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Professor Doutor Rui Pedro Soares de Oliveira

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STATEMENT OF INTEGRITY

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

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

Atividade antioxidante e citotóxica de própolis português

RESUMO

O própolis é uma mistura resinosa e balsâmica natural produzida por abelhas rica em flavonóides e compostos fenólicos, que são geralmente associados às atividades antioxidante, citotóxica e antimicrobiana. Mesmo sendo o própolis um produto reconhecido e bem estudado mundialmente, ainda há poucos estudos sobre o própolis português, sendo este um produto subvalorizado. O presente trabalho é uma extensão de trabalhos realizados anteriormente sobre o própolis português do apiário do Pereiro, Beira Alta. Nesses trabalhos foi observada uma diferença nos efeitos de extratos etanólicos de amostras colhidas em 2010 (P10.EE) e em 2013 (P13.EE) no modelo biológico *Saccharomyces cerevisiae*, sendo que P13.EE apresentava um efeito pouco citotóxico e um efeito antioxidante, enquanto P10.EE se apresentou bastante citotóxico e pouco antioxidante. Estas diferenças entre amostras do mesmo apiário levaram à questão de quais seriam os seus mecanismos de ação e o que os causaria. Os resultados anteriores sugeriram que o P10.EE induz um tipo de morte celular regulada, que poderia ser apoptótica ou necrótica, e, também, que este extrato causa danos no DNA. No presente estudo, no caso do P13.EE, os resultados de viabilidade e de análise da oxidação intracelular por citometria de fluxo com a estirpe parental BY4741 e mutantes deficientes na resposta ao stresse oxidativo demonstraram um efeito anti e pró oxidante dependente da concentração, possivelmente devido à presença de ester fenetil do ácido cafeico, pinobanksina e pinocembrina no extrato, apresentando atividade antioxidante em concentrações menores, que, por sua vez parece estar envolvida com a eliminação direta de radicais livres e a ativação das defesas antioxidantes celulares. Já no estudo de P10.EE, os resultados de ensaios distintos de microscopia de fluorescência com os marcadores 4',6-diamino-2-fenil-indol e iodeto de propídio sugeriram que o extrato não causa morte celular por apoptose, mas sim por uma via necrótica. A análise do ciclo celular por citometria de fluxo indicou que o extrato causa uma paragem reversível nas fases G1/S do ciclo celular. Em suma, P13.EE e P10.EE exibem atividades antioxidante e citotóxica, respetivamente; porém, tais diferenças não estarão relacionadas com o seu teor fenólico, uma vez que os cromatogramas das análises cromatográficas de ultra eficiência de P10.EE e P13.EE não revelaram diferenças entre os dois extratos. Este estudo contribuiu para um maior conhecimento dos seus mecanismos de ação, para o crescimento do valor nacional deste produto português, e para a sua possível aplicação em diversas indústrias.

Palavras-chave: antioxidante, citotoxicidade, mecanismo de ação, própolis, *Saccharomyces cerevisiae*

Antioxidant and cytotoxic activity of Portuguese propolis

ABSTRACT

Propolis is a natural resinous and balsamic mixture made by honeybees, rich in flavonoids and phenolics, which are generally associated with propolis' antioxidant, cytotoxic and antimicrobial activities. Although propolis is generally recognized and well-studied worldwide, Portuguese propolis research is still scarce and this product is nationally undervalued. The present work is an extension to previous works on Portuguese propolis from the Pereiro apiary, located in Beira Alta. Ethanol extracts of Pereiro propolis samples harvested in 2010 (P10.EE) and 2013 (P13.EE) were found to have different effects on the biological model *Saccharomyces cerevisiae*, with P13.EE presenting a low cytotoxic effect and a promising antioxidant effect, while P10.EE exhibited high cytotoxicity and very low antioxidant effects. These intriguing differences in samples from the same apiary led to the question of what their mechanisms of action could be and what could be causing these differences. The previous studies were able to hint on the P10.EE mechanism of action, inferring that the extract induces a type of regulated cell death, that could either be apoptotic or necrotic, and that it also causes DNA damage. In the present work, regarding the study of the P13.EE, results from viability assays and flow cytometry intracellular oxidation analysis with the parent strain BY4741 and derived mutants with a deficient oxidative stress response suggested a dose-dependent anti and pro-oxidant effect, that could be related to the presence of compounds like caffeic acid phenethyl ester, pinobanksin and pinocembrin in the extract, presenting antioxidant activity at lower concentrations, which in turn appears to result from both direct radical scavenging and activation of the cellular antioxidant defenses. Regarding the study of P10.EE, results from separate fluorescent microscopy assays with 4',6-diamidino-2-phenylindole and propidium iodide staining suggested that the extract is not inducing cell death through apoptosis but instead through a necrotic pathway. Cell cycle analysis through flow cytometry indicated that P10.EE causes a reversible arrest in the G1/S cell cycle phases. In conclusion, P13.EE and P10.EE exhibit promising antioxidant and cytotoxic activities, respectively, but these differences are probably not related to their phenolic content, since the chromatograms from the ultra-performance chromatographic analysis of both extracts did not reveal differences between the two. This study was able to successfully uncover parts of their intriguing mechanisms of action, contributing to the growth of Portuguese propolis national value and their possible applications in various industries.

Keywords: antioxidant, cytotoxic, mechanism of action, propolis, *Saccharomyces cerevisiae*

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LIST OF ABBREVIATIONS AND ACRONYMS

$\bullet O_2^-$	superoxide
•OH	hydroxyl radical
ACD	accidental cell death
ANOVA	analysis of variance
Atg	autophagy related gene
BC	before Christ
BF	bright field
BSA	bovine serum albumin
bZIP	basic leucine zipper
C-	negative control
C+	positive control
CAPE	caffeic acid phenethyl ester
CFU	colony-forming unit
DAMP	damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DCF	2',7'-dichlorofluorescein
dH_2O	distilled H_2O
DMCA	3,4-dimethyl-caffeic acid
dNTP	deoxynucleoside triphosphate
DPPH	2,2-diphenyl-1-picrylhydrazyl
EE	ethanol extract
ESI/MS	electrospray ionization/mass spectrometry
EtOH	ethanol
FITC	fluorescein isothiocyanate
GC-MS	gas chromatography-mass spectrometry
GFP	green fluorescent protein
H_2DCF	2',7'-dichlorodihydrofluorescein
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
H_2O_2	hydrogen peroxide
HIV	immunodeficiency virus

HMGB1	high-mobility group box 1
HSV	herpes simplex virus
HU	hydroxyurea
IC_{50}	half maximal inhibitory concentration
IL	interleukin
LPS	lipopolysaccharide
MIC	minimum inhibitory concentration
mRNA	messenger RNA
n-BuOH	n-butanol
NCCD	Nomenclature Committee on Cell Death
n.d.	not determined
NF-κB	nuclear factor κB
Nhp6A	non-histone protein 6A
NK	natural killer
OD_{600}	optical density at 600 nm
OH	hydroxyl ion
P	Pereiro
P.EE	ethanol extract of Pereiro propolis
P10	Pereiro propolis harvested in 2010
P10.EE	ethanol extract of Pereiro propolis harvested in 2010
P13	Pereiro propolis harvested in 2013
P13.EE	ethanol extract of Pereiro propolis harvested in 2013
PBS	phosphate buffered saline
PCD	programmed cell death
pDNA	plasmid DNA
PI	propidium iodide
PS	phosphatidylserine
RCD	regulated cell death
RNR	ribonucleotide reductase
RNS	reactive nitrogen species
ROS	reactive oxygen species
rpm	revolutions per minute

SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SD	standard deviation
SOD	superoxide dismutase
SuHV-1	Suid herpesvirus type 1
TFC	total flavonoid content
TGF	transforming growth factor
TNF	tumor necrosis factor
TORC1	target of rapamycin complex 1
TPC	total phenolic content
UPLC-DAD-ESI/MSn	ultra-performance liquid chromatography with diode array detection coupled to electrospray ionization and mass spectrometry
upH_2O	ultrapure H_2O
UV-Vis	ultraviolet-visible
w/v	weight/volume
YPD	yeast peptone dextrose
YPDA	yeast peptone dextrose agar

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Figure A10. Synchronization of *S. cerevisiae* cell cycle. Percentage of *S. cerevisiae* BY4741 cells in the cell cycle phases G1, S, and G2/M, after synchronization of the cells with HU at 200 mM. Synchronized and unsynchronized cells were prepared for flow cytometry analysis and stained with SYTOX™ Green Nucleic Acid Stain (Invitrogen). These values represent the mean ± SD of n = 3 independent replicates. Two-way ANOVA was performed, followed by Sidak's test for multiple comparisons. Asterisks represent significant differences between synchronized and unsynchronized cells (* $p < 0.05$, **** $p < 0.0001$). 91

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CHAPTER 1. General Introduction

1. Propolis

Propolis is a natural product produced by honeybees (mainly *Apis mellifera*), consisting of lipophilic resinous materials collected from branches, flowers, pollen, buds and exudates of various plant sources, that are partially digested with the bees' salivary enzyme β-glucosidase and then mixed with beeswax and other compounds of bees' metabolism. With a gluey and resinous consistency, propolis displays a strong odor and usually a brown, green, or red tint, depending on the type of propolis (Burdock, 1998; Bankova *et al.*, 2016; Peixoto *et al.*, 2021). Because this natural product is mainly produced from plant material, its precise composition depends greatly on the local flora diversity, being recognized several propolis types (Silva-Carvalho *et al.*, 2015) with diverse chemical compositions that provide different biological activities, such as cytotoxic, antitumoral, antioxidant, and antimicrobial activities (Kujumgiev *et al.*, 1999; Bankova, 2005; Gómez-Caravaca *et al.*, 2006; Castro *et al.*, 2011; Valente *et al.*, 2011; Bankova *et al.*, 2014; Capoci *et al.*, 2015; Silva-Carvalho *et al.*, 2015).

The word propolis comes from the Greek pro- (defense of) and polis- (city), which means defense of the city, or the hive (Ghisalberti, 1979). Due to its mechanical and biological properties, propolis is used by bees in many ways to defend the hive, for example to fix holes and cracks in the honeycombs, to smooth its internal walls, and to protect the hive from intruders – propolis is used to encapsulate the invaders, killing them by asphyxia, but also to conserve their bodies, containing putrefaction and its resultant plagues (Bankova *et al.*, 2002).

