of multi-protein complexes that activate caspase 8. The intrinsic pathway involves permeabilization of the mitochondrial outer membrane, in response to a variety of stresses, allowing the release of *cytochrome c*, apoptosis inducing factor (AIF) and Smac/DIABLO into the cytosol. This release activates, in turn, a cascade of caspases that act via their proteolytic activities to induce the multiple cellular changes associated with the apoptotic program [136, 143, 145]. Additionally, death initiated at the mitochondrial level is regulated by members of the BCL2 family, which can be divided into anti-apoptotic (e.g. Bcl2, Bcl-$x_L$) and pro-apoptotic proteins (e.g. BAX, BAK,BID, Bim, Puma) [143]. The mechanisms that cause apoptosis remain to be fully enumerated, however several cellular sensors and oncoproteins with an important role in tumor development

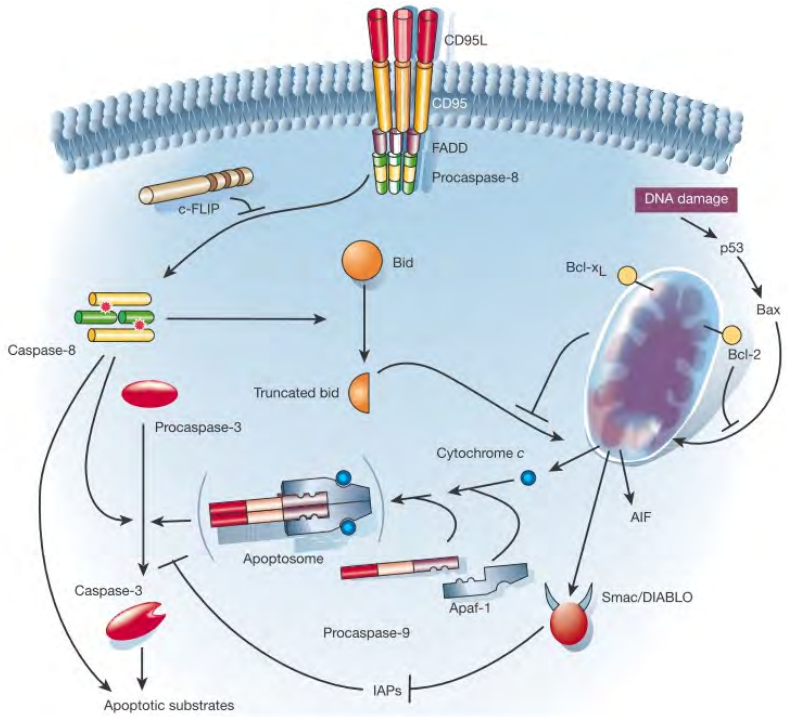



**Figure 2** - Apoptosis, an orchestrated collapse of the cell. In this figure it is possible to see the two major apoptotic pathways in mammalian cells [145].

have been identified, like the sensor of DNA damage which works via TP53 or the oncoproteins c-Myc, E1A or E2F1 [142].

Cancer cells have developed a variety of strategies to resist apoptosis. The most common is the loss of function of the tumor suppressor TP53, which in turn prevents the sensor of DNA damage to induce apoptosis. Additionally, signaling to cell death in response to death receptor ligation or by mitochondrial destabilization can principally be inhibited by mutations in genes that increase the expression of anti-apoptotic proteins ( Bcl-2, Bcl-$x_L$) or survival signals (Igf1/2) or mutations in genes that down regulate the expression of pro-apoptotic proteins (Bax, Bim, Puma, Bak) [146].

### 1.5.3. Induction of Angiogenesis

Similar to normal tissues, tumors need to have means of capturing nutrients and oxygen and excreting the products of their metabolism and carbon dioxide. The tumor-associated vasculature plays a key role in maintaining these functions [136]. In fact, tumors without vascular support may become necrotic or even apoptotic [147]. Angiogenesis, the formation of new blood vessels from pre-existing vasculature, occurs during embryonic development in an active way but in adults it occurs in a transient and highly regulated manner during physiological processes such as wound healing, ovulation and menstruation. In contrast, during tumor progression, angiogenesis is almost always activated and remains in that state to support the expansion of the tumor [136, 148].

Angiogenesis is a complex process and is regulated through production of several pro-angiogenic and anti-angiogenic factors [148, 149]. Different proteins have been identified as angiogenic activators, including vascular endothelial growth factor (VEGF), angiopoietin (Ang), basic fibroblast growth factor (bFGF), angiogenin, transforming growth factors TGF-$\alpha$ and TGF-$\beta$, tumor necrosis factors TNF-$\alpha$, platelet-derived endothelial growth factor, granulocyte colony-stimulating factor (G-CSF), placental growth factor, IL-8, hepatocyte growth factor, and epidermal growth factor. Among these, members of the vascular endothelial growth factor (VEGF) and angiopoietin (Ang) family have a predominant role [147, 149]. Briefly, neovascularization, including in tumor angiogenesis, is basically a four-step process. The angiogenic factors bFGF and VEGF activate endothelial cells, which lead to secretion and activation of matrix metalloproteinases (MMPs) and plasminogen activators. This results in the degradation of the

extracellular matrix, which fills the spaces between cells, allowing endothelial cells to proliferate as they invade the surrounding matrix. Finally, endothelial cells start to organize into hollow tubes that helped by the adhesion factors integrin $\alpha$ or $\beta$ and platelet-derived growth factor (PDGF), evolve gradually into a mature network of blood vessels. Additionally, angiotensin-1 and 2, and their receptor Tie-2 can stabilize and mature the new vessels [147-149]. Due to the uncontrolled tumor angiogenesis, the new vessels in the tumors are typically aberrant, with excessive vessel branching, distorted and enlarged with erratic blood flow, micro-hemorrhages, leakiness and abnormal levels of endothelial cell proliferation and apoptosis [136, 148].

Oncogenes such as Ras and c-Myc can increase the expression of angiogenic factors in some tumors. Additionally, VEGF activity, the major regulator of angiogenesis, is mediated through activation of phosphatidylinositol3-kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK) pathways [136, 148].

### 1.5.4. Mechanisms of Cancer Cell Migration

Vascularization in normal tissue is essential for its growth and evolution, and in tumor tissue is essential for its development, as well as for the metastization of neoplastic cells. Metastasis, a colony of cells that spread from its tissue of origin and subsequent fix and growth in tissues and organs different from the original tissue, is one of the major causes of mortality in cancer patients and occurs as a complex multistep process [150, 151]. To migrate and spread to other tissues, cancer cells use mechanisms that are similar to those used by normal, non-neoplastic cells during physiological processes such as embryonic morphogenesis and wound-healing [152]. Cell motility is a complex and highly coordinated process. In cancer cells this process occurs because there are changes in the expression of several genes that are required for the cell to become motile [150]. To migrate, cancer cells must modify their shape and stiffness as well as their attachment to other cells and to the extracellular matrix (ECM) [136, 152].

Cadherins are transmembrane proteins that play a key role in cell adhesion, making the tissues maintain their cohesion. There are mainly three cadherin types: E-cadherin (present in the epithelia), N-cadherin (present in neurons) and P-cadherin (present in placenta). The best characterized alteration in cancer cells is loss of E-cadherin, a key cell-to-cell adhesion molecule [136]. Additionally, it was revealed that tumor suppressors and oncogenes (e.g.: c-Met, epidermal

growth factor (EGF) receptors, Ras proteins and PTEN (phosphate and tensin homologue)) that have effects on cell proliferation and survival, have also effects on cell motility.

An important question arises when this cancer capability is studied: how do cancer cells move? There are many mechanisms of tumor cell motility, being the mesenchymal cell motility (Figure 3) the best understood of those mechanisms. This type of motility is characterized by an elongated cell morphology and is dependent of proteolytic degradation of the ECM [150]. Briefly, the initiating event for mesenchymal motility is the activation of receptor tyrosine kinases such as c-Met [153]. This leads to the generation of the phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)*P3*) which promotes activation of the small GTPase Rac, and recruitment of the Scar/WAVE family proteins and the Arp2/3 complex, promoting the formation of new F-actin filaments from G-actin. Additionally, oncogenic Ras proteins can also initiate this series of events. These events promote the formation of an actin-rich protrusion [150, 152]. Small integrin-dependent focal contacts are formed after the extension of the protrusion, promoting the attachment to the ECM. Then, some focal contacts develop into large focal adhesions that allow actomyosin contractile force to be transmitted to the ECM [154]. This force and the action of RhoA and its effectors ROCK1 and ROCK2 promote the retraction of the lagging edge of the cell tail, which promotes cell movement. The cell movement is directed by Cdc42 protein, which coordinates actin polymerization [150, 152, 154]. Additionally, mesenchymal cells are able to move through a matrix-filled space by using proteases, such as MMPs [150].

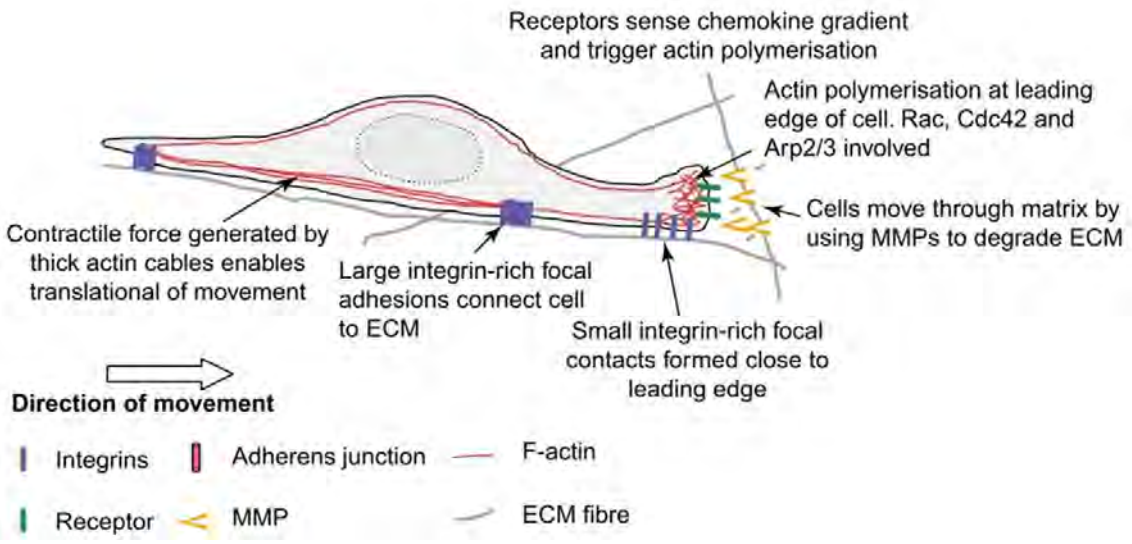



**Figure 3 –** Mechanism of cancer cell motility. Mesenchymal motility is characterized by an elongated cell morphology and is dependent of tumor suppressors and oncogenes activation and proteolytic degradation of the ECM [150].

In the process of invasion and metastasis, this type of movement is extremely important because it allows cancer cells to perform local tissue and lymphatic/blood vessels invasion, which leads to cancer cell extravasation into distant tissues, forming micro metastases that in turn develop into macroscopic tumors (colonization process) [136].

### 1.5.5. Metabolic Reprogramming: An Emerging Hallmark of Cancer

Alteration in cancer cell bioenergetics was the first biochemical hallmark of cancer identified: in fact normal and cancer cells differ largely in energy metabolism [155].

In most normal cells (Figure 4), glucose is the main energy source [156]. In the presence of adequate concentrations of oxygen, complete oxidation of glucose to carbon dioxide is achieved by a process involving cytoplasmic glycolysis, tricarboxylic acid cycle (TCA) and oxidative phosphorylation (OXPHOS) [156-159]. After converting glucose to pyruvate by the process known as glycolysis, pyruvate is first directed into mitochondria where it is oxidized to acetyl-CoA by pyruvate dehydrogenase (PDH). Then, in the TCA the acetyl-CoA is converted into carbon dioxide and reducing equivalents, which are used to produce ATP through oxidative phosphorylation. In this process, normal cells are able to obtain the maximum possible energy through the oxidation of glucose: 32 moles of ATP [156-160].

However, in the absence of oxygen, mitochondrial functions are suppressed, so normal cells perform “anaerobic” glycolysis. In this case pyruvate is shifted from the mitochondrial oxidative pathway and is converted into lactate by lactate dehydrogenase (LDH) [156, 159, 160]. In this process, normal cells are only able to obtain 2 moles of ATP [156].

In contrast, cancer cells (Figure 4), presenting uncontrolled cell growth and proliferation resulting from heritable molecular changes (genetic and epigenetic), depend mostly on the conversion of glucose into lactate for energy production, regardless of the availability of oxygen (Warburg effect). In fact, glucose uptake in this case exceeds the bioenergetic needs of the cell [156, 157, 159, 160]. In most tumor cells, mitochondrial function is suppressed even in the presence of oxygen [156]. This metabolic reprogramming facilitates cancer cell proliferation because it allows not only ATP production but also synthesis of biomass [157].

The observation that cancer cells have higher rates of glucose uptake and lactate production was made by Otto Warburg, in 1956 [161]. Warburg and his colleagues discovered that cancer cells, even under normal oxygen conditions, did not consume more oxygen than normal cells but preferred to perform “aerobic” glycolysis instead of oxidative phosphorylation to produce ATP

[162-164]. Warburg initially assumed that this altered metabolism resulted from mitochondrial defects [163-165], however the hypothesis was later disproved [166]. Different research groups reported that cancer cells did not sacrifice their oxidative phosphorylation to increase the production of lactate [157, 167-169]. In fact, a high mitochondrial membrane potential in a

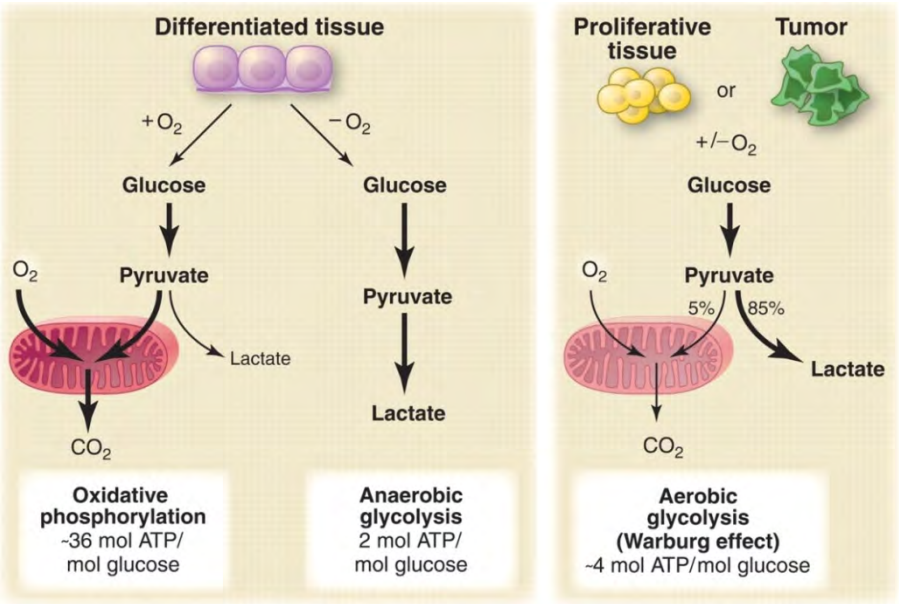



**Figure 4 -** Schematic representation of the differences between oxidative phosphorylation, anaerobic glycolysis, and aerobic glycolysis (Warburg effect) [157].

variety of carcinomas was reported [170], and it was also observed a high mitochondrial membrane potential in cases of chemically or oncogene induced malignant transformation of different cell types [171]. Warburg and his colleagues never considered that mitochondrial metabolism is reprogrammed to meet the challenges of macromolecular synthesis in proliferating cancer cells [165].