Although propolis is a very popular natural remedy nowadays, its usage goes back to at least 300 BC, being recognized by many ancient civilizations (Ghisalberti, 1979): Egyptians used propolis to embalm cadavers due to its anti-putrefactive properties (Kuropatnicki *et al.*, 2013); the Greeks and Romans used it for wound treatment and as mouth disinfectant, due to its antiseptic and healing properties, and it was recognized for its medical properties by many physicians, such as Aristoteles, Hippocrates, Dioscorides, Pliny, and Galen (Castaldo & Capasso, 2002; Kuropatnicki *et al.*, 2013). Arab physicians recognized propolis properties as well, using it for the treatment of eczemas, myalgia and rheumatism (Kuropatnicki *et al.*, 2013). Propolis continued being used for its therapeutic properties during medieval times, being very present in traditional folk medicine in Eastern Europe (Kuropatnicki *et al.*, 2013). It became a very popular drug in Europe between the 17th and 19th centuries, due to its antibacterial and healing properties, and it was also used during the Second World War for tuberculosis treatment, as well as for the treatment of wounds, burns, sore throats and stomach ulcers (Wollenweber *et al.*, 1990; Fokt *et al.*, 2010). Although most propolis application records are due to its medicinal

properties, it can also be used for other purposes, for example, Antonio Stradivari used this bee product in the varnish for musical instruments (Burdock, 1998).

Nowadays, we can easily find an array of products made with propolis in many health-food stores, being available in different formats, such as capsules (either in pure form or combined with other compounds), extracts (hydroalcoholic or glycolic), mouthwash solutions (combined with other substances), creams, powder, and as a purified product (Castaldo & Capasso, 2002; Fokt *et al.*, 2010). Due to its extensive biological and medicinal properties, propolis popularity has been growing, and so has the scientific interest in it, with aims to study it further and possibly apply to human and veterinary medicine, pharmacology, food and cosmetics.

2. Composition of propolis

Propolis is a complex resinous and balsamic mixture, generally composed of resins and plant balsams (50 %), beeswax (30 %), essential and aromatic oils (10 %), pollen (5 %), and other substances and materials, including mineral and organic compounds (5 %) (Bankova 2005; Pasupuleti *et al.* 2017). Phenolic acids (cinnamic and caffeic acid), flavonoids, their esters (flavones, flavanones, flavonols, and dihydroflavonols chalcones), terpenes, aromatic aldehydes and alcohols, fatty acids, stilbenes, and β-steroids are the main organic compounds present in propolis (Huang *et al.*, 2014).

As mentioned above, propolis chemical composition varies among different types of propolis, and is difficult to standardize, since it depends on many different factors, like the geographic location, environmental conditions, vegetation available, and the season of collection (Bankova, 2005). In total, there have been identified over 800 compounds in propolis samples, for example: polyphenols, phenolic aldehydes, sesquiterpenes, quinines, coumarins, amino acids, steroids, and inorganic compounds, but many more are expected to be identified as new samples get chemically characterized (Castro *et al.*, 2011; Huang *et al.*, 2014; Kasote *et al.*, 2022). Due to chemical complexity and diversity, several types of propolis have been classified according to its plant source and chemical composition: Poplar propolis (Europe, North-America, non-tropic regions of Asia, China and New Zealand), Birch propolis (Russia), Green (Alecrim) propolis (Brazil), Red propolis (Brazil, Cuba and Mexico), "Clusia" propolis (Cuba and Venezuela), "Pacific" propolis (Okinawa, Taiwan, Hawaii, Indonesia and Myanmar), "Canarian" propolis (Canary Islands), Mediterranean propolis (Sicily and the Adriatic Coast), Australian propolis, Thailand propolis and African propolis (Bankova, 2005; Silva-Carvalho *et al.*, 2015; Miłek *et al.*, 2022). Propolis chemical profile can be characterized by three parameters: total flavone and flavonols content, total flavanone and dihydroflavonol content, and total phenolics content (Bankova, 2005). Bankova (2005) analyzed various samples of Poplar propolis, the most thoroughly studied type of propolis, and proposed the characteristics of typical poplar propolis as 8 ± 4 % flavones/flavonols, 6 ± 2 % flavanones/dihydroflavonols and 28 ± 9 % of total phenolics.

2.1. Phenolic compounds

Phenolic compounds are greatly present in plants and account for one of the most notable groups of plant secondary metabolites. Their chemical structure is of one or more aromatic rings, with at least one hydroxyl group, being classified as simple phenols if containing a single phenol unit, or as polyphenols if the molecule contains more than one (Roleira *et al.*, 2018). The main groups of phenolic compounds

are flavonoids, phenolic acids, tannins, stilbenes and lignans (reviewed by Ayad & Akkal, 2019) They are commonly found in nature in the form of glycosides, often conjugated with carboxylic acids, organic acids, amines, lipids, and other phenolic compounds (Barba *et al.*, 2014). These compounds are associated with many biological properties, like the antioxidant activity (Miguel-Chávez, 2017), but also anticarcinogenic (Roleira *et al.*, 2018), antimutagenic (Valdez-Morales *et al.*, 2014), and anti-inflammatory activities (Shahidi & Yeo, 2018), making these compounds molecules of interest for cancer prevention and treatment.

2.1.1. Flavonoids

Flavonoids are considered the major constituents of propolis, being responsible for many of its pharmacological properties, such as antioxidant (Kumar & Pandey, 2013), antibacterial (Huang *et al.*, 2014), antiviral (Nijveldt *et al.*, 2001), and anti-inflammatory effects (Bueno-Silva *et al.*, 2013). Depending on its chemical structure, flavonoids can be divided and classified into flavones, flavonols, flavanones, flavanolols, chalcones, dihydrochalcones, isoflavones, isodihydroflavones, flavans, isoflavans and neoflavonoids (Huang *et al.*, 2014).

2.1.1.1. Flavones/flavonols

Flavones and flavonols are commonly found in plants as *O*-glycosides (Prasain *et al.*, 2018). Their structure is very similar, portrayed by a nonsaturated 3-C chain, and a double bond between C-2 and C-3, being the main characteristic that distinguishes them the fact that flavonols have a hydroxyl group at C3 while flavones have a hydrogen (Murkovic, 2003). Although simple, this structural difference between the two molecules is enough to make a change in their physiological and pharmaceutical roles (Murkovic, 2003). Poplar propolis has been shown to be rich in flavones like apigenin, luteolin, and chrysin, and in flavonols like quercetin, kaempferol, and galangin (Figure 1) (Cruz *et al.*, 2016).

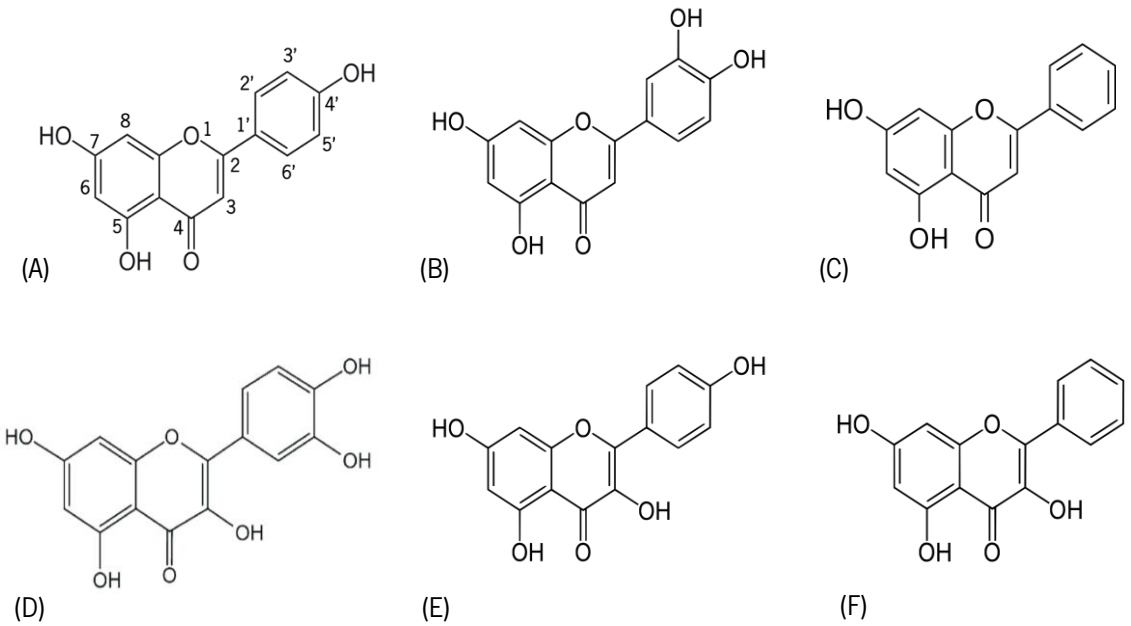

Figure 1. **Structures of common flavones (A, B, C) and flavonols (D, E, F) present in propolis**. Unlike flavones, flavonols have a hydroxyl group in C-3. Represented flavones are apigenin (A), luteolin (B) and chrysin (C). represented flavonols are quercetin (D), kaempferol (E) and galangin (F).

2.1.1.2. Flavanones/dihydroflavonols

Flavanones are mainly found in citrus fruits, tomatoes, and some aromatic plants, like mint (Pérez-Chabela & Hernández-Alcántara, 2018). This group of polyphenols and their 3-hydroxy derivatives, dihydroflavonols, are characterized by the basic 2,3-dihydroflavone structure, without the double bond between C2 and C3 – this particularity makes flavanones chiral at C2 position, which means the B ring is not planar, like it is in conjugated flavones. This molecular orientation difference makes for a different interaction with biological receptors, impacting their biological properties (Figure 2) (Panche *et al.*, 2016; Duodu & Awika, 2019; Nazzaro *et al.*, 2020). Examples of flavanones and dihydroflavonols commonly present in poplar propolis are pinocembrin and pinobanksin, respectively (Cruz *et al.*, 2016).

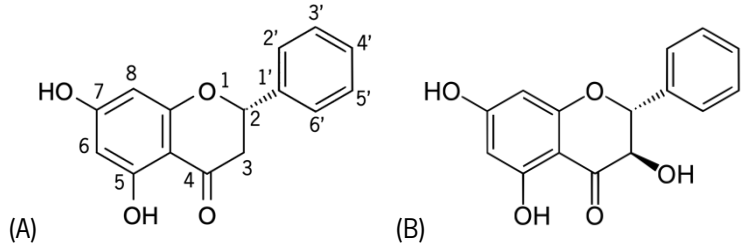

Figure 2. **Structures of common flavanones (A) and dihydroflavonols (B) present in propolis**. Flavanones and their derivatives dihydroflavonols lack a double bond between C2 and C3, making them chiral at the C2 position and causing a twist in the B ring, making this molecule non-planar. (A) Pinocembrin (flavanone), (B) Pinobanksin (dihydroflavonol).

2.1.2. Phenolic acids

Phenolic acids considered one of the main active components of propolis (Medana *et al.*, 2008). They are derivatives of benzoic and cinnamic acids and are found all food groups, being very present in cereals, legumes, oilseeds, fruits, vegetables and herbs (reviewed by Chandrasekara, 2019). Phenolic acids exhibit high antioxidant activity, by scavenging free radicals, but have also been reported to act as reducing agents and are involved in cell signaling pathways (reviewed by Chandrasekara, 2019). Poplar propolis has been shown to be rich in phenolic acids like cinnamic acid, caffeic acid, *p*-coumaric acid, and ferulic acid (Figure 3) (Huang *et al.*, 2014; Cruz *et al.*, 2016).

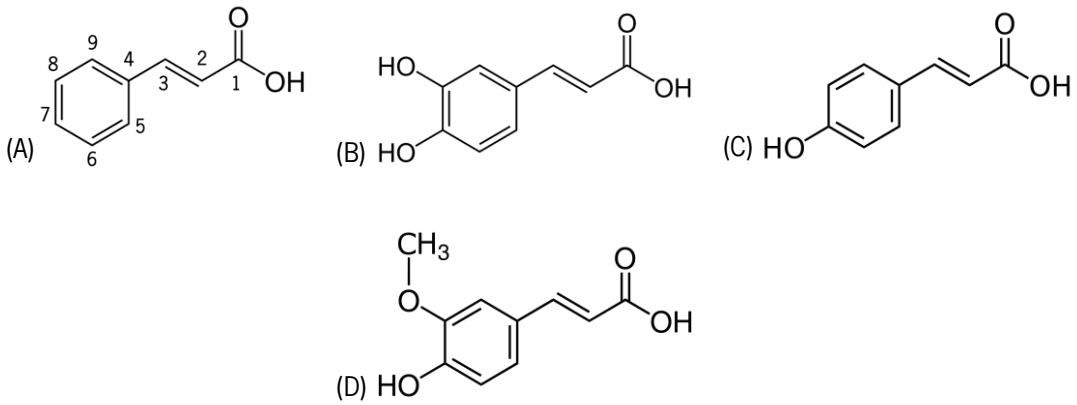

Figure 3. Structures of common phenolic acids. Phenolic acids contain one or more aromatic rings, with at least one hydroxyl group. (A) Cinnamic acid, (B) Caffeic acid, (C) *p*-Coumaric acid, (D) Ferulic acid.

3. Biological activities of propolis

Propolis biological properties have been object of study for many decades, with the first reports appearing in the early 1900's (reviewed by De Castro, 2001). Since then, there have been numerous studies and papers published on this topic, being that of great scientific, medicinal, and pharmaceutical interest (reviewed by Ghisalberti, 1979; Marcucci, 1995; Burdock, 1998; Rivera-Yañez *et al.*, 2020; Hossain *et al.*, 2022; Wieczorek *et al.*, 2022). Although these properties can vary between different types of propolis, several studies have reported that this natural product can have many activities, such as cytotoxic, antioxidant, anti-inflammatory, antitumoral, antimicrobial, antiviral, and immunomodulatory (Marcucci, 1995; Mirzoeva *et al.*, 1997; Burdock, 1998; De Castro, 2001; Hu *et al.*, 2005; Sforcin, 2007; Fokt *et al.*, 2010; Sforcin & Bankova, 2011; Fernandes-Silva *et al.*, 2013; Silva-Carvalho *et al.*, 2015; Zabaiou *et al.*, 2017; Oryan *et al.*, 2018; Ibrahim & Alqurashi, 2022). These biological properties are associated with more than 100 active components present in propolis, such as flavonoids and derivatives of caffeic acid (reviewed by De Castro, 2001). However, the extraction method and solvent have a great influence on propolis activities too, since different solvents can extract different compounds more effectively. Ethanol, methanol, and water are the most common solvents used for these extractions, but since water is less effective in extracting phenolic compounds from propolis and methanol is the most toxic, ethanol is usually the solvent of choice (Cunha *et al.*, 2004b; Miguel *et al.*, 2010).

3.1. Antitumoral, immunomodulatory, and cytotoxic activities

Propolis antitumoral properties have been a topic of interest recently and have already been reported both *in vivo* and *in vitro* (Banskota *et al.*, 2001). *In vitro*, Green propolis from Brazil has been shown to be effective against various tumor cells and Sforcin (2007) suggests that this action is related to inhibition of cell proliferation and apoptosis. *In vivo*, it has been demonstrated that Poplar propolis can decrease lung tumor nodules, and its effectiveness was even greater than that presented by its isolated constituents (caffeic acid, its derivative caffeic acid phenethyl ester (CAPE) and quercetin), suggesting that the antitumoral action observed is a result from the synergy of its components (Oršolić *et al.*, 2004, 2005). Propolis from Portugal has shown cytotoxicity against several human cancer cell lines (Valente *et al.*, 2011; Valença *et al.*, 2013; Silva-Carvalho *et al.*, 2014; Calhelha *et al.*, 2014, Freitas *et al.*, 2022; Oliveira *et al.*, 2022). Sforcin *et al.* (2002) demonstrated that a three-day treatment with Brazilian Green propolis has enhanced the cytotoxicity of natural killer (NK) cells against mice lymphoma, confirming that short-term administration of propolis increases the immunological response (Scheller *et al.*, 1988; Sforcin

et al., 2002). However, the increased cytotoxicity in NK cells could be related to propolis immunomodulatory action. Dimov *et al.* (1991) suggested that propolis modulates innate immunity by activating macrophages, and it has been demonstrated that this bee glue can stimulate mice peritoneal macrophages to produce cytokines (cell-signaling proteins), such as tumor necrosis factor-α (TNF-α) and interleukin-12 (IL-12), which act on NK cells and could be the reason for its increased cytotoxicity (Moriyasu *et al.*, 1994; Sforcin, 2007). Orsi *et al.* (2000) observed that propolis would also cause peritoneal macrophages to increase H_2O_2 production and inhibit NO• generation.

Propolis could potentially be used as a vaccine adjuvant or immunostimulant, since it activates macrophages, stimulating them to produce cytokines, which are responsible for activating T and B lymphocytes. In fact, Fischer *et al.* (2007) observed that an inactivated Suid herpesvirus type 1 (SuHV-1) vaccine associated with Green propolis extract and aluminum hydroxide would result in mice presenting higher levels of antibodies and, when administered with a lethal dose of the vaccine, propolis increased the survival percentage. Propolis adjuvant properties have also been studied in carps, and when administered with an inactivated vaccine of *Aeromonas hydrophila*, the phagocytic activity and number of antibodies were higher when the vaccine was combined with propolis (Chu, 2006).

CAPE, a caffeic acid derivative present in propolis and a specific inhibitor of nuclear factor κB (NF-κB) (Ozturk *et al.*, 2012), has been reported to interfere in cell cycle, provoking an arrest at G2/M phase in the oral cancer cell line OEC-M1 (Lee *et al.*, 2005), but has also shown effectiveness against several cancer cell lines (Ozturk *et al.*, 2012; Liu *et al.*, 2018; Fırat *et al.*, 2019). Apigenin has too been reported to be effective against various cancer cell lines, and described as a promising chemotherapeutic agent, with the possibility of being used in combination with immunotherapy for cancer treatment (Torkin *et al.*, 2005, Chuang *et al.*, 2009; Shukla *et al.*, 2014; Salehi *et al.*, 2019).

3.2. Antioxidant activity

Oxidative stress can be caused either by exogenous agents, such as toxins, drugs, and UV, or by endogenous factors, such as cellular metabolism, which in turn generate reactive oxygen species (ROS), like H_2O_2 and O_2^-, and reactive nitrogen species (RNS), like NO• (Di Meo *et al.*, 2016). These reactive species cause oxidative alterations on biomolecules that affect the cell, leading to its death (Salmon *et al.*, 2004; Viuda-Martos *et al.*, 2008; Sosa *et al.*, 2013), and are linked to the development of many human diseases, such as neurodegenerative diseases (Wu *et al.*, 2010), cancer (Ladiges *et al.*, 2010; Sosa *et al.*, 2013), and diabetes (Sforcin & Bankova, 2011).

Propolis is enriched with phenolic compounds, such as flavonoids and phenolic acids, which are responsible for its antioxidant activity (Scalbert *et al.*, 2005; Almaraz-Abarca *et al.*, 2007; Viuda-Martos *et al.*, 2008). These compounds can scavenge free radicals and protect cells from oxidative stress, DNA strand breaks, and fragmentation of proteins (Oryan *et al.*, 2018). Antioxidant activity can occur by many means, such as inhibiting ROS production, scavenging free radicals, and chelating metal ions (Kurek-Górecka *et al.*, 2014).

Ethanol extracts (EE) of propolis from Portugal have been demonstrated to scavenge free radicals and protect human erythrocytes from lipid peroxidation (Valente *et al.*, 2011), but also to protect yeast cells from H_2O_2-induced oxidative stress and DNA damage (Cruz *et al.*, 2016). Red propolis antioxidant activity has been studied in many tumor cell lines, such as SF-295 (glioblastoma), OVCAR-8 (ovary), and HCT-116 (colon), and it has a powerful scavenging effect of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (Alencar *et al.*, 2007; Mendonça *et al.*, 2015). Ethyl acetate extract of propolis from China and methanol extract of Algerian propolis have both been reported to have a strong scavenging and ferric reducing activity (Yang *et al.*, 2011; Piccinelli *et al.*, 2013). Furthermore, studies in mice revealed that a low concentration of Brazilian green propolis inhibited lipid peroxidation and hemolysis (Noli & Miolo, 2001).