### 1.5.6. Glycolytic Metabolism: Molecular Mechanisms

Proliferating cancer cells exhibit increased glycolysis in order to sustain energy production and proliferation, which consists of an increase of glucose uptake and lactate formation, even in the presence of oxygen (Warburg effect) [140, 164, 167, 172, 173]. Currently, the mechanism responsible for this metabolic change has been revealed. It is known that some stresses that exist in the tumor microenvironment are a result of poorly formed tumor vasculature, leading to low levels of oxygen in the tumor regions (hypoxia) [174]. Hypoxia plays an important role in

various cellular and physiological events that are responsible for the malignant alteration of the entire tumor [175].

A great number of human cancers respond to this condition by expressing, in a high frequency, several genes that are important to glycolysis, leading to the Warburg effect [159, 174].

Hypoxia-inducible factor 1 (HIF-1) is the principal driver of glycolysis in tumor cells [176-178]. This transcriptional factor is constituted by two subunits: HIF-1α, which is inducible by hypoxia and is located in the cytoplasm, and HIF-1β, which is constitutively expressed in the nucleus [159, 163, 174, 176, 179]. Under normal conditions of oxygen, HIF-1α protein is unstable because it is rapidly hydroxylated on proline residue 402 (Pro-402) and/or Pro-564 by prolyl hydroxylase domain protein 2 (PHD2). After hydroxylation, HIF-1α is conjugated to the von Hippel-Landau (VHL) protein complex for poly-ubiquitilation, followed by rapid degradation by the proteasome [159, 179, 180].

Under hypoxic conditions, HIF-1α escapes proteolytic degradation, due to the inactivation of the PHD2 enzyme, and migrates into the nucleus where it binds to the β subunit. This process leads to the formation of the active HIF-1 complex. It is known that HIF-1 has many genes as targets, which are directly involved in many functions, including angiogenesis, inflammation and maintenance of high glycolytic rates [177, 179, 181].

The target genes products of HIF-1 involved in the promotion of the aerobic glycolysis are: glucose transporters 1 and 3 (GLUT1 and GLUT3), hexokinases 1 and 2 (HK1, HK2), phosphofructokinase 1 (PFK1) and 2 (PFK2), aldolases A and C (ALDA, ALDC), phosphoglycerate kinase 1 (PGK1), enolase 1 (ENO1), pyruvate kinase M2 (PKM2), lactate dehydrogenase (LDH-A), pyruvate dehydrogenase kinase-1 (PDK1), monocarboxylate transporter 4 (MCT4), carbonic anhydrases IX (CAIX) and others [159, 163, 177, 179]. In tumor cells, overexpression of GLUT1 and GLUT3 supports the high glucose uptake that is needed to support the glycolytic demands. The following enzymes, HK1-2, PFK1-2, ALDA-C, PGK1, ENO1 and PKM2, that suffer also a transcriptional enhancement, contribute to increase the conversion rates of glucose to pyruvate. The role of PDK1 is to phosphorylate and inactivate the catalytic domain of pyruvate dehydrogenase (PDH). In other words, this enzyme acts in a crucial metabolic control point, because pyruvate is not allowed to enter the mitochondria to be converted to acetyl-CoA and therefore is converted to lactate by LDH-A [159, 177, 179, 181].

In addition to contributing to the glycolytic metabolism, which is important for tumor cells to maintain ATP levels and survive, HIF-1 also contributes to acid-resistant phenotype. The increased lactate production is a consequence of the switch to glycolysis, and in order to avoid a drop in intracellular pH that could damage the cells, HIF-1 up-regulates important pH regulators, such as the $Na^+/H^+$ exchanger 1 (NHE1), which pumps $H^+$ out of the cells coupled to a transmembrane $Na^+$ gradient, carbonic anhydrase IX (CAIX), which catalyses the conversion of carbon dioxide into bicarbonate and monocarboxylate transporters (MCTs) such as monocarboxylate transporter 4 (MCT4), which is responsible for the export of lactic acid from the cell [156, 166, 177, 182, 183]. MCT4 is not only important for the acid-resistant phenotype. In fact, some evidence shows that it is also important for the hyper-glycolytic phenotype by exporting newly formed lactate, as it contributes to the continuous aerobic glycolysis by the conversion of pyruvate to lactate [177].

### 1.5.7. Lactate Efflux. Why is it so Important for Cancer Progression?

Lactate, the end product of glycolysis, should be excreted and transported from the cancer cell to the microenvironment to avoid the problem of intracellular acidification. The acid-resistant phenotype is an important characteristic since maintaining an alkaline intracellular milieu is essential for cancer cell survival, whereas acidification of the extracellular microenvironment is important in tumorigenesis [183, 184]. Acidification of the extracellular microenvironment by accumulation of lactic acid and other acidity sources is associated with tumor growth, proliferation and other malignant features like radio and chemoresistance, mutagenesis/clastogenesis or invasive behavior [167, 185]. In addition to these properties, lactate may induce other properties that will contribute to the malignant behavior of cancer cells. As already mentioned, cancer has the ability to evade immune destruction [136]. In cancer regions, extracellular concentration of lactate is high, which will inhibit the lactate efflux from T cells. As a result, the metabolism and the immune responses of T cells against tumor cells is disrupted, since T cells must perform higher rates of glycolysis and a rapid export of lactate in order to be activated [178, 186].

It was recently described by Sonveaux *et al* [160] that lactate is an important metabolite in the symbiosis between glycolytic cancer cells and oxidative cancer cells, in which oxygenated cells consume lactate that is produced by glycolytic cells.

As already mentioned, some evidence shows that, independently from hypoxia, lactate is important for the maintenance of the hyper-glycolytic phenotype because it can regulate some genes that are induced by hypoxia, through stimulating the accumulation of HIF-1α [187]. Additionally, extracellular accumulation of lactic acid and other acidity sources are associated with up-regulation of various angiogenic molecules (such as VEGF, FGF, and TGF) as well as hyaluronan and its receptor CD44, and also induce signaling pathways (including PLCγ, PI3K, Src, Smad signaling) that result in cancer motility, cell proliferation, increased vascular permeability and cell migration [188-194].

For energy and biomass production, cancer cells can use glycolysis or other energetic pathways, although lactate will always be an important metabolite.

### 1.5.8. Energy Supply and Building Blocks for Cancer Growth

Proliferating cancer cells exhibit increased glycolysis to sustain energy production, live in conditions of fluctuating oxygen tension and enhance the production of intermediates for the synthesis of lipids, proteins and nucleic acids [157]. There is a question that remains, why do proliferating cells switch to a less efficient metabolism? Anaerobic glycolysis is a less efficient metabolism in terms of ATP production, because aerobic respiration produces 18 times more ATP per mole of glucose than anaerobic glycolysis; however, anaerobic glycolysis presents a rate that is 100 times superior to the aerobic respiration [195]. Also, converting all the glucose to $CO_2$ in order to maximize ATP production is something against the needs of a proliferating cell. So, cancer cells choose the most convenient way to increase proliferation. Some glucose is diverted to the synthesis of macromolecular precursors that are important to cell proliferation [157]. It was suggested that the major role of glycolysis in cancer is to provide substrates for the pentose phosphate pathway (PPP) to generate nicotinamide adenine dinucleotide phosphate (NADPH) and ribose-5-phosphate, which is used to synthesize DNA and RNA [196, 197]. PPP consists of two distinct parts: an oxidative, non-reversible part that allows reduction of $NADP^+$ to NADPH while converting glucose-6-phosphate to D-Ribulose 5-phosphate and $CO_2$, and a non-oxidative part that reconverts pentose phosphates into glycolytic intermediates. In cancer cells, ribose, which is important to DNA synthesis, is synthesized from glucose or glycolytic intermediates either via the oxidative branch of the PPP or the non-oxidative branch, and this is very important for proliferation. Cancer cells are more susceptible than normal cells to oxidative stress due to

the defective mitochondrial electron transport system. So, the high glycolytic rates of tumor cells confer advantages since it helps cells to avoid reactive oxygen species (ROS). The NADPH that is obtained in the PPP is used by the cells for glutathione regeneration that is responsible for the maintenance of a low level of oxidative stress in cells and is also used for anabolic pathways such as lipid and cholesterol synthesis [157, 164, 166, 196-198].

Taking into account the described above, it is obvious the need to develop new agents that display antitumoral activity with a sufficiently large therapeutic window to kill tumor cells while sparing normal cells. Throughout history, natural products have provided a great number of compounds that have some applications in the fields of medicine, pharmacy and biology. Propolis and its isolated compounds may be useful in different pathological conditions, especially against cancer. However, before establishing a strategy using this bee product, it will be necessary to perform even more studies in order to overcome the problem of chemical standardization and to understand under which conditions it may promote health.

# Chapter 2: Objectives

Portuguese propolis has been little explored but it appears to have promising biological properties [50-52]. On the other hand, it is necessary to identify natural products which could be sources of new pharmacological agents. Thus, in the present study, we aimed to analyze the antitumoral activity of a Portuguese propolis sample. For that, we used a sample of propolis collected in an apiary (Pereiro) located in the central region of the country (Beira Alta), in the district of Guarda, near the city of Pinhel.

To specifically evaluate propolis antitumor activity, different parameters were analyzed using an ethanol extract:

- ✓ Cell viability of different tumor cell lines - breast, prostate and brain cell lines – assessed by the sulforhodamine B (SRB) assay;
- ✓ Cell proliferation of two cell lines that exhibited a greater sensitivity to propolis, assessed by 5-bromo-2´-deoxyuridine (BrdU) assay;
- ✓ Cell death and cell cycle evaluation in the two selected cell lines, assessed by flow cytometry;
- ✓ Cell migration in the two selected cell lines, assessed by the Scratch-wound healing assay;
- ✓ *In vitro* effect of propolis on angiogenesis, assessed by SRB and BrdU assays in one endothelial cell line and *in vivo* effect of propolis on angiogenesis, assessed by the chicken chorioallantoic membrane (CAM) assay;
- ✓ Glucose and lactate measurement in the chosen cell lines, assessed using a colorimetric assay based on an enzyme-catalyzed reaction;
- ✓ The levels of expression of metabolic related proteins, assessed by Western blotting;
- ✓ The influence of ethanolic extract of Pereiro propolis on paclitaxel activity in both cell lines, assessed by a combinatory drug study.

# Chapter 3: Materials and Methods

## 3.1. Sample

Propolis sample (Figure 6) was collected, by conventional scraping, in an apiary (Pereiro) located in the central region of the country (Beira Alta), in the district of Guarda, city of Pinhel (Figure 5). After collection, propolis sample was appropriately labeled and frozen at -20ºC.

The raw propolis sample (Figure 6) was grounded into small pieces and subsequently extracted with ethanol (EtOH 100%) at room temperature, protected from light and under constant stirring. After 1 hour of stirring, the obtained mixture was filtered and then, the sample residues were re-extracted twice more under the same conditions. In all filtrations, the mass of the filtrate was registered in order to know the total weight of the final product and to get an idea of the extraction efficiency and yield. The resultant filtrates were combined and brought to evaporation at 40°C in a rotary evaporator (Buchi, RE 121), in order to obtain the ethanolic extract (EE) of Pereiro (P) propolis (P10.EE), which was kept at 4ºC.

**Figure 5 –** Propolis sampling site.

**Figure 6** – Crude sample of propolis from Pereiro, obtained in the central region of the country.

## 3.2. Sample Preparation

P10.EE was dissolved in dimethyl sulfoxide (DMSO) to a 500 mg/ml stock solution, from which were prepared the working solutions ate the desired concentrations.

Paclitaxel solution concentrated for infusion (6 mg/ml) was purchased from Mylan® and it was dissolved in PBS 1x to make the different working solutions.

The final concentrations of P10.EE and paclitaxel used to treat the cell lines in the assays were obtained by diluting the stock solutions in serum free medium. DMSO and PBS 1x concentrations never exceeded 1%.

## 3.3. HPLC and Mass Spectrometry Analysis

The phenolic profile of the propolis extract was analyzed by HPLC-DAD-ESI-$MS^n$, a powerful chemistry technique that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry. The principal purpose of HPLC is to separate and identify different components of a mixture. The HPLC separation is influenced by the conditions of the liquid solvent (pressure and temperature), by the chemical interactions between the components of the mixture and the liquid solvent and by the chemical interactions between the particles of the stationary phase and the components of the mixture. Mass spectrometry (MS) is an analytical technique that works by ionizing chemical compounds to generate charged molecules or molecule fragments and measuring their mass-to-charge ratios. It is used for determining masses of particles, for determining the elemental composition of a sample or molecule, and for elucidating the chemical structure of molecules.

In this work, the phenolic profile was performed as described by Falcão *et al* [23, 24] by Susana Cardoso (Instituto Politécnico de Coimbra). Briefly, the HPLC analysis was performed on an Ultimate 3000, Dionex separation module equipped with PDA (Varian Prostar) detector. The column used was a 250 mm x 4,6 mm id, 5 µm bread diameter, end-capped Nucleosil C18 (Macherey-Nagel) and its temperature was maintained at 25 ºC. The mobile phase was composed by two solvents which were previously degassed and filtered: (A) 0.1% (v/v) formic acid in water, and (B) acetonitrile. The solvent gradient started with 80% A and 20% B, reaching 30% B at 10 min, 40% B at 40 min, 60% B at 60 min, 90% B at 80 min and return to the initial conditions. In the analysis, the flow rate was 1 ml/min. Spectral data for all peaks were acquired in the range 200–600 nm. The MS used was an Amazon SL (BrukerDaltonics) ion trap MS

equipped with an ESI source. Control and data acquisition were carried out with the Compass Data Analysis data system (BrukerDaltonics, Bremen, Germany). The nitrogen gas pressure (above 99% purity) was a 520 kPa (75 psi). The instrument was operated in negative-ion mode, with Esi voltage set at 5.00 kV and the dry temperature at 200 ºC. The full scan covered the mass range from m/z 70 to 700.

## 3.4. Cell Culture

The *in vitro* experiments were performed using different cancer cell models: breast (MDA-MB-231, MDA-MB-468 and MCF7), prostate (DU145 and 22RV1), brain (U251 and SW1088) and also a human brain microvascular endothelial cell line (HBMEC). Table 3 shows details on the selected cell lines, all obtained from the American Type Culture Collection (ATCC). Breast and brain cancer cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Invitrogen) supplemented with 10% fetal bovine serum (FBS, GIBCO, Invitrogen) and 1% of antibiotic (penicillin/ streptomycin, 10 µg/ml, Gibco, Invitrogen), at 37ºC in a humidified atmosphere of 5% $CO_2$. Prostate cancer cell lines and HBMEC were maintained in Roswell Park Memorial Institute-1640 medium (RPMI, GIBCO, Invitrogen) supplemented with 10% fetal bovine serum (FBS, GIBCO, Invitrogen) and 1% of antibiotic (penicillin/ streptomycin, 10 µg/ml, Gibco, Invitrogen), at 37ºC in a humidified atmosphere of 5% $CO_2$.

**Table 3** - Characteristics of the cell lines used in this study

| Cell line | Organ | Morphology | Growth Properties | Disease | Composition of the culture medium |
|---|---|---|---|---|---|
| MCF7 | Breast | Epithelial | Adherent | Adenocarcinoma | DMEM supplemented with 10% of FBS and 1% of antibiotic (penicillin/streptomycin) |
| MDA-MB-468 | Breast | Epithelial | Adherent | Adenocarcinoma | |
| MDA-MB-231 | Breast | Epithelial | Adherent | Adenocarcinoma | |
| SW1088 | Brain | Fibroblast | Adherent | Astrocytoma | |
| U251 | Brain | Epithelial | Adherent | Glioblastoma | |
| DU145 | Prostate | Epithelial | Adherent | Carcinoma | RPMI supplemented with 10% of FBS and 1% of antibiotic (penicillin/streptomycin) |
| 22RV1 | Prostate | Epithelial | Adherent | Carcinoma | |
| HBMEC | Brain cortical tissue | Epithelial | Adherent | ———— | |

## 3.5. Cell Viability Assay

Cell viability was analyzed using the sulforhodamine B assay (SRB, TOX-6, Sigma-Aldrich). The SRB assay is a colorimetric assay based on the ability of the dye sulforhodamine B to bind electrostatically and pH dependent to protein basic amino acid residues of trichloroacetic acid-fixed cells. Under mild acidic conditions it binds to the cells and under mild basic conditions it can be extracted from cells and solubilized for measurement. An increase and/or decrease in the cell number (total biomass) results in a simultaneous change in the amount of dye incorporated by the cells in the culture, which indirectly indicates the degree of cytotoxicity caused by the propolis sample tested.