CAPE is a major compound in propolis antioxidant activity, showing a protective effect against peroxidation and a strong scavenging activity for hydroxyl radicals (Russo *et al.*, 2002). Furthermore, compounds like gallic acid, *t*-cinnamic acid, quercetin, chrysin, *p*-coumaric acid, pinocembrin, apigenin and ferulic acid have been linked to a higher antioxidant activity in propolis (Kowalczyk *et al.*, 2017; Okińczyc *et al.*, 2021; Kurek-Górecka *et al.*, 2022).

3.3. Anti-inflammatory activity

Inflammation occurs in response to tissue injury after exposure to external and internal stimuli and it happens in two stages: acute and chronic (Hu *et al.*, 2005). Acute inflammation activates immune system cells, which move to the injury site and release cytokines, ROS, and growth factors. When acute inflammation fails to solve the injury, it becomes chronic (Costa *et al.*, 2012).

Propolis is rich in many compounds with anti-inflammatory properties, such as caffeic acid, CAPE, quercetin, apigenin, and ferulic acid, which suppress the synthesis of prostaglandins and leukotrienes, and the activity of nicotine adenine dinucleotide phosphate (NADPH)-oxidase, ornithine decarboxylase, myeloperoxidase, and tyrosine-protein kinase (Ramos & Miranda, 2007; Araujo *et al.*, 2011; Cho *et al.*, 2014). In contrast to what has been described for propolis immunomodulatory activity, this bee glue has been reported to play the opposite role too, reducing inflammation by inhibiting the release of cytokines

and the proliferation of T cells (Araujo *et al.*, 2011; Oryan *et al.*, 2018). Flavonoids isolated from an ethanol extract of propolis from Nepal have shown an inhibitory effect on the expression of many inflammatory genes in bone marrow-derived mast cells, such as *IL-6*, *TNF-α*, and *IL-13* (reviewed by Silva-Carvalho *et al.*, 2015). Brazilian green propolis has been demonstrated to decrease the number of macrophages and neutrophils in a lipopolysaccharide-induced pulmonary inflammation model, inducing a decrease in IL-6 and TNF-α secretion, and an increase in IL-10 and transforming growth factor-β (TGF-β) (Machado *et al.*, 2012). Additionally, an EE of Portuguese propolis has exhibited anti-inflammatory effects, both *in vitro*, inhibiting bovine serum albumin (BSA) denaturation, and *in vivo*, improving mechanical hyperalgesia and gait pattern parameters in an osteoarthritis-induced model (Araújo et al, 2022).

3.4. Antimicrobial activity

Antimicrobial activity is one of the most studied properties in propolis and it has been well documented against bacteria, fungi, protozoa, and viruses (Sforcin *et al.*, 2000; Gekker *et al.*, 2005; Búfalo *et al.*, 2009; Sforcin & Bankova, 2011; Silva-Carvalho *et al.*, 2015; Asfaram *et al.*, 2021).

3.4.1. Antibacterial activity

Bacterial resistance to antibiotics is a rising concern nowadays and investigating natural products with antibacterial properties is crucial to cope with such problem (Campos *et al.*, 2015). Propolis has long been a contender for this purpose, due to the vast reports on its antibacterial activity (reviewed by Silva-Carvalho *et al.*, 2015). This property of propolis depends greatly on its chemical composition and place of collection, but it is mostly due to its flavonoids, aromatic acids and esters (Marcucci, 1995; Hegazi *et al.*, 2000; Seidel *et al.*, 2008). Compounds such as galangin, pinocembrin and pinostrobin have been strongly associated with propolis antibacterial activity, but caffeic and ferulic acid play a role in it too (Dimov *et al.*, 1992; Marcucci, 1995).

Propolis is much more active against Gram-positive than Gram-negative bacteria, which could be a consequence of the differences in cell wall structure of these two bacterial types, since Gram-negative bacteria have a negatively charged lipopolysaccharide (LPS) layer that acts as a barrier (Kujumgiev *et al.*, 1999; Cushnie & Lamb, 2005; Fokt *et al.*, 2010;). Mirzoeva *et al.* (1997) proposed that propolis can affect the ion permeability of the inner bacterial membrane, leading to the dissipation of the membrane potential and inhibiting bacterial motility. Propolis has been reported to be effective against many Gram-

positive bacteria, such as *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, several species of the genera *Streptococcus*, *Enterococcus* spp., *Micrococcus luteus*, among others (Popova *et al.*, 2009; Righi *et al.* 2011; Schmidt *et al.*, 2014; Bittencourt *et al.*, 2015; Campos *et al.*, 2015; AL-Ani *et al.*, 2018). Although not common in many propolis extracts, Turkish and Brazilian propolis ethanol extracts have shown significant activity against *Escherichia coli*, a Gram-negative bacterium (Fernandes Jr. *et al.*, 2006; Katircioğlu & Mercan, 2006). Moreover, synergetic effects between propolis and several antibiotics have been reported (Orsi *et al.*, 2006; Al-Waili *et al.*, 2012; Lavigne *et al.*, 2020; Freitas *et al.*, 2021; Ramata-Stunda *et al.*, 2022), suggesting that a combination of both could potentiate the antibiotics' effect and allow for dose reduction.

3.4.2. Antifungal activity

Much like the antibacterial, propolis antifungal activity is affected by its chemical composition and site of collection (Kujumgiev *et al.*, 1999). This property of propolis has been studied among several fungi, and has been mostly ascribed to propolis volatile compounds and flavonoids, such as pinocembrin and galangin (Quiroga *et al.*, 2006; Fokt *et al.*, 2010; Bankova *et al.*, 2014; Silva-Carvalho *et al.*, 2015). Propolis has been reported to be effective against several *Candida* strains, including *Candida albicans*, but also *Pichia ohmeri*, *Rhodotorula* spp., *Trichosporon* spp., *Aspergillus fumigatus*, several species of the genera *Trichophyton* and *Microsporum*, *Epidermophyton floccosum*, *Malassezia* spp. among others (Fernandes Jr. *et al.*, 1995; Oliveira *et al.*, 2006; Wagh, 2013; Falcão *et al.*, 2014; Cerqueira *et al.*, 2022). The effectiveness against *C. albicans* is of great interest, and Brazilian propolis EE has been reported to inhibit the growth and biofilm formation of this strain in vulvovaginal candidiasis (Dota *et al.*, 2011; Capoci *et al.*, 2015). It has been proposed that propolis affects *C. albicans* via metacaspase and by the Ras pathway (Castro *et al.*, 2013). Moreover, Portuguese propolis EEs have been reported to act as biocontrol agents in fruit fungal infections, such as brown spot disease in pear, caused by the fungus *Stemphylium vesicarium* (Loebler *et al.*, 2020), and apple blue mold, caused by the fungus *Penicillium expansum* (Pereira *et al.*, 2022), being a great candidate for agri-food applications.

3.4.3. Antiprotozoal activity

Propolis antiprotozoal activity, although not as deeply investigated as its other antimicrobial activities, is of great importance, as protozoan parasitic infections represent a worldwide public health issue (Sundar & Chakravarty, 2013). Propolis has been reported to be effective against a few protozoan

parasites: *Leishmania* spp., the causer of leishmaniasis – propolis inhibits the proliferation of the parasite and has an anti-inflammatory action through the inhibition of $NO^{\bullet}$ production (Asfaram *et al.*, 2021); *Plasmodium* spp., the causer of malaria – propolis inhibits the parasitemia (quantitative measure of parasites in the blood), which causes an improvement in the anemia caused by the infection, but also controls the infection through immunomodulation (Olayemi, 2014; AlGabbani *et al.*, 2017); *Trypanosoma* spp., the causer of trypanosomiasis – propolis has been reported to act in a similar way as for *Plasmodium* spp. (Nweze *et al.*, 2017, Asfaram *et al.*, 2019); *Blastocystis* spp., a common lower intestinal opportunistic pathogen – propolis could be activating apoptotic mechanisms (Asfaram *et al.*, 2021).

A phenolic extract of propolis from Central Portugal was reported to be active against *Plasmodium falciparum*, presenting a half maximal inhibitory concentration (IC_{50}) of 8.83 μg/mL (Falcão *et al.*, 2014), while Brazilian green propolis presented an IC_{50} of 20 μg/mL (Filho *et al.*, 2009). Samples of propolis from North-eastern and central Portugal both presented an IC_{50} of 8.11 μg/mL against *Leishmania infantum* (Falcão *et al.*, 2014), while two Turkish samples presented an IC_{50} of 125 and 325 μg/mL (Duran *et al.*, 2011) and Brazilian green propolis an IC_{50} of 49 μg/mL (Filho *et al.*, 2009). Propolis samples from North-eastern and central Portugal presented an IC_{50} of 6.16 and 8.59 μg/mL against *Trypanosoma cruzi*, respectively (Falcão *et al.*, 2014), while in Bulgarian and Brazilian propolis samples IC_{50} ranged from 108.8 to 1065 μg/mL (Prytzyk *et al.*, 2003) and from 421 to 1437 μg/mL (Cunha *et al.*, 2004a), respectively. Finally, propolis from North-eastern Portugal was reported to be very active against *Trypanosoma brucei*, presenting an IC_{50} of 1.70 μg/mL (Falcão *et al.*, 2014), which fits in the value range of the antitrypanosomal reference drug suramin (Otoguro *et al.*, 2012). These disparities in activities of propolis samples from different countries are caused by their distinct chemical compositions, although it is not yet clear what propolis compounds are behind it (Paula *et al.*, 2021). Otogura *et al.* (2012) observed that a ß-phenylethyl caffeate isolate from propolis presented a considerable activity against *T. brucei*, with an IC_{50} of 0.013 μg/mL, but it is suggested that propolis antiprotozoal activity originates from a combination of its antibacterial, anti-inflammatory, and immunomodulatory activities (Paula *et al.*, 2021).

3.4.4. Antiviral activity

Propolis from different geographic locations has been reported to be active against several viruses, such as influenza, herpes simplex (HSV) types 1 and 2, hepatitis B, and even immunodeficiency

virus (HIV) (Amoros, *et al.*, 1992a,b; Serkedjieva *et al.*, 1992; Marcucci, 1995; Gekker *et al.*, 2005; Shimizu *et al.*, 2008; Schnitzler *et al.*, 2010; Sartori *et al.*, 2012).

Many compounds present in propolis have been associated with its antiviral action: quercetin, kaempferol, CAPE, and isoprenyl caffeate have been shown to act against HIV by inhibiting the enzyme HIV-1 integrase, which is essential for viral DNA integration into the host genome (Fesen *et al.*, 1993; Harish *et al.*, 1997; Xu *et al.*, 2000; Chiu & Davies, 2004). Flavonoids have also been associated with propolis action against picornavirus, preventing the decapsidation of viral particles and the release of RNA in the cells (Tait *et al.*, 2006). Propolis from Czech Republic was tested against HSV-1, and galangin and chrysin proved to be the main bioactive compounds (Schnitzler *et al.*, 2010). Isopentyl ferulate, an ester of a substituted cinnamic acid, inhibited the activity of influenza virus A (Serkedjieva *et al.*, 1992).

Recently, propolis has been proposed to be effective against SARS-CoV-2, although this has only been tested *in silico* (molecular docking), and further studies would be necessary to confirm it (Berretta *et al.*, 2020). Many propolis components, such as CAPE, galangin, chrysin, and caffeic acid could serve as potential drugs against this virus, as they are predicted to interact with MPRO, an enzyme essential for coronavirus' life cycle and processing of proteins (Hashem, 2020). SARS-CoV-2 also binds firmly to the enzyme ACE2, using it as receptor and for the replication in host cells (Hoffmann *et al.*, 2020; Zhou *et al.*, 2020); Guler *et al.* (2021) used molecular docking to test if some propolis flavonoids and phenolic compounds would bind to it: although rutin had the most favourable binding energy to ACE2, myricetin, CAPE, hesperetin and pinocembrin were great candidates too.

4. Cellular effects of propolis and its mechanism of action - *Saccharomyces cerevisiae* as a biological model

The biological model chosen in this work to study propolis effects is the unicellular yeast *Saccharomyces cerevisiae*. It is among the most commonly used eukaryotic model organisms, mainly because of its cellular structure and simplicity to work with, its fully sequenced genome that allows the researcher to easily delete or replace specific genes, and the fact that many genes present in this yeast's genome are orthologues to genes associated with human diseases and, for most of them, the mammalian homologue is functional (Goffeau *et al.*, 1996; Heinicke *et al.*, 2007; Karathia *et al.*, 2011; Wloch-Salamon & Bem, 2013). *Saccharomyces cerevisiae* has been used as a model to study many biological processes, such as cell cycle, metabolism, cell death, regulation of gene expression, and neurodegenerative disorders, among others (Nasheuer *et al.*, 2002; Brocard-Masson & Dumas, 2006; López-Mirabal & Winther, 2008; Owsianowski *et al.*, 2008; Karathia *et al.*, 2011; Wloch-Salamon & Bem, 2013; Scariot *et al.*, 2018). It can be grown on a small volume of defined media and its cell cycle lasts between 90 and 120 min, allowing for a rapid growth of vast cell populations (Goffeau *et al.*, 1996; Hanson, 2018).

Saccharomyces cerevisiae has been used as a model on several studies with propolis regarding its biological properties (Castro *et al.*, 2011; Marques, 2015; Cruz *et al.*, 2016; Alves, 2018; Freitas *et al.*, 2019; Peixoto *et al.*, 2021). As mentioned above, propolis biological properties depend greatly on its composition. Castro *et al.* (2011) reported a cytotoxic effect from Green propolis (from Brazil) in *S. cerevisiae*, inducing cell death by apoptosis and even necrosis, when the exposure to propolis is longer. Green propolis was also reported to induce vacuolar acidification and the translocation of Atg8p (a hallmark of autophagy) to the vacuole – this pH imbalance could lead to necrosis, since pro-necrotic proteases are active in acidified cytosol (Eisenberg *et al.*, 2010). *Saccharomyces cerevisiae* was later reported to have many cell cycle related genes with a decreased messenger RNA (mRNA) accumulation, when exposed to this propolis, suggesting an effect on transcriptional checkpoint controls (Castro *et al.*, 2012).

5. Portuguese propolis

Presently, as reported on the Portugal National Beekeeping Program 2020-2022 (Gabinete de Planeamento, Políticas e Administração Geral, 2019), there are 11,883 registered beekeepers in Portugal, corresponding to around 42,000 apiaries and 768,000 hives. Although beekeeping activity is spread along the whole national territory, most beekeepers are centered in Northern and Central Portugal, and their main focus is on honey production and trade, and not so much on other bee products, such as propolis.

Despite propolis' broad list of valuable properties related to human health and well-being, Portuguese propolis has not deserved much attention and it still is an undervalued product, most of the times being discarded or stored without being used (Peixoto *et al.*, 2021). Propolis production is limited, reaching from 10 to 300 g per hive per year. Yet, propolis economic value in Portugal is still very low, compared to other countries, since studies regarding its chemical composition and biological properties are scarce and still at an early stage (Dias *et al.*, 2012; Tsagkarakis *et al.*, 2017).

As mentioned above, propolis chemical composition can vary depending on the local flora, climate conditions and season of harvest, even in the same country (Falcão *et al.*, 2012). Differences between Portuguese samples harvested from the same apiary but from different years or seasons have been reported (Miguel *et al.*, 2010; Marques, 2015; Peixoto *et al.*, 2022) – these differences have also been reported for other propolis samples worldwide (Simões-Ambrosio *et al.*, 2010; Isla *et al.*, 2012; Neto *et al.*, 2017).

Falcão *et al.* (2013) characterized the phenolic compounds present in 40 propolis samples from different regions of Portugal – north, central interior, central coast, south, Azores archipelago, and Madeira island – and separated the samples in two groups: the ones that resemble common temperate propolis, containing several compounds typically found in poplar propolis, such as flavonoids and their methylated forms, and phenylpropanoid acids and their esters (Falcão *et al.*, 2010) (Type I); and the ones with an uncommon composition (Type II), that besides common flavonoids, also contained quercetin and kaempferol glycosides, with many of them not previously described in propolis. These differences in composition are likely due to a different plant origin other than poplar buds and could be related to diversified biological activities (Dias *et al.*, 2012; Falcão *et al.*, 2013; Silva-Carvalho *et al.*, 2015).

Portuguese propolis has been associated with many biological properties, such as antimicrobial, antioxidant, and antitumoral activities (Moreira *et al.*, 2008; Fokt *et al.*, 2010; Miguel *et al.*, 2010; Valente *et al.*, 2011; Dias *et al.*, 2012; Valença *et al.*, 2013; Calhelha *et al.*, 2014; Silva-Carvalho *et al.*, 2014,

2015; Falcão *et al.*, 2014; Cruz *et al.*, 2016; Freitas *et al.*, 2019; Peixoto *et al.*, 2021, 2022; Oliveira et al, 2022).

Regarding antimicrobial activity, Portuguese propolis has been mainly reported to be active against bacteria and fungi (Silva *et al.*, 2012; Freitas *et al.*, 2019). Ethanol extracts of propolis from Gerês harvested from 2011 to 2014 have been reported to exhibit antibacterial activity against Gram-positive bacteria, with a strong effect against the spore forming bacteria of *Bacillus* genus, presenting minimum inhibitory concentration (MIC) values of 50 μg/mL (Freitas *et al.*, 2019). Falcão *et al.* (2014) reported that although propolis EEs from Bragança and Leiria were not very effective against the yeast *Candida albicans*, they exhibited an effect against the Gram-positive bacterium *Staphylococcus aureus* and the fungus *Trichophyton rubrum*.

The most studied biological property of this natural product in Portugal is definitely its antioxidant activity. Cruz *et al.* (2016) reported that an EE of propolis from Côa has a protective effect in yeast cells against H_2O_2-induced oxidative stress at a concentration of 100 μg/mL and has also caused a decrease in H_2O_2-induced intracellular oxidation, in a dose dependent manner (100 and 300 μg/mL). Propolis EEs from Bornes and Fundão were able to reduce lipid peroxidation and hemolysis caused by peroxyl radicals in human erythrocytes in a dose dependent manner, starting at 5 μg/mL all the way to 40 μg/mL (Valente *et al.*, 2011), which are very low concentrations, when compared to the already tested concentrations for other types of propolis (Campos *et al.*, 2014; Bonamigo *et al.*, 2017). Moreover, many extracts of propolis from all over the country, such as Algarve, Gerês, Bragança and Leiria have shown high antioxidant activity in *in vitro* assays like DPPH scavenging activity (Falcão *et al.* 2014; Peixoto *et al.*, 2021, 2022), O_2^- scavenging activity and iron chelating activity (Miguel *et al.*, 2010; Freitas *et al.*, 2019).

A property that has recently been receiving more and more attention in Portuguese propolis studies because of its importance in health sciences is its antitumoral and cytotoxic activity. Valente *et al.* (2011) tested the ability of Bornes and Fundão propolis EE to suppress the proliferation of human primary renal cancerous cells, with an MTT assay, reporting 85 % (Bornes) and 91 % (Fundão) cell proliferation inhibition at an extract concentration of 100 μg/mL. Chloroform and residual ethanol fractions from Azores propolis EE were shown to decrease the proliferation of the human colon carcinoma cell line HCT-15 in a dose-dependent manner, starting at the concentrations of 10 μg/mL for the chloroform fraction and 15 μg/mL for the residual ethanol fraction, but also caused a disturbance in HCT-15 glycolytic metabolism (Valença *et al.*, 2013). Phenolic extracts of propolis from Bragança and São Miguel, Azores, were too shown to exhibit high cytotoxicity against the HCT-15 cell line, with the concentrations necessary to inhibit 50 % of the net cell growth of 10 ± 1 (Bragança) and 9 ± 1 μg/mL (São Miguel; Calhelha *et al.*,

2014). A Beira Alta propolis EE from 2010 was reported to decrease proliferation and migration of the cancer cell lines MDA-MB-231 (breast cancer) and DU145 (prostate cancer), in a dose-dependent manner and starting at the concentrations of 15 μg/mL for MDA-MB-231 cell line and 7 μg/mL for the DU145 cell lines (Silva-Carvalho *et al.*, 2014). Recently, an n-butanol (n-BuOH) fraction of Gerês propolis EE from 2018 was found to show high cytotoxicity on BRAF-mutated cell lines, one of the most aggressive melanoma types, with an IC_{50} of 8.14 ± 0.03 μg/mL for the A375 cell line and of 11.22 ± 1.66 μg/mL for the WM9 cell line – this fraction has high quantities of CAPE, a compound known for its anticancer properties, suggesting its involvement in Gerês propolis cytotoxicity against melanoma (Oliveira *et al.*, 2022). An ethyl acetate fraction of the same Gerês propolis EE was also shown to be highly cytotoxic against the A498 and 786-O renal cell carcinoma cell lines, with an IC_{50} of 0.162 μg/mL for the A498 cell line and of 0.271 μg/mL for the 786-O cell line (Freitas *et al.*, 2022).