All cell lines were plated into 96-well plates, at a density of 15000, 20000 and 13000 cells per well for MDA-MB-231, MDA-MB-468 and MCF7 respectively, 8000 cells per well for DU145 and 22RV1 and 5000 cells per well for SW1088, U251 and HBMEC, and allowed to adhere overnight in complete medium. The effect of the studied propolis ethanol extract on cell number (total biomass) was determined upon P10.EE treatment (0.005-0.1 mg/ml) at 24, 48 and 72 h. After reaching the specific time points, cell culture medium was aspirated and the wells were washed with PBS 1x. Then, 100 µl of cold TCA 10% were added per well and the plate was incubated during 1 hour at 4 ºC. Subsequent to the incubation, the wells were washed three times with water and allowed to air dry thoroughly (at least 24 hours). When the wells were dried, 50 µl of sulforhodamine B were added and the plate was incubated for 30 minutes at room temperature. When the incubation was over, wells were quickly rinsed four times with acetic acid 1%, and allowed to dry during 30 minutes until no moisture was visible. To finalize the assay, 100 µl of Tris 10 mM were added per well and, in a shaker, the plate was allowed to incubate for 5 minutes. The absorbance was read at 490 nm, with a background absorbance of 655 nm. $IC_{50}$ values (concentration of P10.EE that corresponds to 50% of cell growth inhibition) were estimated from 3 independent experiments, each one in triplicate, using Graph Pad Software version 5.03.

## 3.6. Cell Proliferation Assay (BrdU)

The 5-bromo-2´-deoxyuridine assay is a precise, fast, and simple colorimetric assay to measure cell proliferation, which is based on the measurement of BrdU incorporation during DNA synthesis in replicating cells. Using this assay, the P10.EE effect on cell proliferation was assessed. The most sensitive cell lines MDA-MB-231 and DU145 were plated in a 96-well culture

plate, at a cell density of 10000 and 7000 per well, respectively. Cells were allowed to adhere overnight. For the plates containing the MDA-MB-231 cells, the medium was changed to DMEM medium (without FBS) containing 0.0075 mg/ml and 0.015 mg/ml of P10.EE and for the plates containing the DU145 cells, the medium was changed to RPMI medium (without FBS) containing 0.0035 mg/ml and 0.007 mg/ml of P10.EE. The plates were allowed to incubate for 18 hours at 37 ºC in a humidified atmosphere of 5% $CO_2$ and 5 µl of 5-bromo-2´-deoxyuridine (BrdU, 400µM, Roche Applied Science) were added per well. Cells were re-incubated for an additional 6 hours (total of 24 hours of treatment). Following incubation, the labeling medium was removed by suction and 100 µl/well of FixDenat was added to the cells. The cells were incubated for 30 minutes at room temperature. The FixDenat solution was removed and 100 µl/well of anti-BrdU-POD working solution (1:100) were added. After 90 minutes incubation, the antibody conjugate was removed and the wells were washed five times with 200-300 µl of PBS 1x. Finally, the washing solution was removed and 100 µl/well of substrate solution were added. After 5-30 minutes incubation, the reactions were stopped using 25 µl of $H_2SO_4$ 1M (Sigma) and the absorbance was measured in a micro-plate reader (Infinit M200, Tecan) at 450 nm with a reference wavelength of 690 nm.

## 3.7. Metabolism Assay

Metabolism was assessed by glucose and lactate quantification using two colorimetric assays. For that, MDA-MB-231 and DU145 cells were seeded in 48-well plates (Nunc, Thermo Scientific) at a density of 30000 and 28000 cells per well, respectively, and allowed to adhere overnight in complete medium. For the plate containing MDA-MB-231 cells, the medium was changed to DMEM medium (without FBS) containing 0.0075 mg/ml or 0.015 mg/ml of P10.EE. For the plate containing DU145 cells, the medium was changed to RPMI medium (without FBS) containing 0.0035 mg/ml or 0.007 mg/ml of P10.EE. After 24 and 48 hours, 100 µl of supernatant were collected from each well and transferred to a 96-well plate (Nunc, Thermo Scientific) for glucose (Roche Applied Science) and lactate (Spinreact) quantification.

### 3.7.1. Extracellular Glucose Measurement

In this colorimetric assay, the enzyme glucose oxidase (GOD) catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide ($H_2O_2$). The $H_2O_2$ is detected by a chromogenic

oxygen acceptor, phenol, 4-aminophenazone (4-AP) in the presence of peroxidase (POD) (Figure 7). The intensity of the colour formed is proportional to the glucose concentration in the sample.

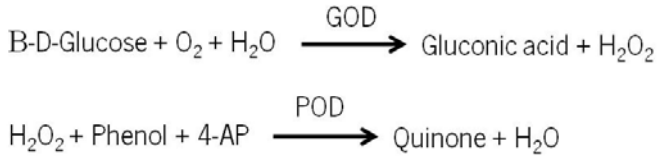



**Figure 7** - Principle of the quantitative glucose determination method.

In a 96-well plate, 10 µl of supernatant were diluted in 90 µl PBS 1x (1:10). After that, 10 µl of the first dilution were diluted in 90 µl of PBS 1x (total dilution 1:100). A calibration curve based in a range of glucose solutions with different concentrations was performed. Finally, 100 µl of glucose reagent was added to the wells and the plate was incubated for 20 minutes. The blank was made with 100 µl PBS and 100 µl of glucose reagent. Absorbance was read at 490 nm.

### 3.7.2. Extracellular Lactate Measurement

In this colorimetric assay, the enzyme lactate oxidase catalyses the oxidation of lactate to pyruvate and hydrogen peroxide ($H_2O_2$). The formed pyruvate and hydrogen peroxide under the influence of peroxidase (POD), 4-aminophenazone (4-AP) and 4-chlorophenol form a red quinone compound (Figure 8). The intensity of the colour formed is proportional to the lactate

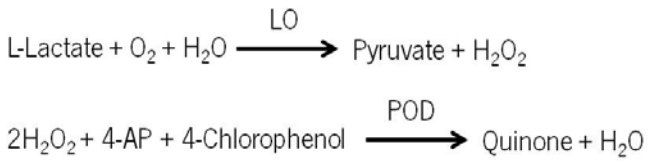



**Figure 8 -** Principle of the quantitative lactate determination method.

concentration in the sample. In a 96-well plate, 2 µl of supernatant were added per well. A calibration curve based in a range of lactate solutions with different concentrations was performed. After that, 200 µl of lactate reagent were added to the wells and the plate was incubated for 10 minutes. The blank was made only with 200 µl of lactate reagent. Absorbance was read at 490 nm.

## 3.8. Cell Migration/Wound Healing Assay

The Scratch Wound Healing Assay is a simple and inexpensive technique used to study the effects of a variety of experimental conditions, like gene-knockdown or chemical compound

treatment, on cell migration. In this assay, a "wound gap" is created by a scratch in a cell monolayer, followed by monitoring the "healing" of this gap by cell migration. This assay helped us to understand the migratory capacity of the cells after P10.EE treatment and it was performed as previously described [199]. The cell lines MDA-MB-231 and DU145 were seeded in a six-well culture plate (Nunc, Thermo Scientific), at a density of $10^6$ cells/well. After reaching 95 % of confluence, the cell monolayer was "wounded" by scraping it with a 200 µl pipette tip and washed once with PBS 1x. Next, MDA-MB-231 cells were covered with DMEM medium (without FBS) containing P10.EE at concentration of 0.0075 mg/ml and 0.015 mg/ml and DU145 cells were covered with RPMI medium (without FBS) containing P10.EE at concentration of 0.0035 mg/ml and 0.007 mg/ml. Specific scratching sites of the wound areas were analyzed and photographed at 0, 24 and 48 hours. The relative migration distances were analyzed using the software QWound (developed at the ICVS by the biomedical engineering team) and data were expressed in % of migration.

## 3.9. Apoptosis Assay

Apoptotic and necrotic cell populations were determined by Annexin V-FLOUS Apoptosis Kit (RocheDiagnostics). This assay is based on the estimation of cell membrane changes during apoptosis and ability of the protein annexin V, a $Ca^{2+}$-dependent phospholipid-binding protein, to bind to phosphatidylserine exposed on the apoptotic cells outer membrane. A simultaneous combination of annexin V and propidium iodine (PI), allows discrimination of apoptotic from necrotic cells.MDA-MB-231 and DU145 cell lines were seeded in T25 flasks (Nunc, Thermo Scientific) at a density of 100000 cells/ml in DMEM and RPMI culture medium, respectively, and allowed to adhere and grow overnight at 37 ºC, 5% $CO_2$. MDA-MB-231 cells were then covered with DMEM medium (without FBS) containing 0.0075 mg/ml or 0.015 mg/ml P10.EE and DU145 cells were covered with RPMI medium (without FBS) containing 0.0035 mg/ml or 0.007 mg/ml P10.EE. After 48 hours of incubation, the culture supernatant was recovered from each flask and treated cells were trypsinized. The trypsinized cells were recovered with new medium and placed with previously recovered supernatant from respective cells. Cell suspensions were centrifuged at 1000 rpm for 5 min, and the supernatant was discarded. Each pellet was re-suspended in 1ml binding buffer (10 mM Hepes pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$). For compensation of fluorochrome spectral overlap, cells were divided in equal volume parts, centrifuged at 1000 rpm for 5 min, and subject to different staining conditions: non staining (300

µl binding buffer), Annexin V staining (5 µl Annexin V + 300 µl binding buffer) and Annexin V/PI staining (5 µl Annexin V + 15 µl PI + 300 µl binding buffer). Cells at these different staining conditions were incubated for 15 min at room temperature. The percentage of cell death was assessed by flow cytometry (LSRII model, BD Biosciences) with a total of 20000 events, and the results were analyzed using the FlowJo software (version 7.6; Tree Star, Inc).

## 3.10. Cell Cycle Analysis

Cell cycle analysis by DNA content quantification is one of the recent applications of flow cytometry. Using the DNA-binding dye propidium iodine (PI), which binds in proportion to the amount of DNA present in the cell, it is possible to discriminate four distinct phases in a proliferating cell population: the G1-, S- (DNA synthesis phase), G2- and M-(mitosis) phase. Cells that are in S phase have more DNA than cells in G1 and will take up proportionally more dye and will fluoresce more brightly until they have doubled their DNA content. MDA-MB-231 and DU145 cell lines were seeded in T25 flasks at a density of 100000 cells/ml in DMEM and RPMI culture medium, respectively, and allowed to adhere and grow overnight at 37 ºC, 5% $CO_2$. Then, cells were put in starvation to ensure synchronization of the cell cycle. For that, during 6 hours, the medium was changed to a medium without FBS. After starvation, MDA-MB-231 cells were covered with DMEM medium (without FBS) containing 0.0075 mg/ml or 0.015 mg/ml P10.EE and DU145 cells were covered with RPMI medium (without FBS) containing 0.0035 mg/ml and 0.007 mg/ml P10.EE. After 48 hours of incubation, the culture supernatant was recovered from each flask and treated cells were trypsinized. The trypsinized cells were recovered with new medium and placed with previously recovered supernatant from the respective cells. Cell suspensions were centrifuged at 1000 rpm for 5 min, and the supernatant was discarded. In constant and slow stirring, each pellet received gradually 800 µl of ethanol 70% for cell fixation. Cells were maintained 30 minutes on ice. Then, the cell suspensions was washed with PBS 1x and centrifuged at 1200 rpm during 5 min. The supernatant was carefully discarded and the cells were re-suspended in 0.3 ml of marking solution (20 µg/ml PI (Sigma), 250 µg/ml RNase (Invitrogen) in PBS/0.01% Triton X), followed by 1 hour incubation at 50 ºC in the dark and with constant stirring. Cell cycle was assessed by flow cytometry (LSRII model, BD Biosciences) with a total of 15000 events, and the results were analyzed using the FlowJo software (version 7.6; Tree Star, Inc).

## 3.11. Drug Combinatory Assay

Drug combination assay is a simple technique that allows to know whether, and to what extent a treatment with a particular combination of two or more drugs is effective. A density of 15000 MDA-MB-231 and 8000 DU145 cells per well were seeded into 96-well plate and allowed to adhere overnight in complete medium. Then, treatments with (i) paclitaxel (0.01 - 1 µM), (ii) P10.EE (0.005 - 0.1 mg/ml), (iii) paclitaxel (0.01 - 1 µM) + P10.EE (0.0075 or 0.0035 mg/ml) were done for 48 hours. Additionally, a different type of treatment was performed treating cells firstly with P10.EE (0.0075 or 0.0035 mg/ml) during 24 hours followed by 24 hours of treatment where P10.EE was removed and the cells were treated with paclitaxel (0.01 - 1 µM). The effect of the drugs on cell viability (total cell biomass) was evaluated using the sulforhodamine B assay, as described above. The combined effect of the drugs was determined using the CalcuSyn Software (Biosoft). For quantification of the synergism or antagonism effect for the two drugs the combinatory effect (CI) was calculated. CI < 1, CI = 1, and CI> 1 indicate synergism, additive and antagonism effects, respectively [200].

## 3.12. Chicken Chorioallantoic Membrane (CAM) Assay

The chicken chorioallantoic membrane (CAM) assay is a quick, technically simple, and inexpensive assay. After its development, the blood vessel network can be easily accessed, manipulated and observed providing an optimal setting to study the effect of different compounds, like propolis, in the formation of new blood vessels (angiogenesis). However, a major problem is the large number of eggs that are required to obtain consist results.

The CAM assay was performed as previously described [199, 201]. Fertilized chicken eggs (Pinto Bar) were incubated at 37 ºC. On day 3 of development, after puncturing the air chamber, a hole in a specific region of the eggshell was performed and eggs were sealed with tape and returned to the incubator. On the 10$^{th}$ day of development, a plastic ring was placed on the CAM and different concentrations of P10.EE were placed inside the ring. The control group received 30 µl of 1% DMSO in PBS 1x and the treated group received 30 µl of 0.1 mg/ml of P10.EE in PBS 1x. Treated eggs were tapped and returned to the incubator. The effect of P10.EE on CAM vascularization was assessed on days 14 and 17 of development. In the 14$^{th}$ day, digital images of the egg were taken in a stereomicroscope (Olympus S2 × 16), using a digital camera (Olympus DP71). After taking the digital images, the eggs received again the same treatment of

the day 10. At the 17th day of development, digital images of the egg were taken as described above and, after that, the chicken embryos were sacrificed by 10 min incubation -80 ºC and the CAMs were dissected in order to take *ex ovo* digital images.

## 3.13. Western Blotting

The Western blot is an analytical technique used to detect specific proteins in sample extracts. Gel electrophoresis is used to separate denatured proteins and the proteins are transferred to a membrane, being labeled with specific antibodies to target protein(s). Propolis-treated MDA-MB-231 and DU145 cells were grown to 80% confluence. Protein samples were prepared by collecting the cells in lysis buffer containing 1% Triton-X, 1% NP-40, 0,1 mM EDTA, 50 mM Tris pH 7.5, 150 mM NaCl and 1/7 protease inhibitor cocktail, for 15 minutes and then centrifuged at 13.000 rpm, 15 minutes at 4 ºC. The supernatant was collected and the protein concentrations determined according to the Bio-Rad Dc Protein Assay (500-0113, Bio Rad). Aliquots of 20 µl of total protein were separated on 10 % (w/v) polyacrylamide gels by SDS-PAGE and transferred to nitrocellulose membranes (Hybond-c Extra, Amersham Bioscience) using a wet system. Membranes were blocked with 5% milk, in TBS/TW 0.1% Tween for 1 hour at room temperature. After incubation overnight at 4 ºC with the primary polyclonal antibodies for MCT1 (1:500 dilution, sc-365501, Santa Cruz Biotechnology); MCT4 (1:2000 dilution, sc-50329, Santa Cruz Biotechnology); CD147 (1:200 dilution, sc-71038, Santa Cruz Biotechnology); HIF-1α (1:1000 dilution, 610958, BD Bioscience); PDK (1:2000 dilution, ab110025, Abcam); LDH-A (1:2000 dilution, ab101562, Abcam); PDH (1:300 dilution, ab67592, Abcam); GLUT1 (1:800 dilution, ab15309-500, Abcam); CAIX (1:1000 dilution, ab15086, Abcam); HKII (1:2000 dilution, ab104836, Abcam); CD44 (1:500 dilution, MCA2726, AbDSerotec), membranes were washed in TBS/0.1% Tween and incubated with the respective secondary antibodies attached to horseradish peroxidase (SantaCruz Biotechnology). Signals of the bound antibodies were detected by chemiluminescence (Supersignal West Femto kit, Pierce, Thermo Scientific). β-Actin was used as loading control at 1:300 dilution (sc-1616; Santa CruzBiotechnology).

## 3.14. Statistical Analysis

All graphs and statistical analysis were performed with the Graph Pad Prism 5 software. The results were expressed as mean ± SEM. Statistical significance between two groups was assessed by t-test. P values ≤0.05, 0.01 and 0.001 were considered statistically significant.

# Chapter 4: Results

## 4.1. Chemical Characterization of P10.EE by UV-visible Spectrophotometry and HPLC-DAD-ESI-MS$^n$

In order to establish the chemical profile of Pereiro propolis, different parameters were analyzed using different procedures.

Total flavonoid and total phenolic content were measured by spectrophotometric methods using folin-ciocalteu reagent and aluminium chloride ($AlCl_3$) respectively.

**Table 4** - Total flavonoids and total phenolic contents of P10.EE. [a] Expressed as mg quercetin equivalent (QE) per g of propolis (unpublished data from Márcia Andreia Oliveira Cruz).

| P10.EE | Flavonoids (mgQE/g)[a] | Total Phenols (mgQE/g)[a] |
|---|---|---|
| | 51.26231 | 252.4199 |

Table 4 shows that ethanolic extract of Pereiro propolis has a high concentration of phenolic compounds but identical flavonoids content to those found in other propolis samples of different geographic origin, like propolis from Bragança and Algarve, north and south of Portugal respectively, propolis from Turkey and Slovenia, among others [8, 50, 51, 65].

The HPLC-DAD-ESI-MS$^n$ analysis of P10.EE sample allowed the identification of phenolic compounds by the determination of their chromatographic behavior, UV spectra and mass spectrometry information.

As represented in the chromatographic profile at 280 nm (Figure 9), the HPLC analysis allowed the collection of 36 fractions. Within the 36 fractions, only the main fractions were

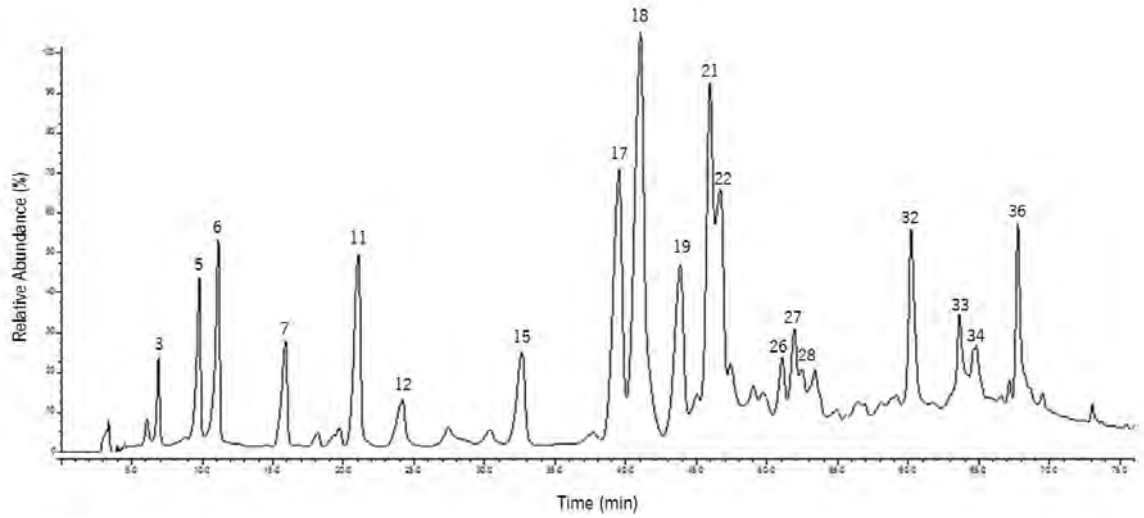



**Figure 9 –** Chromatographic profile at 280 nm of the ethanolic extract of Pereiro propolis sample. The numbers in the figure correspond to the fractions collected that were further analysed by ESI-MS$^n$.

identified by electrospray ionization–mass spectrometry (ESI–$MS^n$). The identification of the compounds in each HPLC fraction was carried out in negative ion mode and was based on the search of the $[M - H]^-$ deprotonated molecule together with the interpretation of its $MS^n$ fragmentations.

The mass spectrometry analysis of the principal fractions allowed the identification of 20 phenolic compounds (Table 5), which included not only phenolic acids and flavonoids already reported but also new compounds not yet identified and never referred in the literature.

The fractions 3, 5, 6 correspond to simple phenolic acids that were eluted in the first 11.5 min (Table 5 and Figure 10). Further analyze by ESI-$MS^n$ allowed the identification of caffeic acid (m/z 179, fraction 3), *p*-coumaric acid (m/z 163, fraction 5) and ferulic acid (m/z 193, fraction

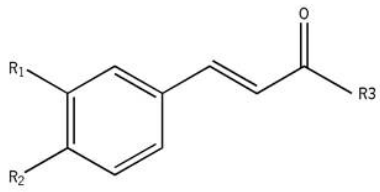
**Table 5 –** Identification of HPLC-eluting fractions from P10.EE and results obtained by mass spectrometry analysis.

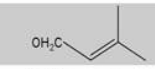
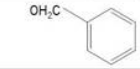
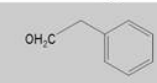
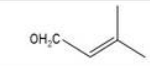
| Peak | $t_R$ (min) | λmax (nm) | $[M - H]^-$ m/z | HPLC-ESI-$MS^n$ m/z (% base peak) | Compound |
|---|---|---|---|---|---|
| **3** | 6.91 | 240, 323 | 179 | $MS^2$ [253]: 135, 163 | Caffeic acid |
| **5** | 9.8 | 231, 309 | 163 | $MS^2$ [163]: 119 | *p*-Coumaric acid |
| **6** | 11.5 | 241, 323 | 193 | $MS^2$ [193]: 177, 149, 133 | Ferulic acid |
| **7** | 15.9 | 242, 322 | np | | Unknown |
| **11** | 21.0 | 234, 308 | 177<br>315 | np<br>$MS^2$ [315]: 299, 269, 271 | *p*-Coumaric acid methyl ester<br>Quercetin-3-methyl ether |
| **12** | 24.2 | 292 | 271 | $MS^2$ [271]: 253, 225, 125 | Pinobanksin |
| **15** | 32.6 | 243, 311 | 233<br>299 | $MS^2$ [233]: 178, 134<br>$MS^2$ [299]: 284 | Unknown<br>Kaempferol-methyl-ether |
| **17** | 39.5 | 243, 325 | 247 | $MS^2$ [247]: 179, 135 | Caffeic acid isoprenyl ester |
| **18** | 41.1 | 242, 325 | 247<br>269 | $MS^2$ [247]: 179, 135<br>$MS^2$ [269]: 178, 134, 221, 265 | Caffeic acid isoprenyl ester (isomer)<br>Caffeic acid benzyl ester |
| **19** | 43.8 | 234, 290 | 255<br>285 | $MS^2$ [255]: 211, 213, 145, 151<br>$MS^2$ [285]: 165, 119 | Pinocembrin<br>Unknown |
| **21** | 45.9 | 268, 308 | 253 | $MS^2$ [253]: 151, 179, 249, 208 | Chrysin |
| **22** | 46.7 | 293 | 313<br>283 | $MS^2$ [313]: 253<br>$MS^2$ [283]: 179, 135 | Pinobanksin-3-*O*acetate<br>Caffeic acid phenylethyl ester |
| **26** | 51.0 | 244, 306 | 231<br>311 | $MS^2$ [231]: 163, 119<br>$MS^2$ [311]: 275, 157, 187 | *p*-coumaric acid isoprenyl ester<br>Unknown |
| **27** | 51.9 | 246, 303 | 231<br>403 | $MS^2$ [231]: 161, 163, 119<br>$MS^2$ [403]: 293, 385, 281, 255 | *p*-coumaric acid isoprenyl ester<br>pinocembrin-*O*4–hydroxyphenylpropanoyl |
| **28** | 52.5 | 247, 295, 318 | 295 | $MS^2$ [295]: 178, 134, 251, 211 | caffeic acid cinnamyl ester |
| **32** | 60.2 | 246, 289 | 315<br>417 | $MS^2$ [315]: 297, 171<br>np<br>np | Unknown<br>Unknown<br>Unknown |
| **33** | 63.6 | 269 | 319<br>271 | np<br>np | Unknown<br>Unknown |
| **34** | 64.8 | 271, 291 | 315<br>565 | $MS^2$ [315]: 179, 135<br>$MS^2$ [565]: 283, 417, 517 | Caffeic acid derivative<br>*p*-Coumaric acid-4-hydroxyphenylethyl ester dimer |
| **36** | 67.8 | 236, 278 | 205<br>297<br>377<br>579 | $MS^2$ [205]: 201<br>np<br>np<br>$MS^2$ [579]: 543, 469 | Unknown<br>Unknown<br>Unknown<br>Unknown |

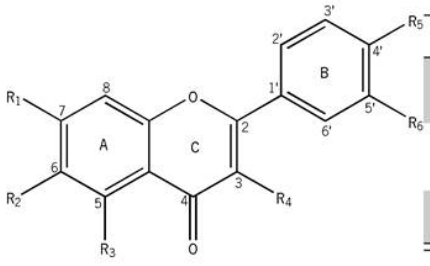
Np – not performed

6). Additionally, some esterified and/or methylated derivatives of caffeic acid and *p*-coumaric acid were also identified. These included five esters of caffeic acid - the caffeic acid isoprenyl ester and its isomer (m/z 247, fraction 17 and m/z 247, fraction 18, respectively), the caffeic acid benzyl ester (m/z 269, fraction 18), the caffeic acid phenylethyl ester (m/z 283, fraction 22) and the caffeic acid cinnamyl ester (m/z 295, fraction 28,) - and also included three esters of *p*-coumaric acid - the *p*-coumaric acid methyl ester (m/z 177, fraction 11), the *p*-coumaric acid isoprenyl ester (m/z 231, fraction 27) and the *p*-coumaric acid-4-hydroxyphenylethyl ester dimer (m/z 565, fraction 34). In addition, one caffeic acid derivative (m/z 315, fraction 34) was detected in P10.EE.

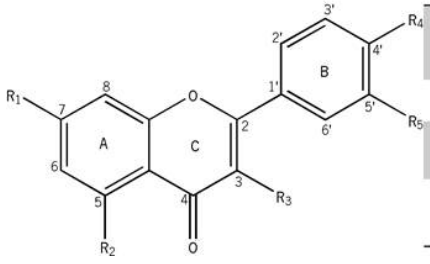
The analysis of the phenolic extract allowed also the detection of dihydroflavonols, flavones,




| Phenolic acids | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| Caffeic acid (3) | OH | OH | OH |
| *p*-Coumaric acid (5) | H | OH | OH |
| Ferulic acid (6) | OH | $OCH_3$ | OH |
| *p*-Coumaric acid methyl ester (11) | H | OH | $OCH_3$ |
| Caffeic acid isoprenyl ester (17) | OH | OH |  |
| Caffeic acid benzyl ester (18) | OH | OH |  |
| Caffeic acid phenylethyl ester, CAPE (22) | OH | OH |  |
| *p*-coumaric acid isoprenyl ester (26, 27) | H | H |  |
| Caffeic acid cinnamyl ester (28) | OH | OH |  |




| Flavonoids | | | | | | |
|---|---|---|---|---|---|---|
| **Flavones and Flavonols** | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | $R_6$ |
| Kaempferol-methyl-ether (15) | OH | H | $OCH_3$ | H | H | H |
| Chrysin (21) | OH | H | OH | H | H | H |




| **Flavonones and Dihydroflavonols** | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ |
|---|---|---|---|---|---|
| Quercetin-3-methyl ether (11) | OH | OH | $OCH_3$ | OH | OH |
| Pinobanksin (12) | OH | OH | OH | H | H |
| Pinocembrin (19) | OH | OH | H | H | H |
| Pinobanksin-3-*O*-acetate (22) | OH | OH | $OCOCH_3$ | H | H |

**Figure 10 -** Structures of several phenolic acids and flavonoids isolated from Pereiro propolis. The number in parentheses represents the number of the fractions according to Figure 9.

flavanones and flavonols in its free form or methylated and/or esterified or hydroxylated forms (Table 5 and Figure 10). In particular it is possible to identify pinobanksin (m/z 271, fraction 12), pinocembrin (m/z 255, fraction 19) and chrysin (m/z 253, fraction 21), the esterified derivative pinobanksin-3-*O*-acetate (m/z 313, fraction 22), the hydroxylated derivative pinocembrin-*O*-4-hydroxyphenylpropanoyl (m/z 403, fraction 27) and the mono-methyl ether derivative quercetin-3-methyl ether (m/z 315, fraction 11) and kaempferol-methyl ether (m/z 299, fraction 15). This analysis allowed also elution of some compounds which occurrence has never been referred in the literature (fractions 7, 15, 19, 26, 32, 33, 36).

## 4.2. Effect of P10.EE in the Viability of Different Cancer Cell Lines

To characterize the antitumoral activity of P10.EE, a screening for cell viability using the Sulforhodamine B assay was first performed, using different cancer cell lines derived from breast, prostate and brain tissue. $IC_{50}$ values were calculated to understand the sensitivity of the different cell lines to the studied propolis sample.

The response of the various cancer cell lines to P10.EE was estimated after 24, 48 and 72 hours of treatment using the following concentrations: 0.005, 0.01, 0.025, 0.05 and 0.1 mg/ml. Table 6 shows the calculated $IC_{50}$ values for all the tested cancer cell lines along time.

**Table 6 -** Concentration of P10.EE that inhibits 50% of cell growth ($IC_{50}$) in different cancer cell lines.

| Cell Lines | $IC_{50}$ (mg/ml) | | |
|---|---|---|---|
| | **24h** | **48h** | **72h** |
| MDA-MB-231 | 0.034 | 0.015 | 0.013 |
| MDA-MB-468 | 0.022 | 0.011 | 0.04 |
| MCF7 | 0.019 | 0.017 | 0.02 |
| DU145 | 0.028 | 0.007 | 0.007 |
| 22RV1 | 0.023 | 0.01 | 0.01 |
| SW1088 | 0.033 | 0.015 | 0.029 |
| U251 | 0.022 | 0.013 | 0.007 |

All the cancer cell lines suffered a decrease in total biomass after propolis treatment (Figure 11); nevertheless, there are cells that are more sensitive than others.