It is crucial to keep studying Portuguese propolis, diving deeper into its properties and exact composition, in order to promote its use and increase its national value.

6. Aim of the dissertation

Because of their many biological properties, natural products have been on the forefront of pharmaceutical, cosmetic, agricultural and food research (Che & Zhang, 2019; Atanasov *et al.*, 2021; González-Manzano & Dueñas, 2021; Goyal & Jerold, 2021). Propolis is a natural product that presents many biological properties, such as antioxidant, antimicrobial and cytotoxic activities. Although it is gaining attention worldwide for its multiple purposes, its chemical composition varies greatly from region to region, presenting different biological activities, which is a setback for its application on health, cosmetic and food industries, since it needs to be chemically standardized. Portuguese propolis, besides the mentioned standardization problem, is yet to be recognized for its value by beekeepers and consumers.

Ethanol extracts of Portuguese propolis samples from the Pereiro apiary harvested in 2010 (P10.EE) and 2013 (P13.EE) were reported to exhibit very different activities, as P13.EE exhibits antioxidant activity and P10.EE exhibits cytotoxic activity. The present work aims to evaluate these P13.EE and P10.EE bioactivities, in order to better understand their different mechanisms of action, and therefore contributing to the valorization of Portuguese propolis, that has so much potential and is many times overlooked.

For this purpose, the biological model *S. cerevisiae* was used, and the work was divided in two parts, the study of the antioxidant properties of P13.EE, and the study of the cytotoxic properties of P10.EE. For the study of P13.EE, viability assays with *S. cerevisiae* BY4741 and mutant strains were done, to assess cytotoxicity. Intracellular oxidation was also analyzed, by flow cytometry, for the same yeast strains, to assess the extract's antioxidant activity. For the study of P10.EE, viability assays with *S. cerevisiae* BY4741 strain were done, to confirm cytotoxicity, and, to assess its effect on regulated cell death, two fluorescence microscopy assays were done: chromatin condensation and fragmentation (apoptotic morphological features) was evaluated with 4',6-diamidino-2-phenylindole (DAPI) staining; loss of plasma membrane integrity (common necrosis marker) was evaluated with propidium iodide (PI) staining. P10.EE effect on the yeast cell cycle was also studied, by flow cytometry, to assess if the extract's cytotoxicity was causing an arrest or delay in the cell cycle.

7. References

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CHAPTER 2. Antioxidant activity of Pereiro propolis

1. Introduction

1.1. Reactive oxygen species

Although aerobic eukaryotes depend on oxygen to survive, this molecule can also present a danger to their survival, when in supraphysiological concentrations. In fact, atomic oxygen has two unpaired electrons, making it vulnerable to the formation of radicals (Bardaweel *et al.*, 2018). Reactive oxygen species (ROS) is an umbrella term used to describe the reactive molecules and free radicals that originate from molecular oxygen, such as superoxide ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\bullet OH$) and hydroxyl ion (OH^-) (Sies & Jones, 2020).

ROS can be formed naturally in many ways and from various sources, and even though the mitochondrial electron transport chain contributes greatly to their formation, it has been evidenced that ROS are also produced by peroxisomes and some cellular enzymes, such as NADPH oxidases, cytochrome P450 oxidases and xanthine oxidases (Izyumov *et al.*, 2010; Lassègue *et al.*, 2012; Lismont *et al.*, 2015; Bezawork-Geleta *et al.*, 2017; Fransen *et al.*, 2017; Zhao *et al.*, 2019; Al-Shehri *et al.*, 2020; Wolf *et al.*, 2020; Zhang *et al.*, 2020). Although not a strong oxidizing agent, $\bullet O_2^-$ is the precursor of all cellular ROS and is easily dismutated to H_2O_2 and O_2 by superoxide dismutases (SOD) (Sies & Jones, 2020; Herb *et al.*, 2021). Hydrogen peroxide is considered to be the main ROS in redox regulation of biological activities, contributing to many cell processes, namely cell proliferation, differentiation, migration and angiogenesis (Sauer *et al.*, 2001; Jones & Sies, 2015; Sies *et al.*, 2017; Winterbourn, 2018). It is also involved in the formation of $\bullet OH$, the most reactive ROS and an initiator of lipid peroxidation, by reacting with iron-sulfur (Fe-S) clusters through a metal-catalyzed Fenton reaction (Liochev & Fridovich, 1999; Imlay, 2003; Koppenol & Hider, 2019):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$$

When in physiological concentrations, ROS play many important cellular roles, such as intracellular redox signaling and contribution to blood pressure regulation, cognitive functions and immune responses (Yang *et al.*, 2013; Li *et al.*, 2017; Togliatto *et al.*, 2017; Zarse & Ristow, 2021). However, these are very reactive molecules, and a detoxification of ROS is crucial for cell survival, as they could cause harm when in uncontrolled concentrations – an imbalance between ROS cellular levels and the cell's antioxidant response is referred to as oxidative stress (Bardaweel *et al.*, 2018). Antioxidant defense mechanisms have evolved in cells, allowing them to combat oxidative stress. As mentioned above, SOD converts $\bullet O_2$ into H_2O_2 and O_2, but the enzyme catalase converts H_2O_2 into H_2O and O_2 in the peroxisomes (Alfonso-Prieto *et al.*, 2009). Glutathione peroxidase is another enzyme that also reduces

H_2O_2 into H_2O (Chu *et al.*, 2020). Besides enzymatic defenses, there are many small molecule antioxidants that play a part in combating oxidative stress, such as glutathione, vitamin C (ascorbic acid), vitamin E (α-tocopherol), carotenoids and flavonoids (Valko *et al.*, 2007). Regardless of the many defense systems, living aerobic organisms still suffer from oxidative damage, and oxidative stress is linked to a large variety of degenerative processes, diseases and syndromes (Pizzino *et al.*, 2017; Bardaweel *et al.*, 2018). Oxidative stress affects numerous biological mechanisms and structures, causing protein modification, inflammation, cell death, mitochondrial malfunction, among others, which can lead to and/or worsen serious pathologies, like Alzheimer's disease, type 2 diabetes mellitus, chronic obstructive pulmonary disease, hypertension and cancer (Zinellu *et al.*, 2016; Butterfield & Halliwell, 2019; Oguntibeju, 2019; Barnes, 2020; Hayes *et al.*, 2020; Touyz *et al.*, 2020 Arfin *et al.*, 2021; Forman & Zhang, 2021; Misrani *et al.*, 2021; Bhatti *et al.*, 2022; Tain & Hsu, 2022).

1.2. Antioxidant activity of Pereiro propolis

In this chapter, the focus will be on Pereiro propolis harvested in 2013 (P13). An ethanol extract of this sample (P13.EE) has been studied in previous works and has exhibited antioxidant properties (Marques *et al.*, 2015; Barroso *et al.*, 2017). P13.EE showed little effect on the viability of the yeast *S. cerevisiae* at the concentrations of 100, 200, and 500 μg/mL, but presented cytotoxicity at 750 μg/mL. These results were reported by Marques (2015) and their constancy was confirmed by Barroso (2017). Barroso (2017) also tested this extract mutagenicity with an Ames test and since P13.EE showed no significant differences when compared to the negative control, the author concluded that this extract is not mutagenic.

Marques (2015) studied the effects of P13.EE on yeast viability under H_2O_2-induced oxidative stress, showing that this extract (at the concentrations of 50, 100 and 200 μg/mL) caused an increase in *S. cerevisiae* viability when compared to the H_2O_2 control, suggesting an antioxidant activity. Barroso (2017) assessed the antioxidant activity of P13.EE through *in vitro* assays, namely the DPPH radical scavenging activity at the concentrations of 100, 1000 and 10000 μg/mL, showing similar activity to the positive control ascorbic acid at the same concentrations of the extract, and the reducing power of Fe^{3+}/ferricyanide complex to ferrous form, at the concentrations of 100, 500 and 1000 μg/mL, and although the results were significantly lower when compared to the positive control gallic acid (at the same concentrations of the extract), the extract still presented reducing power at all the tested concentrations. Both activities tested were shown to be dose-dependent and several studies have linked them to the presence of phenolic compounds and flavonoids (Moreira *et al.*, 2008; Petelinc *et al.*, 2013;

De Marco *et al.*, 2017). Barroso (2017) also tested the P13.EE Fe^{2+} chelating activity and plasmid DNA (pDNA) protection against Fe^{2+} damage. Remarkably, P13.EE was not able to chelate Fe^{2+} but presented a protective effect against the damage it caused on pDNA even at the lowest concentration of 10 μg/mL. It was suggested by the author that the DNA protection mechanism could be related to the extract ability to scavenge free radicals, since it is not able to directly chelate Fe^{2+}.

The combination of these results suggest that P13.EE has promising antioxidant properties, but it would be interesting to dive deeper into the study of such bioactivity. The aim of this chapter is to continue and complement this study, using the biological model *S. cerevisiae* to better understand the mechanism of action behind this antioxidant activity.

2. Materials and methods

2.1. Propolis samples and ethanol extract preparation

The propolis sample used in this work was obtained from the Pereiro (P) apiary located at Beira Alta, Pinhel city, Guarda district (N 40° 44'57.135", W 7° 0'59.403", elevation: 672.6 m) – and was collected in the year 2013 (P13) from *A. mellifera* beehives by conventional scraping. An ethanol extract was then prepared from this sample, obtaining P13.EE, according to Alves (not published). Briefly, 100 mL of absolute ethanol (EtOH) (CARLO ERBA Reagents) were added to 15 g of raw propolis sample P13 in an Erlenmeyer and kept in an orbital shaker at 25 °C and 130 revolutions per minute (rpm), in the dark, for 24 h. This suspension was then filtered with Macherey-Nagel filter papers, utilizing a Buchner funnel and a Kitasato combined with a vacuum pump. The remaining residue went through a new extraction with 35 mL of absolute EtOH. The resulting filtrates were mixed and the EtOH was evaporated in a Büchi Rotavapor RE 121 with a water bath (Büchi 461), at 40 °C and 46 rpm. The dried ethanol extract was then kept in the dark, at -20 °C, until usage (Marques, 2015). Stock solutions were prepared with EtOH at the necessary concentrations for the different assays whenever necessary and kept at -20 °C.