Regarding the results for breast cancer cell lines (Figure 11A-C), it is possible to observe that MCF7 cell line (Figure 11B) is the most sensitive of the three after 24 hours of treatment;

however it becomes less sensitive after 72 hours. The same happens to the MDA-MB-468 cell line (Figure 11C). In opposition, MDA-MB-231 cells (Figure 11A) become more sensitive to propolis over time.

Relatively to the prostate cancer cell lines, DU145 (Figure 11D) and 22RV1 (Figure 11E), both become more sensitive along time, being the DU145 the most sensitive one; however, between 48 and 72 hours, there are no differences in the $IC_{50}$ values. Regarding the brain cell lines, U251 (Figure 11F) and SW1088 (Figure 11G), it is possible to observe that U251 cells become more sensitive along time, whereas SW1088 cells are less sensitive.

For subsequent studies, the concentration of propolis used were the $IC_{50}$ values at 48 hours and two cell lines were chosen on the basis of their sensitivity to P10.EE: DU145, the most sensitive cell line and MDA-MB-231 which is also very sensitive to P10.EE but presents an $IC_{50}$ 2-fold higher than the $IC_{50}$ value calculated for DU145 cells.

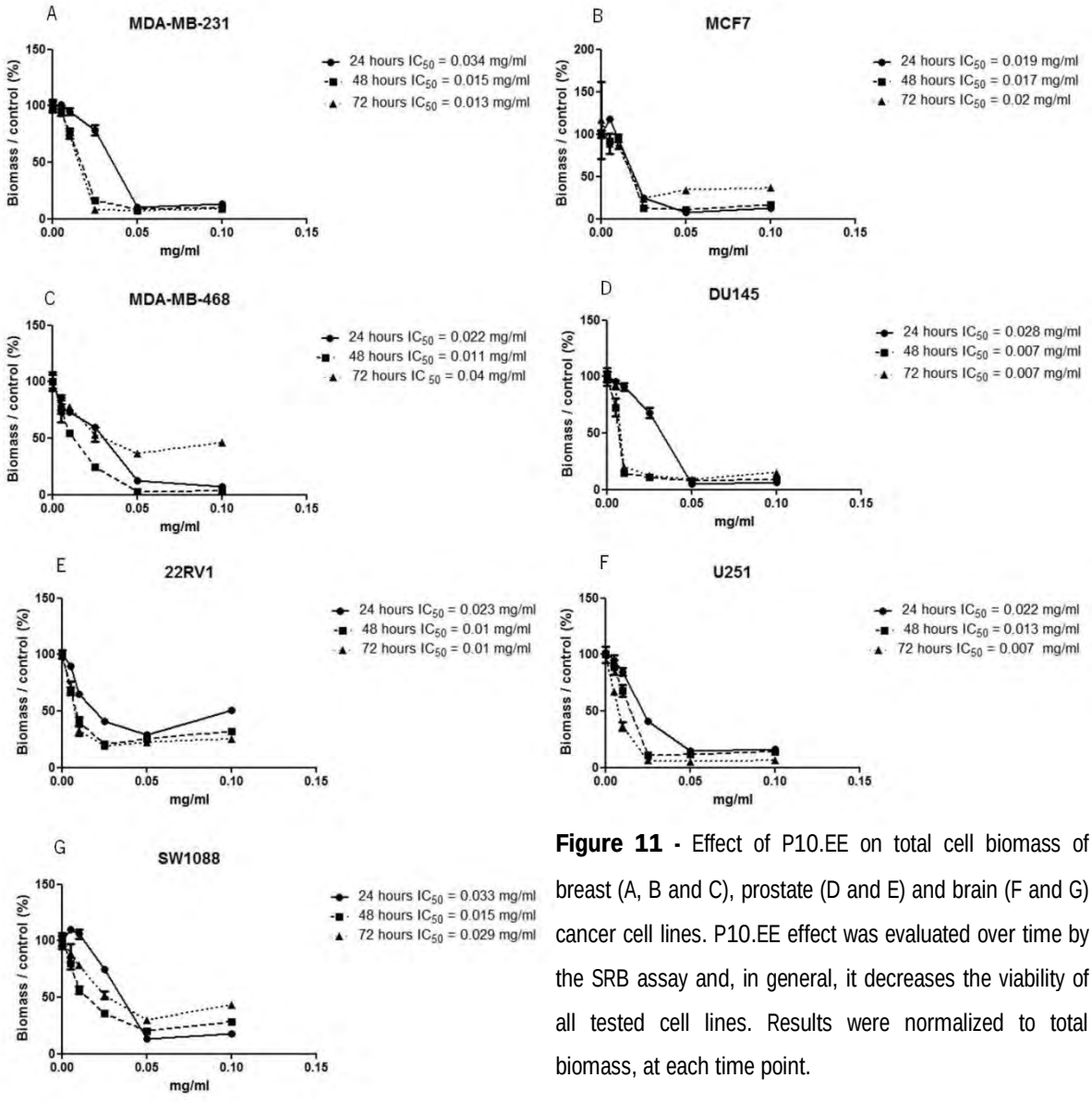



**Figure 11 -** Effect of P10.EE on total cell biomass of breast (A, B and C), prostate (D and E) and brain (F and G) cancer cell lines. P10.EE effect was evaluated over time by the SRB assay and, in general, it decreases the viability of all tested cell lines. Results were normalized to total biomass, at each time point.

## 4.3. Effect of P10.EE on the Proliferation and Cell Cycle of Breast and Prostate Cancer Cell Lines

The effect of P10.EE on the proliferation of breast (MDA-MB-231) and prostate (DU145) cancer cell lines was analysed by the BrdU assay, by measuring BrdU incorporation during DNA synthesis (Figure 12A and B) in propolis-treated cells. In Figure 12A, it is possible to observe that different concentrations of P10.EE (0.0075 and 0.015 mg/ml) promoted a significant reduction in MDA-MB-231 cell line proliferation compared to the control, being this effect concentration-dependent. Additionally, DU145 cell line also suffers a significant decrease in proliferation, comparing to control, after P10.EE treatment at different concentration (0.0035 and 0.007

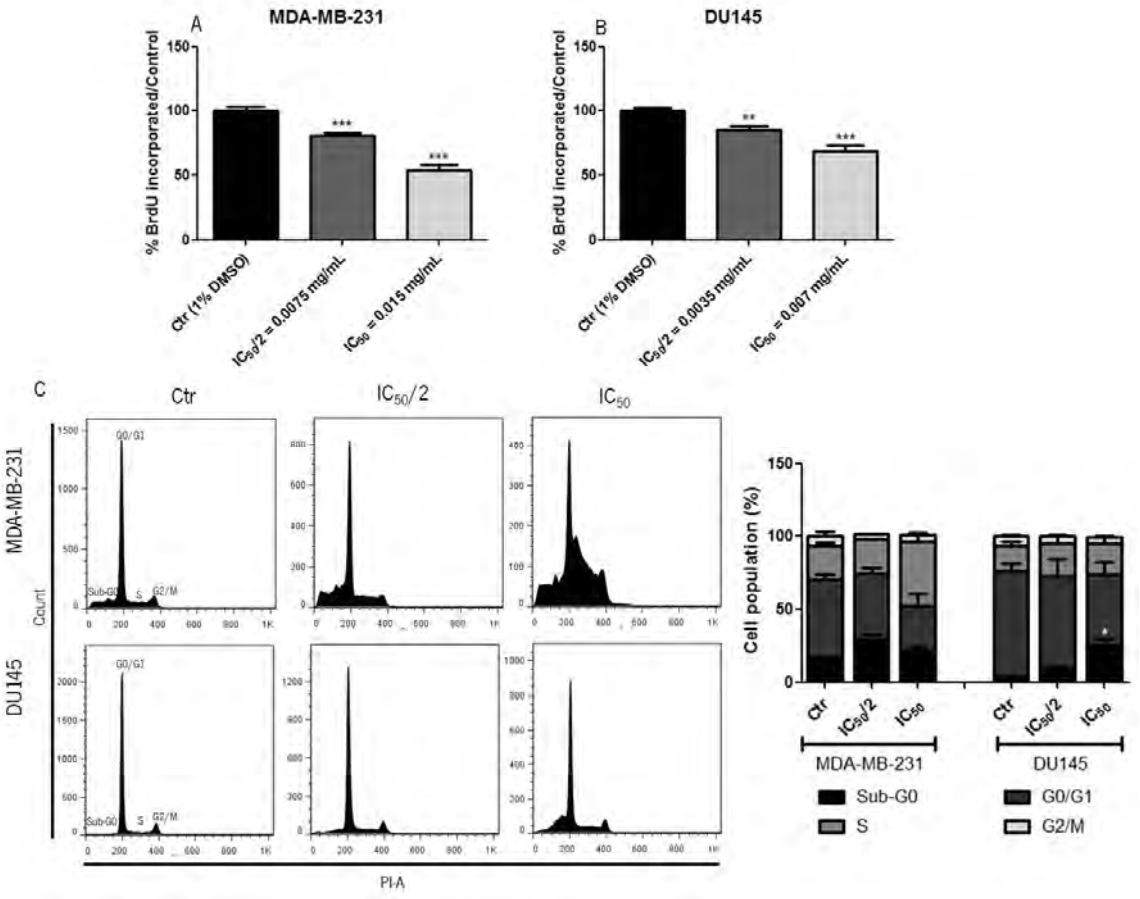



**Figure 12 –** Effect of P10.EE on breast (A) and prostate (B) cancer cell lines proliferation, determined by BrdU assay and on cell cycle distribution (C), determined by flow cytometry of iodide propidium stained cells. **(A** and **B)** P10.EE decreased significantly the proliferative capacity of MDA-MB-231 and DU145 after 24 h of treatment. **$p \leq 0.01$; ***$p \leq 0.001$, comparing treatment conditions with control. Results are expressed as the mean ± SEM of at least three independent experiments, each in triplicate. **(C)** Cell cycle studies were performed after 48 h of P10.EE treatment, at corresponding $IC_{50}$ values, for MDA-MB-231 and DU145. P10.EE induced a decrease in G0/G1 with an increase in S phase population in MDA-MB-231 and significantly increased the sub-G0 phase population in DU145 cells. *$p \leq 0.05$, comparing treatment conditions with controls. Results are expressed as mean ± SEM of two independent experiments

mg/ml) (Figure 12B). This effect was also concentration-dependent.

The effect of P10.EE in the cell cycle of the two chosen cancer cell lines was analysed by flow cytometry. MDA-MB-231 cells were treated with 0.0075 and 0.015 mg/ml of P10.EE and DU145 cells were treated with 0.0035 and 0.007 mg/ml of P10.EE. As seen in Figure 12C, MDA-MB-231 presented a decrease in the cell population of G0/G1 phases and G2/M phases which was accompanied with an increase in the cell population of S phase, compared to the control. These alterations are visible upon treatment with 0.0075 mg/ml of P10.EE, becoming more evident with 0.015 mg/ml of P10.EE. Additionally, an increase in cell population of sub-G0 phase was observed. In this case it is possible to observe an implicit tendency of the results, which could become statistically significant by performing more assays. However, in the DU145 cells, there was a significant increase in the cell population of sub-G0 phase with a decrease in the cell population of the G0/G1 phases, compared to the control, being the greatest effect obtained at the highest concentration tested. Also, comparing to control, it is possible to observe a slight increase in the cell population of S phase.

## 4.4. Effect of P10.EE on the Cell Death of Breast and prostate Cancer Cell Lines

Many natural compounds can inhibit tumor cell growth, but not all of them can trigger apoptosis. In order to determine whether apoptosis was induced by P10.EE, we performed flow cytometry analysis with the Annexin V/PI assay. Annexin V-FITC-negative, PI-negative (Annexin V-FITC (-) PI (-)) cells were considered to be viable, Annexin V-FITC-positive, PI-negative (Annexin V-FITC (+) PI (-)) cells were considered to be early apoptotic and Annexin V-FITC-positive, PI-positive (Annexin V-FITC (+) PI (+)) cells were considered to be late apoptotic/necrotic (Figure 13). By observing Figure 13, it is possible to conclude that 0.015 mg/ml of P10.EE induced cell death in MDA-MB-231 cells by a significant increase in early apoptotic and late apoptotic/necrotic cell population, comparing to control. Despite non-significant, this effect was also observed when the cells were treated with 0.0075 mg/ml of P10.EE. Nevertheless, treatment of DU145 cells with 0.007 mg/ml of P10.EE only promoted a slight increase in late apoptotic/necrotic cell population.

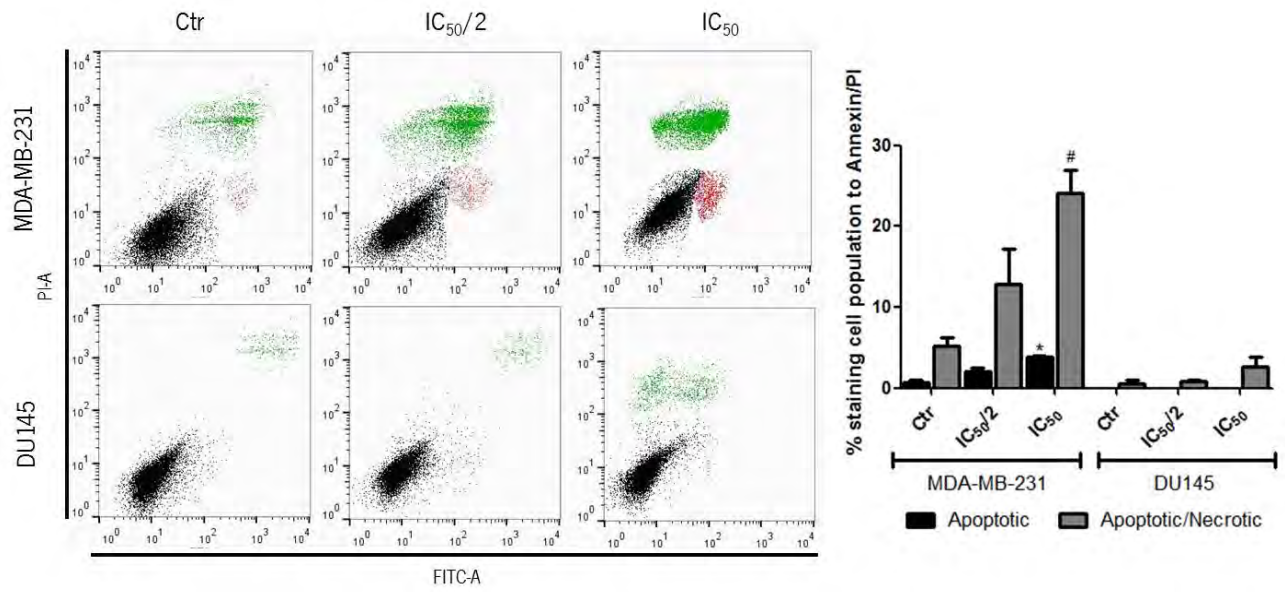



**Figure 13 –** Effect of P10.EE on breast and prostate cancer cell death. Cell death analysis was performed by Annexin V/PI (flow cytometry) after 48 h of treatment with $IC_{50}/2$ and $IC_{50}$ values of P10.EE. In MDA-MB-231 cells a significant increase in cell death was observed whereas there were no differences for DU145. Representative dot plot of cell population distribution stained for Annexin V and PI are shown (cell population in bottom/left = viable cells; the cell population in down/right = dead cells [early apoptosis] and the cell population in upper/right = dead cells [late apoptosis/necrotic]). *p≤0.05 or #p≤0.05 , comparing to control. Results are expressed as mean ± SEM of two independent experiments.

## 4.5. Effect of P10.EE on the Migration of Breast and Prostate Cancer Cell Lines

Cancer cells have the ability to migrate from one organ or part to another non-adjacent organ or part, which turns the treatment more difficult and increases mortality rates. In this study, we examined the effect of P10.EE on cell migration performing the scratch-wound healing assay in MDA-MB-231 and DU145 cells treated for 24 and 48 hours with different concentrations of propolis (Figure 14). In MDA-MB-231 (Figure 14A) it is possible to observe that after 24 and 48 hours both concentrations of P10.EE (0.0075 and 0.015 mg/ml) decreased the capacity of wound closure by the cells comparing to the control, being the effect greater for 0.0015 mg/ml. This decrease was only significant for both concentrations after 48 hours of treatment and only 0.015 mg/ml of P10.EE caused a significant decrease in cell migration from 24 to 48 hours.

Regarding DU145 cells (Figure 14B), both concentrations of P10.EE (0.0035 and 0.007 mg/ml) significantly decreased the capacity of wound closure by the cells and this effect seems to be dose-dependent. Nevertheless, there are no significant differences between the treatment conditions of 24 and 48 hours.

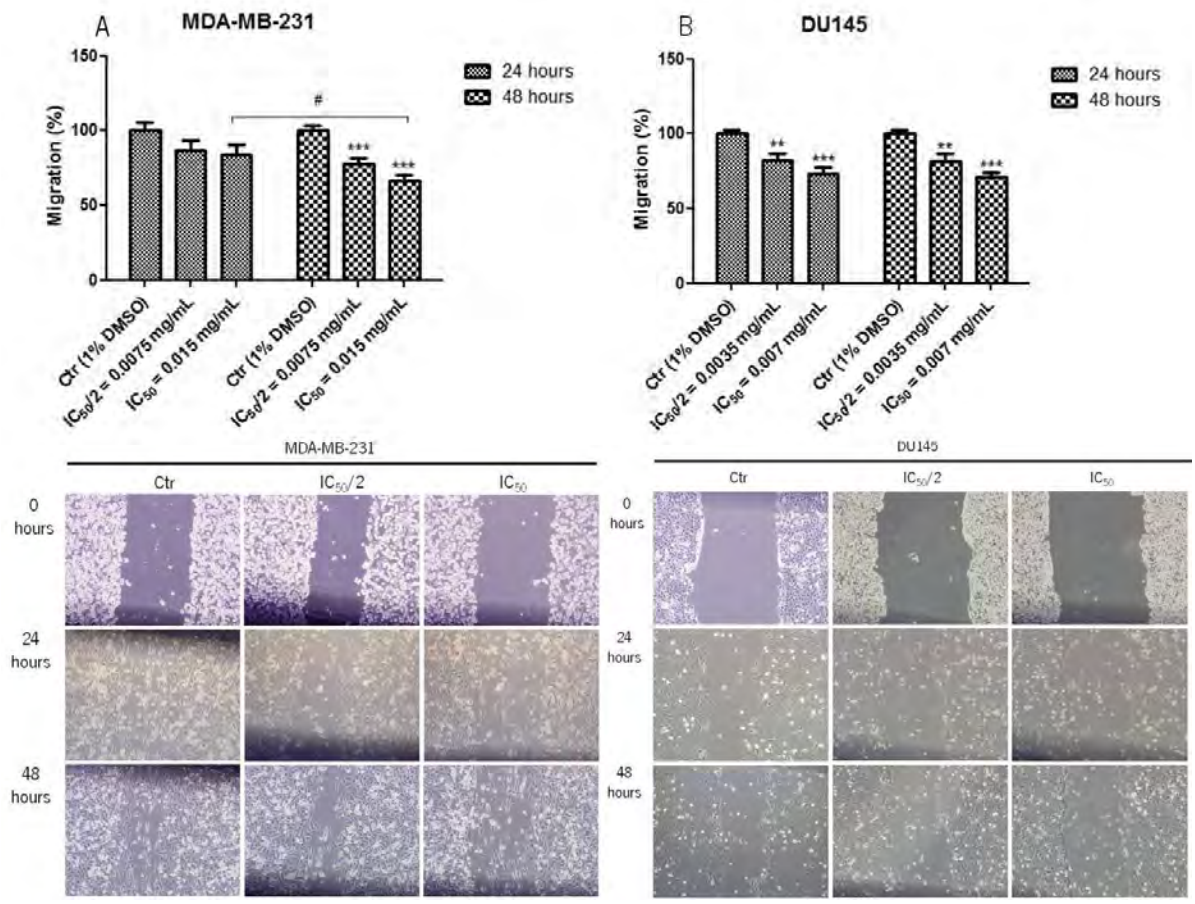



**Figure 14** - Effect of P10.EE on breast (A) and prostate (B) cancer cell lines migration, determined by the scratch-wound healing assay. **(A)** In MDA-MB-231 cells, only 48 h of P10.EE treatment decreased significantly cell migration and only the concentration of 0.015 mg/ml caused a significant decrease in cell migration from 24 to 48 h. **(B)** In DU145 cells, P10.EE significantly decreased the cell migratory capacity; however, no significant differences were observed between different treatment times. **$p \leq 0.01$; ***$p \leq 0.001$, comparing to control and #$p \leq 0.05$, comparing 24 and 48 h treatment conditions. Results are expressed as mean ± SEM of at least three independent experiments, each in triplicate.

## 4.6. Effect of P10.EE on Angiogenesis

In order to evaluate the effect of P10.EE on angiogenesis, both *in vitro* and *in vivo* studies were performed. For the *in vitro* study a brain endothelial cell line (HBMEC) was used to evaluate the effect of P10.EE on cell viability and proliferation. In Figure 15A it is possible to observe a reduction in total biomass and that this cell line is sensitive to propolis over time, being the $IC_{50}$ values equal to 0.015 mg/ml for 24 hours and 0.008 mg/ml for 48 and 72 hours. In what concerns proliferation (Figure 15B), P10.EE at 0.004 and 0.008 mg/ml significantly decreased the proliferative capacity of the cells, being the greatest effect obtained for the highest concentration tested.

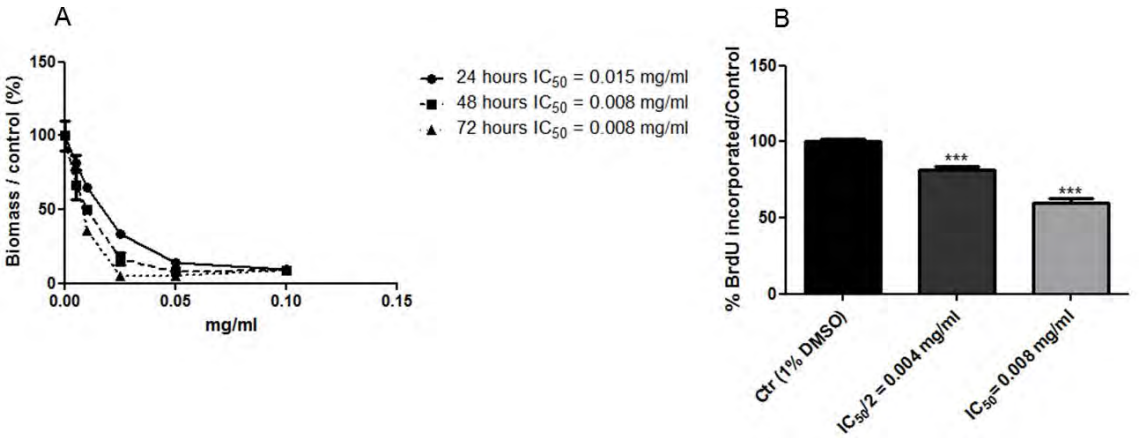



**Figure 15 -** Effect of P10.EE on HBMEC viability (A) and proliferation (B), using the SRB and the BrdU assays, respectively. **(A)** P10.EE induced a dose- and time-dependent reduction of HBMEC cell viability. **(B)** P10.EE decreased significantly the proliferative capacity of HBMEC after 24 h treatment. ***p≤0.001, comparing to control. Results are expressed as mean ± SEM of at least three independent experiments, each in triplicate

The potential of P10.EE in inhibiting angiogenesis was also evaluated using the *in vivo* chicken chorioallantoic membrane assay (Figure 16). Treatment with 30 µl of 0.1 mg/ml of P10.EE began after 10 days of embryo development. After 7 days of treatment, P10.EE appears to affect the naturally occurring neovascularization from existing vessels in the CAM. In fact, in *ex*

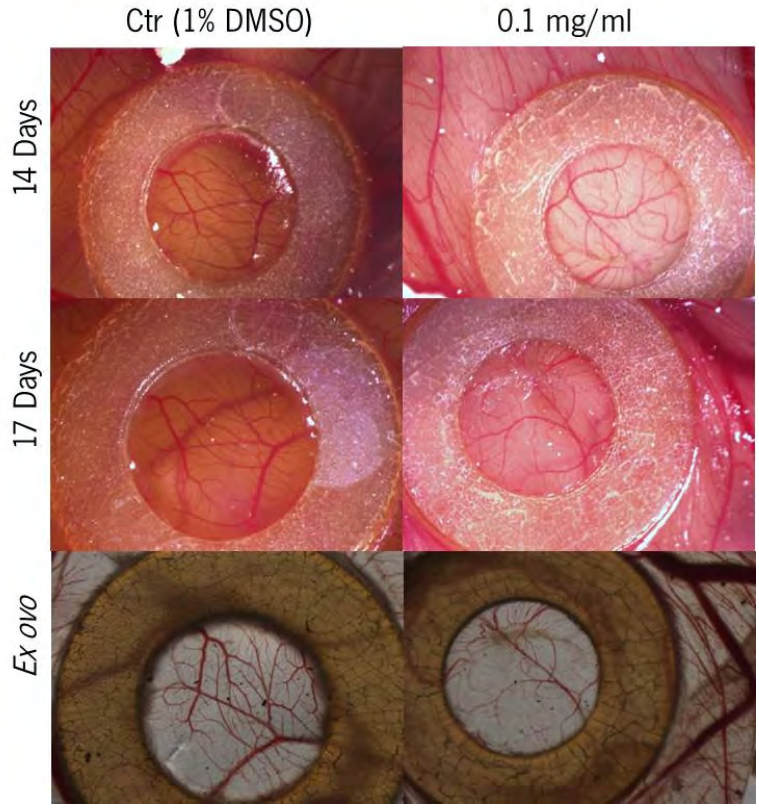



**Figure 16 –** *In vivo* effect of P10.EE on the vascularization of CAM of the chicken embryo. At day 10 of development, 0.1 mg/ml of P10.EE was placed in the CAM for 7 days. The CAM was photographed at days 14 (*in ovo*) and 17 (*in* and *ex ovo*) of development (12.5x magnification). P10.EE treatment appears to affect the natural sprouting of new vessels from the existing vessels, comparing to control.

*ovo* digital images it is visible a slight decrease in the sprouting vessels and it seems that the morphology is slightly altered (decreased vessel width) comparing to the control. Nevertheless, despite this interesting observation, it will be important to increase the number of eggs to confirm these results.

## 4.7. Effect of P10.EE on the Glycolytic Metabolism of Breast and Prostate Cancer Cell Lines

In this study, the effect of P10.EE on the glycolytic metabolism was also evaluated in both cell lines. For that, glucose consumption and lactate production were measured after 24 and 48 hours treatment with 0.0075 mg/ml and 0.015 mg/ml of P10.EE for MDA-MB-231 and 0.0035 mg/ml and 0.007 mg/ml of P10.EE for DU145 cell line (Figure 17).

Results for MDA-MB-231 showed a significant increase in glucose consumption either for 24 or for 48 hours treatment with 0.015 mg/ml P10.EE and in lactate production after 24 hours treatment with 0.015 mg/ml P10.EE and after 48 hours for both P10.EE concentrations. Additionally, it is possible to observe that only 0.015 mg/ml of P10.EE caused a significant increase in lactate production from 24 to 48 hours (Figure 17A). Regarding Figure 17B, which represents the results for DU145 cell line, it is possible to observe a significant increase in glucose consumption after 24 and 48 hours treatment with 0.007 mg/ml P10.EE, and in lactate production only after 48 hours of the same treatment. Comparing treatment conditions from the different time points, only the highest concentration of P10.EE caused a significant increase in lactate production from 24 to 48 hours in this cell line.

To further complement and confirm the above results, the expression of several markers that are important to the glycolytic phenotype - HIF-1α, PDK, GLUT1, HKII, LDH-A, PDH, MCT1, MCT4, CD147, CD44, CAIX – was analysed by Western blotting (Figure 18). Consistent with the induction of glycolytic metabolism, an increase in HIF-1α, PDK, GLUT1, LDH-A and CAIX, was observed in MDA-MB-231 cells treated with P10.EE. Nevertheless, there are no alterations in the expression of MCT4, MCT1 and their chaperones (CD147 and CD44), which are also important to the promotion of aerobic glycolysis. For DU145 cell line, despite the increase in the glycolytic metabolism after P10.EE treatment, no alterations were observed in the expression of these proteins.

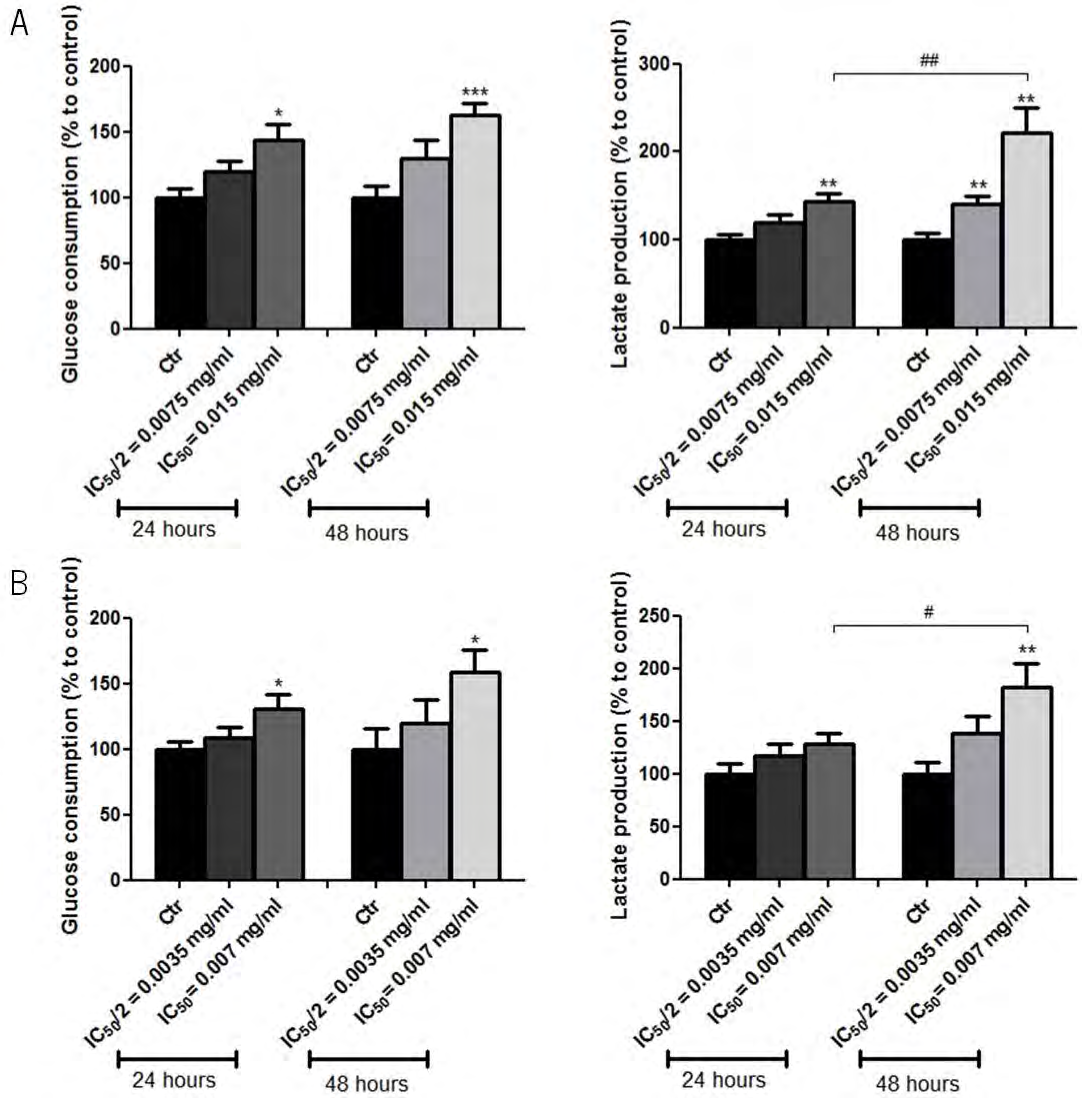



**Figure 17 -** Effect of P10.EE on the glycolytic metabolism of breast (A) and prostate (B) cancer cell lines. **(A)** In the MDA-MB-231 cells 0.015 mg/ml P10.EE significantly increased glucose consumption and lactate production after 24 and 48 h treatment and only the highest concentration caused a significant increase in lactate production from 24 to 48 h. **(B)** In DU145 cells, P10.EE at 0.007 mg/ml significantly increased the glucose consumption after 24 and 48 h and only after 48 h it increased lactate production significantly. Additionally, only this highest concentration causes a significant increase in lactate production from 24 to 48 h. *p≤0.05; **p ≤0.01; ***p≤0.001 comparing to control and #p≤0.05; ##p≤0.01, comparing the treatment conditions at 24 h with 48 h. Results are expressed as mean ± SEM of at least three independent experiments, each in triplicate.