2.2. *Saccharomyces cerevisiae* strains and conditions of media and culture growth

The effects of P13.EE were evaluated using *Saccharomyces cerevisiae* BY4741 (Table 1). Mutants derived from this parent strain (*Δyap1, Δsod1, Δsod2*) were also used in some assays (Table 1).

Table 1. Yeast strains used in this work and their respective genotypes and origin.

Strain	Genotype	Origin
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
Δyap1	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YML007w::kanMX4*	Euroscarf
Δsod1	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YJR104c::kanMX4*	Euroscarf
Δsod2	*BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YHR008c::kanMX4*	Euroscarf

Cultures of the selected *S. cerevisiae* strains were prepared in YPD medium (1 % (w/v) yeast extract (DB Bacto™), 2 % (w/v) peptone (DB Bacto™), 2 % (w/v) glucose (Scharlau)) with incubation at 30 °C, 200 rpm and growth was monitored by optical density at 600 nm (OD_{600}). Inocula for the liquid

cultures were obtained from single colonies cultured on solid YPDA medium (YPD medium complemented with 2 % (w/v) agar (LabChem Inc.)) at 30 °C for 48 h. Cultures in solid media were kept at 4 °C.

To prepare cells for the different assays, liquid cultures were grown overnight at 30 °C and 200 rpm and then diluted in fresh medium to an OD_{600} of 0.1. The culture was then incubated under the same conditions for 4 h, guaranteeing two generations at exponential phase (OD_{600} between 0.4 and 0.8).

2.3. Evaluation of cellular viability

A *S. cerevisiae* BY4741 YPD-culture was prepared as described above and after OD_{600} confirmation, aliquots of the cell suspension were placed in microtubes and different volumes of P13.EE were added, at the final concentrations of 200, 500 or 750 μg/mL. A negative control was prepared using the same volume of EtOH as for the 750 μg/mL P.EE. The cells were then incubated at 30 °C and 200 rpm for 90 min and at time-points 0, 30, 60 and 90 min, aliquots of 100 μL were collected and serially diluted from 10^{-1} to 10^{-4} in sterilized distilled H_2O (dH_2O) and 5 μL drops of each dilution were placed on YPDA medium plates, which were inverted and incubated at 30 °C for 48 h, in the dark. Photographs were made using ChemiDoc™XRS. Viability assays were also performed with *S. cerevisiae* BY4741 mutants *Δyap1*, *Δsod1* and *Δsod2*, to analyze the effect of P13.EE at the same concentrations tested for the parent strain.

2.4. Evaluation of intracellular oxidation

A *S. cerevisiae* BY4741 YPD-culture was prepared as described above and, after OD_{600} confirmation, it was centrifuged at 4 °C and 4,400 *g*, for 3 min (Eppendorf Centrifuge 5804 R), and the pellet washed two times with and resuspended in PBS (137 mM NaCl (Honeywell Fluka™), 2.7 mM KCl (Honeywell Fluka™), 4.3 mM Na_2HPO_4 (PanReac AppliChem), 1.47 mM KH_2PO_4 (Merck KGaA); pH 7.4). The cell suspension was diluted to an OD_{600} of 0.04 in a volume of 10 mL, and 2 mL were collected for autofluorescence controls. Fifty micromolar 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes) was added to the remaining suspension and it was incubated at 30 °C and 200 rpm, in the dark, for 60 min. The cell suspension was then distributed into microtubes, P13.EE was added to the final concentrations of 50, 100, 200 or 500 μg/mL, and H_2O_2 (CARLO ERBA Reagents) was added to a final concentration of 5 mM. Negative and positive controls were prepared, respectively, with absolute EtOH (same volume as for the 500 μg/mL P.EE) and 5 mM H_2O_2. The cells were then incubated at 30 °C and 200 rpm, in the dark, for 20 min. The samples were placed on a flat bottom 96-well plate and

analyzed in a flow cytometer (Beckman Coulter CytoFLEX V0-B4-R2), using a blue laser for excitation at 488 nm and the FITC filter. Data analysis was done with the program CytExpert 2.5.

2.5. Chemical characterization of phenolic content from P13.EE

Extract P13.EE phenolic content was determined following the Folin-Ciocalteu colorimetric method, as described by Silva-Carvalho *et al.* (2014), through an UPLC-DAD-ESI/MS analysis, performed as described by Freitas *et al.* (2019) on an Ultimate 3000 (Dionex Corporation) equipped with an Ultimate 3000 Diode Array Detector (Dionex Corporation) and coupled to a mass spectrometer (Thermo Fischer Scientific Inc. LTQ XL Linear Ion Trap) equipped with an ESI source. The chromatographic system comprehended a quaternary pump, an autosampler, a photodiode array detector and automatic thermostatic column compartment. The analysis was carried on an endcapped Hypersil Gold (Thermo Fischer Scientific Inc.) C18 column (100 mm length, 2.1 mm diameter, 1.9 μm particle diameter), kept at 30 °C. The mobile phase was composed of (A) 0.1 % of formic acid (v/v) and (B) acetronile. The solvent gradient started with 20 % of solvent (B), then 40 % at 25 min, 60 % at 35 min, 90 % at 50 min, and finally returning to the initial conditions. The flow rate was 0.1 mL/min and UV-Vis spectral data for all peaks were accumulated in the range of 200-500 nm, while chromatographic profiles were recorded at 280 nm. The instrument was operated in negative-ion mode, with ESI needle voltage set at 5.00 kV and an ESI capillary temperature of 275 °C. Data acquisition was carried out with the Xcalibur Qual Browser data system. Compounds were identified through the retention times of standards and by comparison of the ESI-MS with the data published in the literature (Falcão *et al.*, 2010; Silva-Carvalho *et al.*, 2014; Freitas *et al.*, 2019; Oliveira *et al.*, 2022). This analysis was carried out at Aveiro University, with the support of Doctor Susana Cardoso.

2.6. Statistical analysis

Unless otherwise stated, the experiments were done in three independent replicas, and the results are either presented as one representative experiment or as the mean ± standard deviation (SD). For all of the statistical tests, a 95 % confidence interval was assumed. Data was analyzed with a One-way analysis of variance (ANOVA), followed by *post hoc* tests for multiple comparisons (Dunnett). The significance of differences between the mean values is represented by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Data was analyzed and graphs were created using GraphPad Prism 8.0.

3. Results and discussion

3.1. Evaluation of cellular viability

3.1.1. P13.EE maintains its low effect on the viability of *Saccharomyces cerevisiae*

As mentioned, P13.EE was shown to have a low cytotoxicity effect on *S. cerevisiae* cells in previous studies (Marques, 2015; Barroso, 2017). Cytotoxic activity in propolis has been well described (Burdock *et al.*, 1998; Alencar *et al.*, 2007; Campos *et al.*, 2015), inclusively in Portuguese propolis (Valente *et al.*, 2011; Valença *et al.*, 2013; Calhelha *et al.*, 2014; Silva-Carvalho *et al.*, 2014; Freitas *et al.*, 2022; Oliveira *et al.*, 2022), although contrasting intensities of this activity have been observed in different propolis samples, being linked to differences in the chemical composition of this natural product (Simões-Ambrósio *et al.*, 2010; Fernandes-Silva *et al.*, 2013; Shehata *et al.*, 2020). Marques (2015) assessed the cytotoxic effect of several Pereiro propolis samples from different years through viability assays, treating a YPD-culture of *S. cerevisiae* BY4741 cells with P.EE, collecting aliquots after 0, 30, 60 and 90 min of treatment and plating 10^{-1} to 10^{-4} dilutions of each aliquot on YPDA plates. In order to verify if P13.EE maintained its effect on the viability of *S. cerevisiae*, after so many years of being stored in a Falcon® tube at -20 °C, this assay was replicated in the present study (Figure 4A). A cytotoxic effect of P13.EE can be observed by a decrease in number of colonies when compared to the negative control (C; cells treated with absolute EtOH at the same volume as for the 750 μg/mL P13.EE), which is very clear at 60 and 90 min incubation with 500 and 750 μg/mL.