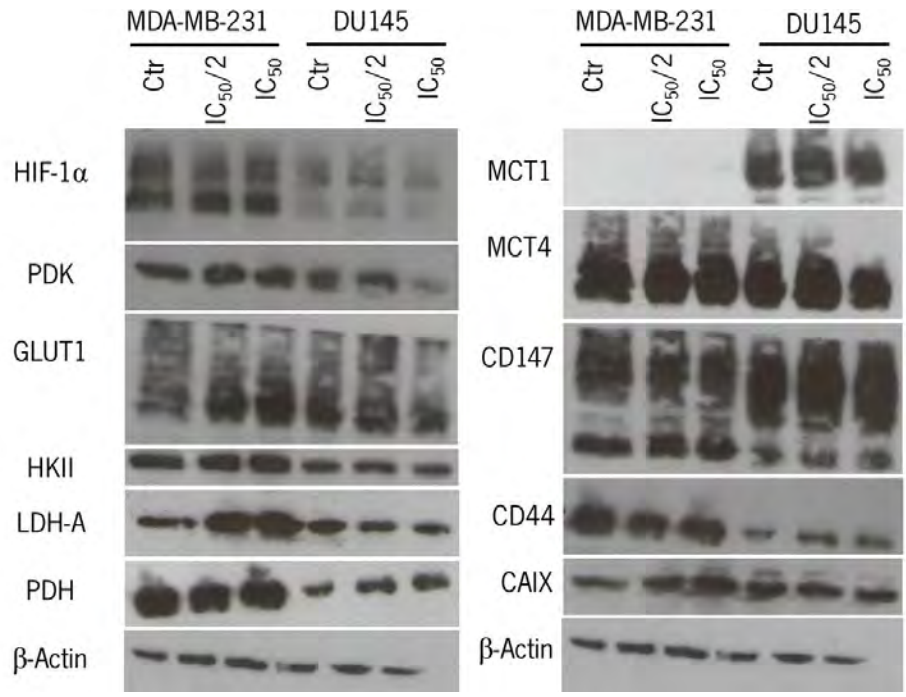



**Figure 18 -** Characterization of the expression of several proteins important to the glycolytic metabolism in breast and prostate cancer cell lines. Western Blot analysis of HIF-1α, PDK, GLUT1, HKII, LDH-A, PDH, MCT1, MCT4, CD147, CD44, CAIX shows different levels of expression in both cell lines. The molecular weights (kDa) are the following: 120 kDa for HIF-1α, 44 kDa for PDK, 55 kDa for GLUT1, 102 kDa for HKII, 37 kDa for LDH-A, 43 kDa for PDH, 43 kDa for MCT1, 43kDa for MCT4, 52 kDa for the highly glycosylated and 43 kDa for low glycosylated form of CD147, 90 kDa for CD44, 55 kDa for CAIX.

## 4.8. Combinatory Effect of P10.EE with Paclitaxel on Breast and Prostate Cancer Cell Lines

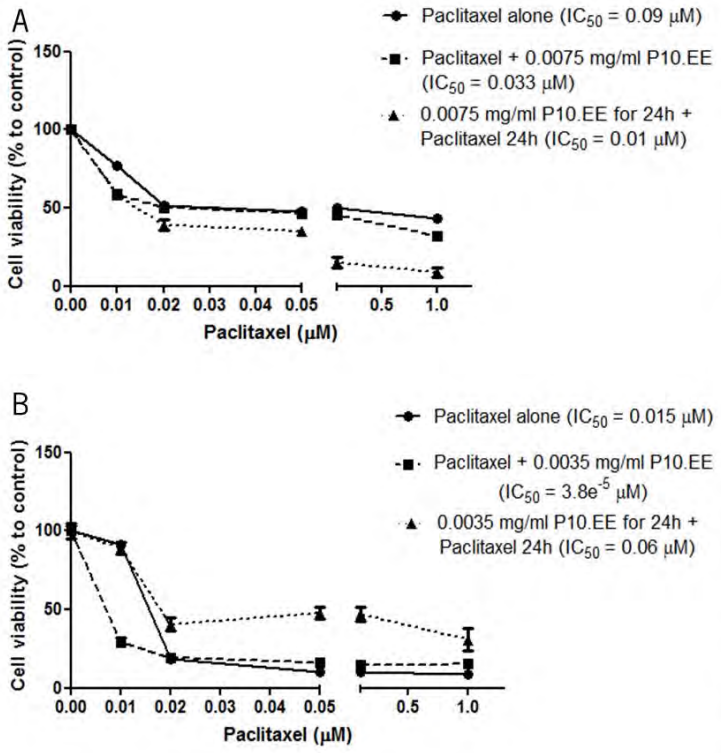
The effect of P10.EE in the treatment of MDA-MB-231 and DU145 cells lines with the cancer drug paclitaxel was analyzed. To evaluate this drug combinatory effect, total cell biomass was assessed with the SRB assay (Figure 19).

As mentioned above, P10.EE induced a significant dose-dependent reduction in cell viability (Figure 11). The P10.EE concentrations established for this study were based on the comparative analysis of the effects of different concentrations of P10.EE- 0.0075 mg/ml ($IC_{50}/2$ for MDA-MB-231 cell line) and 0.0035 mg/ml ($IC_{50}/2$ for DU145 cell line). In this study, cells were treated for 48 hours with paclitaxel alone (0.01, 0.02, 0.05, 0.1, 1 μM) and with paclitaxel (0.01, 0.02, 0.05, 0.1, 1 μM) + P10.EE (0.0075 or 0.0035 mg/ml). The effect of pre-sensitization with P10.EE (0.0075 or 0.0035 mg/ml) during 24 hours, followed by further treatment with paclitaxel (0.01, 0.02, 0.05, 0.1, 1 μM) for 24 hours, was also evaluated. As seen in Figure 19, paclitaxel

alone decreased cell viability of both cell lines after 48 hours ($IC_{50}$ = 0.09 µM for MDA-MB-231 and 0.015 µM for DU145 cell lines), being DU145 cell line the most sensitive.

For MDA-MB-231 cells treated with the combination of P10.EE and paclitaxel for 48 hours it is possible to see a decrease in the $IC_{50}$ value (0.033 µM), although no synergistic or additive effect was obtained (CI > 1). When cells were first sensitized with P10.EE for 24 hours and then treated for additional 24 hours with different concentrations of paclitaxel, it is possible to see that P10.EE potentiated the effect of paclitaxel, decreasing its $IC_{50}$ value (0.01 µM). Additionally, a synergistic effect (CI < 1) was obtained when cells were treated with 0.02 µM of paclitaxel and additive effect was obtained when cells were treated with 0.01, 0.05 and 0.1 µM of paclitaxel.

In DU145 cells (Figure 19B) treated with the combination of P10.EE and paclitaxel for 48 hours it is possible to see that P10.EE potentiated the effect of paclitaxel, decreasing its $IC_{50}$ value ($3.8e^{-5}$




**Combination Index (CI)**

**MDA-MB-231**

| Paclitaxel (µM) | 0.0075 mg/ml P10.EE (48h) | Sensitizing with 0.0075 mg/ml P10.EE (24h) |
|---|---|---|
| 0.01 | | CI = 1 |
| 0.02 | | CI < 1 |
| 0.05 | CI > 1 | CI = 1 |
| 0.1 | | CI = 1 |
| 1 | | CI > 1 |

**DU145**

| Paclitaxel (µM) | 0.0035 mg/ml P10.EE (48h) | Sensitising with 0.0035 mg/ml P10.EE (24h) |
|---|---|---|
| 0.01 | CI > 1 | |
| 0.02 | CI < 1 | |
| 0.05 | CI < 1 | CI > 1 |
| 0.1 | CI > 1 | |
| 1 | CI > 1 | |

**Figure 19 –** Effect of P10.EE on paclitaxel treatment of breast (A) and prostate (B) cancer cell lines, determined by SRB at 48 h. Cells were treated with paclitaxel alone, paclitaxel and P10.EE simultaneously, or treated with P10.EE during 24 h after which P10.EE was removed and replaced by paclitaxel, being cells incubated for further 24 h. Growth curves for paclitaxel and P10.EE in monotherapy were compared with the different types of combination to determine the combination index (CI) for each concentration of paclitaxel (table). CI values < 1, = 1 and > 1 indicate synergistic, additive and antagonism efects, respectively. **(A)** For MDA-MB-231 cells, synergistic and additive effects were obtained in the sensitizing treatment. **(B)** For DU145 cells, synergistic effect was obtained with the mixture of the drugs. Results are representative of three independent experiments, each in triplicate.

μM). Additionally, a synergistic effect (CI < 1) was obtained when cells were treated with 0.02 and 0.05 μM paclitaxel. When cells were first sensitized with P10.EE for 24 hours and then treated for further 24 hours with different concentrations of paclitaxel it is possible to see an increase in the $IC_{50}$ value (0.06 μM) and concomitantly no synergistic or additive effect was obtained (CI > 1).

# Chapter 5: Discussion

The paradigm of cancer therapy has changed significantly in recent years. This change only occurred due to a greater and deeper understanding of tumor biology, which allowed the identification of new therapeutic targets. It is now clear that cancer cells have distinctive and complementary capabilities that allow tumor unlimited proliferation potential, self-sufficiency in growth signals, resistance to anti-proliferative and apoptotic stimuli, reprogramming the metabolism and resistance to destruction by the immune system. In addition, apparently normal cells contribute to the acquisition of tumor changes and constitute a "tumor microenvironment". Genomic instability, which is the basis of genetic diversity associated with tumors, is associated with all these changes. Recognition and knowledge of these concepts will increasingly affect the development of new methods and therapeutic agents with increased specificity for tumor cells, associated with a considerably lower toxicity as well [136, 138].

Over the years, natural products have been a rich and a promising source for discovery of new pharmaceutical agents that are important in medicine [3]. In fact, more than half of the currently available drugs are natural compounds or related, and in the case of cancer this proportion surpasses 60% [1-3]. This area of research is continually growing and is of enormous interest because the different structural range of natural compounds can provide lead compounds for therapeutic improvement by molecular modification [4]. Natural products have been conveniently used in the treatment of various diseases since they are the source of many active compounds, such as phytochemicals, like polyphenols, terpenoids, alkaloids and other nitrogen compounds, carbohydrates and lipids [202].

Propolis, a natural product that has been used since almost immemorial times, is a modified plant product, more specifically by bees, and is composed by a complex mixture of phenolic compounds [6]. Propolis maintained the popularity for a long period of time and has been receiving an increased scientific interest; however it is not considered a therapeutic agent in conventional medicine because the standardization of its chemical composition and biological activity is lacking. Standardization of these two parameters is absolutely essential for acceptance of this natural compound in the health system [6, 9]. Several *in vitro* and *in vivo* studies have shown antitumoral activity of propolis from different geographic origin and of some of its isolated compounds on various tumor cells [8, 16, 52, 54, 102-132, 134, 135], nevertheless, one single

study concerning the antitumor activity of two Portuguese propolis samples in human renal cell carcinoma has been published so far [52].

Taking that into account, in the present study we analyzed the chemical composition and the antitumoral activity of a Portuguese propolis sample, which has been little explored and appears to have promising biological properties, collected in an apiary located in the central region of the country (Pereiro, Guarda). In addition to the scientific knowledge obtained, such studies allow valuing a product that is often neglected even by the beekeepers, therefore helping to value an important sector of the Portuguese economy.

More than 300 compounds have been identified in propolis, being mainly phenolic compounds. In general phenolic compounds or polyphenols can be divided into at least 10 types depending on their basic structure. Flavonoids and phenolic acids constitute the most important class. Such compounds have been identified after extraction with appropriate solvents. Water, methanol, hexane, acetone and ethanol are the most used solvents [203]. In the present work we performed extraction with ethanol 100% to obtain the P10.EE.

After performing a more complex analysis of the chemical composition of the propolis sample under study, total flavonoid and total phenolic content were first analyzed by a spectrophotometric method. Results show that the ethanolic extract of Pereiro propolis has a high concentration of phenolic compounds and of flavonoids, identical to those found in other propolis samples of different geographic origin, more specifically samples of European origin [8, 50, 51, 65].

Upon HPLC-DAD-ESI-$MS^n$ analysis, the chromatographic profile at 280 nm of Pereiro propolis was similar to the ones obtained for several other Portuguese ethanolic extracts already characterized, allowing the inclusion of the studied sample in the common temperate propolis type [23, 24]. Indeed, according to Falcão *et al* [24], Portuguese propolis samples could be arranged in two distinct groups: the common temperate propolis type which contains the typical poplar phenolic compounds such as the main phenolic components (caffeic acid, CAPE, pinobanksin, *p*-coumaric acid, Chrysin) and uncommon temperate propolis type, which in addition to the typical poplar flavonoids, also contains significant amounts of unusual flavonoid glycosides.

The analysis of about 36 fractions collected from HPLC analysis (Table 5) in combination with the results obtained from the total flavonoid and total phenolic contents (Table 4), suggest that this propolis sample contains a large variety of phenolic compounds. Considering the

complexity of the sample, the overall chromatographic separation (Figure 9) can be considered satisfactory.

The identification of the compounds from the main HPLC fraction was made by electrospray ionization-mass spectrometry in the negative ion mode because this mode promotes a high sensitivity in the detection of the distinct classes of phenolic compounds [43]. In general, the analytical approach used in this study based on the search of the $[M - H]^-$ deprotonated molecule together with the interpretation of its $MS^n$ fragmentations, allowed the identification of 20 phenolic compounds (Table 5) that have already been reported in the literature [23, 24, 36]. These included 13 phenolic acids and their derivatives (caffeic acid, *p*-coumaric acid, ferulic acid, caffeic acid isoprenyl ester, caffeic acid isoprenyl ester isomer, caffeic acid benzyl ester, caffeic acid phenylethyl ester, caffeic acid cinnamyl ester, *p*-coumaric acid methyl ester, *p*-coumaric acid isoprenyl ester, *p*-coumaric acid-4-hydroxyphenylethyl ester dimer and one caffeic acid derivative) and 7 flavonoids and their derivatives (pinobanksin, pinocembrin, chrysin, pinobanksin-3-*O*-acetate, pinocembrin-*O*-4-hydroxyphenylpropanoyl, quercetin-3-methyl ether and kaempferol-methyl ether). Additionally, this analysis allowed also elution of some compounds which occurrence has never been referred and still remain to be identified (fractions 7, 15, 19, 26, 32, 33, 36).

Thus, Pereiro propolis has a chemical composition very similar to the assigned poplar propolis type. Nevertheless, it is important to further analyze this sample to obtain a more exhaustive characterization, identifying the unidentified fractions.

Once elucidated the chemical profiling of the propolis ethanol extract under study, we focused our interest in one of the most studied propolis biological properties, the antitumoral activity. In order to study the possible antitumoral activity of P10.EE, a screening was performed in different cancer cell lines to evaluate its susceptibility to propolis treatment - breast (MDA-MB-231, MDA-MB-468 and MCF7), prostate (DU145 and 22RV1) and brain (U251 and SW1088). Overall, all the cancer cell lines suffer a dose-dependent decrease in total biomass after propolis treatment (Figure 11, Table 6) although some cells are more sensitive than others. These results are in agreement with the literature. Using propolis samples of different geographic origin but chemically similar to Pereiro sample (for example, all share one of the main phenolic acid active compound, the caffeic acid phenethyl ester or CAPE), Wu *et al* [114], Chuu *et al* [111] and Markiewicz-Zukowska *et al* [204] also showed, although not in all the cancer cell lines used in this study, that breast, prostate and brain cancer cell lines, respectivelly, are very sensitive to

propolis, and this sensitivity was manifested by gradual reduction of cell viability. From these results and for subsequent studies, two cell lines were selected on the basis of a greater effect of P10.EE after 48 hours: DU145 and MDA-MB-231.

As reviewed earlier, propolis can promote its effect by blocking specific oncogene signaling pathways, which in turn affects and leads to a decrease in cell proliferation and growth and can also act by increasing apoptosis, exhibiting antiangiogenic effects and modulating the tumor microenvironment [54, 102, 103]. So, we analyzed the effect of P10.EE on proliferation, cell cycle, apoptosis, migration and metabolism of the chosen cancer cell lines and also, using the endothelial cell line HBMEC, propolis effect on angiogenesis.

Tumors are diverse and heterogeneous, but all share the ability to proliferate beyond the restrictions that limit the growth in normal tissue. Briefly, proliferation consists of two consecutive processes, mainly characterized by DNA replication and segregation of replicated chromosomes into two separate cells. This cancer ability is promoted by alterations in the regulation of a restricted number of key pathways that control cell proliferation and survival. Deregulated cell proliferation together with suppressed apoptosis, constitutes the minimal common platform that dictate tumorigenesis [138, 141, 142].

The proliferation of MDA-MB-231 cell line was significantly decreased in a dose-dependent way by P10.EE treatment (Figure 12A), an effect that was evident after 24 hours of treatment. Additionally, P10.EE at the used concentrations affected the cell cycle of this cancer cell line (Figure 12C). Propolis seems to start promoting a cell cycle arrest at the S phase because we observed a significant increase in the cell population of S phase with a concurrent decline in the G0/G1 and G2/M phases. Regarding apoptosis, P10.EE induced cell death in MDA-MB-231 cells by a significant increase in early apoptotic and late apoptotic/necrotic cell population (Figure 13).

There are many studies showing the effect of propolis or its components in breast cancer cell lines [114, 116, 120, 121, 205]. The results obtained after treating the MDA-MB-231 cell with Pereiro propolis are in agreement with the observations of Wu *et al* [114]. These authors showed that CAPE, one of the main components of propolis, promoted a decrease in MDA-MB-231 proliferation, a S phase cell cycle arrest and apoptosis.

The NF-κB signal transduction pathway is deregulated in a variety of human cancers. Blocking NF-κB has been shown to stop tumor cells from proliferating, to die, or to become more sensitive to the action of anti-tumor agents [206, 207]. CAPE down-regulates the activation of NF-κB, which in turn affects MDA-MB-231 cell proliferation, cell cycle and death. Additionally, the

authors showed that CAPE promotes cell cycle arrest by an increase in the expression of p21 and p27 proteins and gene down-regulation of Rb, TP53, cyclins G1, D1, E1 and C, CDK2 and apoptosis by a decreased expression of anti-apoptotic proteins. Together, these results suggest that the proliferation decrease induced by CAPE is partially due to both cell cycle arrest and apoptosis [114]. As the propolis sample under study contains as main component CAPE, we can postulate that the effects observed in MDA-MB-231 cell line may be triggered in a manner similar to the one described by Wu *et al* [114].

Concerning DU145 cell line, it was observed that P10.EE significantly decreased in a dose-dependent way the proliferative capacity (Figure 12B), an effect evident after 24 hours of treatment. However, the effect of P10.EE in DU145 cell cycle differs greatly from the effect in the MDA-MB-231 cells. Propolis-treated DU145 cells presents a significant increase in the cell population of sub-G0 phase with a slight increase in the cell population of S phase and a decrease in the cell population of the G0/G1 phases (Figure 12C).

As in breast cancer cell lines, there are many studies evaluating the effect of propolis or its components in prostate cancer cell lines [111, 117-119, 122, 208].

Such results on the anti-proliferative effects of P10.EE in DU145 cells are in agreement with the study of Chuu *et al* [111]. The authors showed that CAPE treatment inhibits cell signaling networks, more especially inhibition of p70S6K (an intermediary of the PI3K/AKT pathway responsible for protein synthesis) and other Akt-related protein signaling networks, in a manner that resulted in a decrease in cell proliferation. So, regarding that, we can postulate that our propolis sample could promote the anti-proliferative effects by promoting alterations in the expression of important proteins in the AKT signaling network. Additionally, the authors said that CAPE significantly reduces cells in the S-phase and increases cells in G0/G1 population phases, and this occurs due to the down-regulation of cyclin A, c-Myc, Skp2, phosphorylated Rb, and Cdk2 concomitantly with the increased expression of phospho-c-Raf (S259), $p21^{Cip}$ and$p27^{Kip1}$. In contrast, the herein studied propolis sample promoted a significant increase in the sub-G0 cell population which possible corresponds to an increase of senescence or apoptotic state cell population. Regarding the apoptosis results (Figure 13), P10.EE only promoted a slight increase in late apoptotic/necrotic DU145 cell population.

McEleny *et al* [122] demonstrated that CAPE induced apoptosis in a dose-dependent manner in PC3 prostate cancer cells, and that this effect was associated with the loss of expression of the inhibitors of apoptosis cIAP-1, cIAP-2 and XIAP. Additionally, Szliszka *et al* [117,

118] proved that propolis, more specifically from Brazil, sensitizes DU145 cell line to a TRAIL-induced death. Possibly, P10.EE can induce cell death in a manner similar to what was described, however further studies are needed. Regarding the results obtained for P10.EE treated DU145 cells it is possible to say that apoptosis and alterations in cell cycle have only a secondary role in this cell line proliferation decrease.

The cancer microenvironment has received growing attention over time. It has been known that cancer microenvironment, which is formed by stromal, endothelial, immune cells, soluble factors, signaling molecules, extracellular matrix is very important in the life cycle of a tumor. In fact, it can support tumor growth and invasion, protect the tumor from host immunity, and provide places for dormant metastases to prosper. Intervening in the symbiosis of cancer microenvironments can be achieved by using natural products, like propolis and their constituents [54, 209].

Overall, after treatment of MDA-MB-231 and DU145 cell lines with different concentrations of P10.EE a significant dose-dependent decrease in cells migratory capacity was observed (Figure 14). Lee [124] and Hwang *et al* [125], obtained similar results using human hepatocellular carcinoma cells (SK-Hep1) and human fibrosarcoma cells (HT1080), respectively. They observed that CAPE could effectively suppress the motility and cell migration potential by inhibiting the expression of MMP-2 and MMP-9, and significantly the NF-κB. Taking into consideration all the results, it can be postulate that Pereiro propolis can promote an anti-metastatic action in a similar way, since it has CAPE.

In addition to its importance in the process of tumor growth and migration, tumor microenvironment has also an active role in the angiogenesis, an important process which plays a limiting role in tumor progression. Both tumor and stromal cells can secrete pro-angiogenic factors, like VEGF, that stimulate the formation and maintenance of new vessels that are important to provide oxygen and nutrients to cancer in order to sustain rapid uncontrolled proliferation and metastization [127].

In order to evaluate the effect of P10.EE in angiogenesis both *in vitro* and *in vivo* studies were performed. There are many *in vitro* and *in vivo* studies that shows the anti-angiogenic effect of different samples of propolis [128, 129, 131, 132, 134, 135]. In a resumed way, it is said that the anti-angiogenic effect of propolis is mainly mediated by decreasing the proliferative capacity of the endothelial cells, by inducing apoptosis in tube-forming endothelial cells and by inhibiting VEGF expression.

A brain endothelial cell line (HBMEC) was used in order to evaluate the *in vitro* effect of P10.EE on cell viability and proliferation. Briefly, results are in agreement with the literature [134] because P10.EE induces a dose- and time-dependent reduction in total biomass of HBMEC and additionally it significantly decreases the proliferative capacity of the cells after 24 hours (Figure 15A and B). In the *in vivo* CAM assay, P10.EE appears to affect the natural occurring neovascularization from existing vessels (Figure 16), as observed in other study [135]. Nevertheless, despite this interesting observation, it is necessary to increase the number of eggs in order to confirm the results.

The study of the effect of a Portuguese propolis sample on cancer cell metabolism was never reported in the literature, to the best of our knowledge. In fact there are only a few studies regarding the influence of propolis and its constituents in the glucose uptake by skeletal muscle cells [210-212]. It is said that Brazilian propolis ethanol extract increase skeletal muscle cells glucose uptake by promoting GLUT4 translocation through both PI3K- and AMPK-dependent pathways [212].

It is known that tumor cells have higher rates of glycolytic metabolism than normal cells. Glycolysis is a complex process where a molecule of glucose is reduced to pyruvate in a series of enzyme-catalysed reactions. In normal cells, during the sequential reactions of glycolysis, some of the free energy released from glucose is conserved in the form of ATP and NADH. In the presence of oxygen, mitochondria can further convert pyruvate to $CO_2$, $H_2O$ and energy catabolites. Proliferating cancer cells exhibit increased glycolysis in order to sustain energy production and to proliferate, which consists of an increase of glucose uptake and lactate formation, even in the presence of oxygen (Warburg effect) [156-160].

In the present study, the effect of P10.EE on the glycolytic metabolism was evaluated in MDA-MB-231 and DU145 cell lines (Figure17). Overall, it was observed that P10.EE at different concentrations promoted in both cell lines a significant increase in glucose consumption and lactate production. Curiously, these results are in agreement to the ones obtained by Ueda *et al* [212], although some caution should be taken in the comparison of the two studies as the authors tested normal cells instead of cancer cells as in the present study.

Hypoxia plays an important role in various cellular and physiological events that are responsible for the malignant alteration of the entire tumor [175]. A great number of human cancers respond to this condition by expressing, in a high frequency, several genes that are important to glycolysis, leading to the Warburg effect [159, 174].

To further complement such results, the expression of several markers that are important to the glycolytic phenotype - HIF-1α, PDK, GLUT1, HKII, LDH-A, PDH, MCT1, MCT4, CD147, CD44, CAIX - was analysed (Figure 18). In MDA-MB-231 cells treated with P10.EE, HIF-1 α, PDK, GLUT1, LDH-A and CAIX expression increased, which is consistent with induction of glycolytic metabolism, because overexpression of HIF-1 α in tumor cells promotes the expression of other proteins, like GLUT1 (transporter that supports the high glucose uptake), that promote an increase of the glycolytic phenotype. Despite being also seen an increase in the glycolytic metabolism in DU145 cell line, alterations in the expression of these proteins were not observed after P10.EE treatment. Until now, we cannot understand how this cancer cell line suffers those metabolic alterations, so, further studies are needed in order to unravel this outcome.

Drug combinations have been used for treating diseases and reducing suffering, since the earliest days of history. Normally, both Western and traditional Chinese medicine perform treatments for different diseases with a combination of agents. An important question arises when this type of combinatory treatments are used: why drug combination? The use of multiple drugs with different modes of action may target multiple targets, multiple sub-populations, or multiple diseases simultaneously or the effect can be directed against a single target or a disease and treat it more effectively [54, 200]. There are a few studies that investigate the additive or synergistic effect of propolis extracts in some anticancer therapies [204, 213]. Aside from potentiating the anticancer effect, propolis can also help to reduce toxicity induced by some cytotoxic agents [214].

A combinatory drug study was performed to assess the influence of P10.EE in Paclitaxel activity in both cancer cell lines previously selected. Markiewicz-Zukowska *et al* [204] observed that an ethanolic extract of propolis containing CAPE and Chrysin display cytotoxic properties and may cooperate with termozolomide synergistically enhancing its growth inhibiting activity against glioblastoma U87MG cell line. Regarding this study we thought that our propolis sample, which is also composed by CAPE and Chrysin, could also cooperate with an anticancer drug.

For MDA-MB-231 cells, synergistic and additive effects were only obtained in the sensitizing treatment and when cells were treated, respectively, with paclitaxel 0.02 µM and 0.01 and with 0.05 and 0.1 µM. For DU145 cells, a synergistic effect was obtained with the mixture of P10.EE with paclitaxel 0.02 and 0.05 µM. Such conditions of treatment – in terms of concentrations of P10.EE and paclitaxel - potentiate the decrease of the MDA-MB-231 and DU145 cells viability.

Overall, apart from the increase in glycolytic metabolism, P10.EE appears to be a good candidate for cancer drug development because it affects important characteristics that dictate tumorigenesis - cell proliferation, migration and angiogenesis - while also promotes cancer cell death.

# Chapter 6: Conclusion

With this study, it was possible to draw the following conclusions:

Regarding the results of the chemical characterization, we observed that the chromatographic profile of Pereiro propolis was similar to several other Portuguese ethanolic extracts studied and we have identified 20 phenolic compounds that have already been reported in the literature for other samples, such as caffeic acid, *p*-coumaric acid, Chrysin, CAPE.

Then, what concerns antitumoral activity we first observed that P10.EE affects the cell viability of different tumor cell lines, being the MDA-MB-231 (breast) and DU145 (prostate) two of the most sensitive ones after 48 hours of treatment. Additionally, P10.EE significantly decreases the cell migration and proliferation of these two cell lines, which are accompanied by alterations in the cell cycle and an increase in cell death.

In this study, it was also analyzed and for the first time the effect of a Portuguese propolis sample on cancer cell metabolism. Results show that P10.EE significantly increases glucose consumption and lactate production, which is explained, in the MDA-MB-231 cells, by the increased expression of HIF-1$\alpha$, PDK, GLUT1, LDH and CAIX.

It was additionally observed that P10.EE induces a decrease in total biomass and proliferation of HBMEC cells, appearing to have antiangiogenic effect as it affects the natural occurring neovascularization from existing vessels in chicken chorioallantoic membrane. Furthermore, P10.EE can cooperate with the anticancer drug paclitaxel.

In conclusion, and taking into account all the results, we think that P10.EE seems a good candidate for cancer drug development since it affects important characteristics that dictate tumorigenesis; however further studies are needed to fully chemically and biologically characterize Pereiro propolis as well as to unravel its mode of action for acceptance of this natural compound in the health system.

In fact, propolis extracts may be important economically and would allow a relatively inexpensive cancer treatment; nevertheless, in order to more easily become a new drug or a lead for modern medicine, propolis from different regions should not be used as a mixture of all constituents but as isolated compounds to warrant standardization.

# Chapter 7: Future Perspectives

To complement our view of the importance of Pereiro propolis as a candidate for cancer drug development supplementary experiments must be undertaken. So, in the near future, it is our purpose to:

- ✓ Further analyze the P10.EE sample by HPLC-DAD-ESI-$MS^n$ to obtain a more complete and comprehensive chemical characterization of the sample;

- ✓ Analyze the effect of P10.EE at the molecular level, namely its effect on the signaling pathways that are responsible for cell proliferation, cell cycle, apoptosis, migration, metabolism and angiogenesis, in order to unveil the molecular mechanism involved in propolis action;

- ✓ Test the main compounds of the P10.EE sample on the cell lines in order to understand the role that each compound has in promoting the antitumoral activity;

- ✓ Perform the same study using other samples of propolis of different geographic origin. Due to the chemical variability of propolis this type of study should not be limited to a single specimen.

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