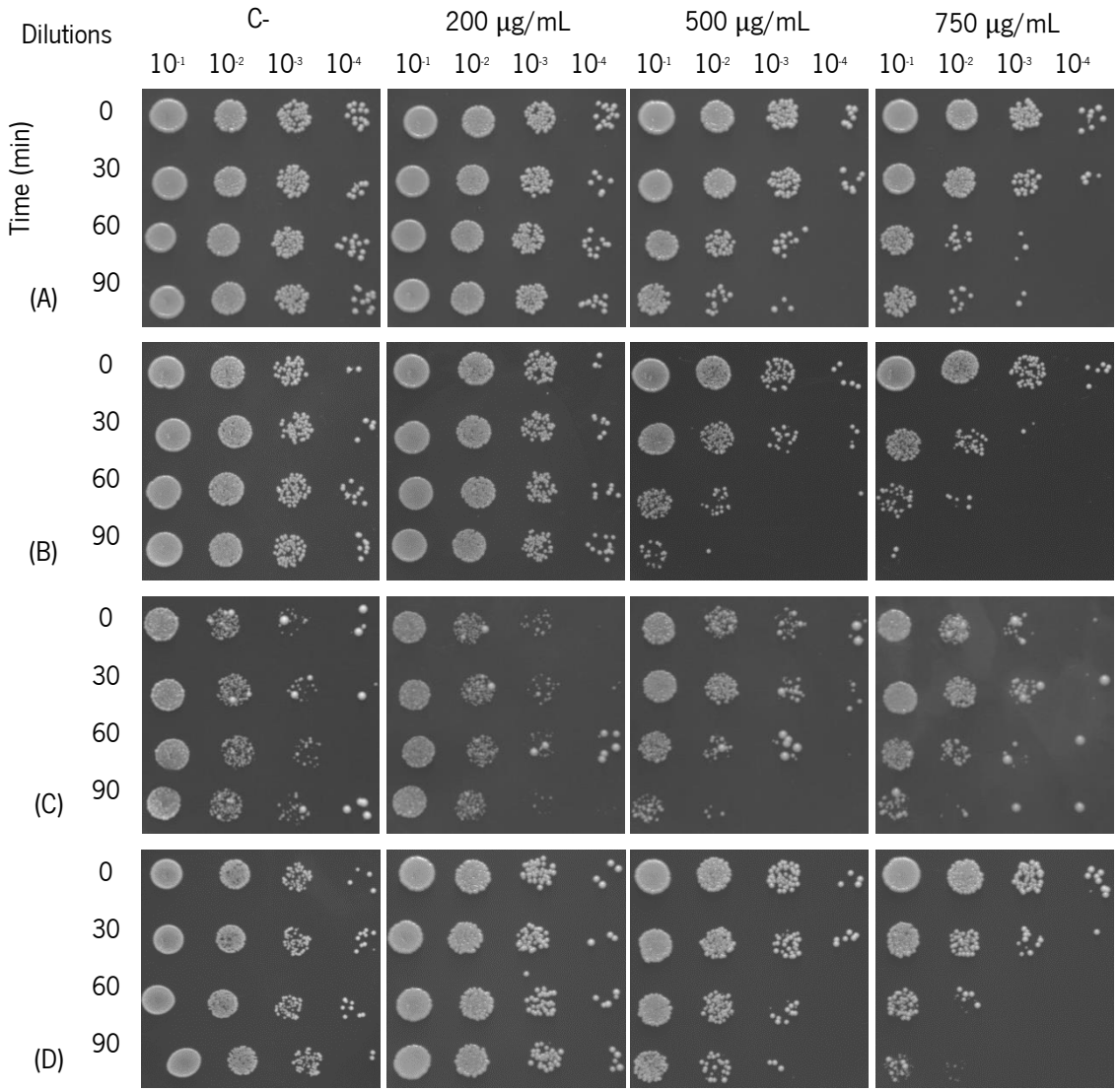

Figure 4. **Differences in susceptibility of *S. cerevisiae* BY4741 and its mutant strains *Δyap1*, *Δsod1* and *Δsod2* to P13.EE.** Cells from *S. cerevisiae* BY4741 strain (A) and its mutant strains *Δyap1* (B), *Δsod1* (C), and *Δsod2* (D) were treated with P13.EE at the concentrations of 200, 500, or 750 µg/mL. For the negative control (C-) cells were treated with absolute EtOH, at the same volume as for the 750 µg/mL P13.EE. Aliquots were collected after 0, 30, 60, and 90 min, and serially diluted from 10^{-1} to 10^{-4}, and 5 µL drops of each dilution were placed on YPDA plates and incubated at 30 °C for 48 h. Cytotoxicity is observed by a decrease in number of colonies when compared to C-. These results are from one representative experiment, out of three independent replicates.

As observed in Figure 4A, and in accordance with the results obtained by Marques (2015), P13.EE was able to maintain its low cytotoxicity over the years of storage at -20 °C, showing only a slight effect on the viability of *S. cerevisiae* at the concentration of 500 µg/mL and a higher effect at the concentration of 750 µg/mL.

3.1.2. Oxidative stress response mutants from *Saccharomyces cerevisiae* are more susceptible to P13.EE

Since Marques (2015) and Barroso (2017) reported a considerable antioxidant activity from P13.EE, and in an attempt to better understand its mechanism of action, the same viability assay was done with the *S. cerevisiae* oxidative stress response mutants *Δyap1* (Figure 4B), *Δsod1* (Figure 4C), and *Δsod2* (Figure 4D). The gene *YAP1* encodes a basic leucine zipper (bZIP) transcription factor essential for the cell response to oxidative stress, being activated by the presence of ROS and moving from the cytoplasm to the nucleus (Kuge *et al.*, 1997; Moye-Rowley *et al.*, 1989). The genes *SOD1* and *SOD2*, both essential in the response to oxidative stress, encode SODs, which catalyze the conversion of O^{2-} into O_2 and H_2O_2 (Miller, 2011). The gene *SOD1* encodes the copper/zinc SOD, located in the cytoplasm (Chang *et al.*, 1991), and *SOD2* encodes the manganese SOD, located in the mitochondrial matrix (Van Loon *et al.*, 1986; Zyrina *et al.*, 2017).

As observed in Figure 4, all of the tested mutant strains appear to be more susceptible to P13.EE when compared to the parent strain, especially at the concentration of 750 μg/mL. At the concentration of 500 μg/mL, the mutant *Δsod2* behaves in a similar manner to the parent strain, but the mutants *Δyap1* and *Δsod1* exhibit a slight decrease in viability. These results suggest that in higher concentrations, such as 750 μg/mL, P13.EE could be exhibiting a pro-oxidant effect, as opposed to the antioxidant properties reported for the extract in lower concentrations. These dose-dependent antagonistic effects have also been reported in a Portuguese propolis sample from Côa (Cruz *et al.*, 2016), and the authors linked it to the presence in the extract of compounds that interfere with DNA synthesis and/or cell proliferation, such as CAPE and chrysin, and of antioxidants, such as kaempferol, pinobanksin and pinocembrin. Flavonoids in propolis, famous for their antioxidant activity, have been reported to exhibit a pro-oxidant activity, and Tsai *et al.* (2012) proposed that this is because flavonoids can act as temporary electron carriers, aiding in the formation of ROS.

3.2. Evaluation of intracellular oxidation

3.2.1. P13.EE decreases H_2O_2-induced intracellular oxidation in *Saccharomyces cerevisiae*

In order to complement and contribute to the previous results pointing to the antioxidant activity of P13.EE, the extract's effect was investigated in a cellular context by assessing intracellular oxidation of *S. cerevisiae*. For this assay, *S. cerevisiae* BY4741 cells were loaded with H_2DCFDA, a cell-permeant fluorescent probe. This cell permeant probe enters the cells and is deacetylated by cellular esterases to

its reduced non-fluorescent form 2'-7'-dichlorodihydrofluorescein (H_2DCF), which is oxidized and converted to fluorescent 2'-7'-dichlorofluorescein (DCF) by intracellular ROS, serving as an intracellular oxidation indicator (Chang *et al.*, 2013). After loading, cells were treated with P13.EE and H_2O_2 at the concentration of 5 mM, to evaluate the extract's capacity of reducing the intracellular oxidation provoked by H_2O_2. For the C-, cells were treated with absolute EtOH at the same volume as for the P13.EE, and for the positive control (C+), cells were treated with H_2O_2 at the concentration of 5 mM. The treated cells were incubated at 30 °C for 20 min and then analyzed by flow cytometry (Figure 5A). With the CytExpert,2.5 software, a gating was drawn on the C+ peak, as shown on Figure 5A, and the cells to the left of this gating were counted as cells with less intracellular oxidation, as they displayed less fluorescence emission. Graphical representation of these cells is shown in Figure 5B.

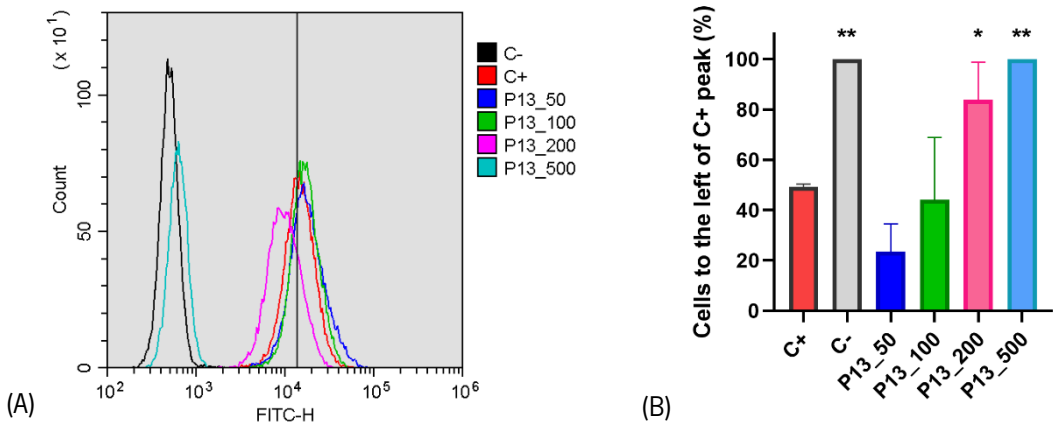

Figure 5. P13.EE decreases intracellular oxidation of cells from *S. cerevisiae* BY4741 strain. H_2DCFDA-loaded *S. cerevisae* BY4741 cells were treated with P13.EE at 50 (P13_50), 100 (P13_100), 200 (P13_200), or 500 µg/mL (P13_500) and with 5 mM H_2O_2, and incubated at 30 °C for 20 min. For the negative control (C-) cells were treated with absolute EtOH, at the same volume as for 500 µg/mL P13.EE, and for the positive control (C+) cells were treated with 5 mM H_2O_2. (A) Histogram of one representative experiment out of three independent replicates. (B) Percentage of cells with decreased intracellular oxidation. A gating was drawn at the peak of C+ to obtain the percentage of cells exhibiting less fluorescence emission (cells to the left of the gating). These values represent the mean ± SD of n = 3 independent replicates. One-way ANOVA was performed, followed by Dunnett's test for multiple comparisons. Asterisks represent significant differences between treatments and C+ (* $p < 0.05$, ** $p < 0.01$).

As expected, cells of C- displayed very low levels of fluorescence, however, cells exposed to H_2O_2 (C+) exhibited higher fluorescence, suggesting that they were under oxidative stress. When cells were incubated with H_2O_2 and P13.EE, fluorescence emission was similar to C+ for the 50 and 100 µg/mL extract concentrations, however, cells treated with the extract concentrations of 200 and 500 µg/mL exhibited significantly less fluorescence than the C+ cells. The concentration of 500 µg/mL exhibits an even higher significant difference than 200 µg/mL, with 100.00 ± 0.00 % of the cells to the left of the C+

peak. The results presented in Figure 5 showcase the ability of P13.EE to reduce intracellular oxidation caused by H_2O_2, both in the concentrations of 200 and 500 μg/mL.

3.2.2. P13.EE may enhance the antioxidant defenses of *Saccharomyces cerevisiae*

In an attempt to better understand the mechanism of action behind the antioxidant activity exhibited by P13.EE, the same intracellular oxidation assay was done with the oxidative stress response mutants used for the viability assays, *Δyap1* (Figures 6A and 6B), *Δsod1* (Figures 6C and 6D), and *Δsod2* (Figures 6E and 6F).

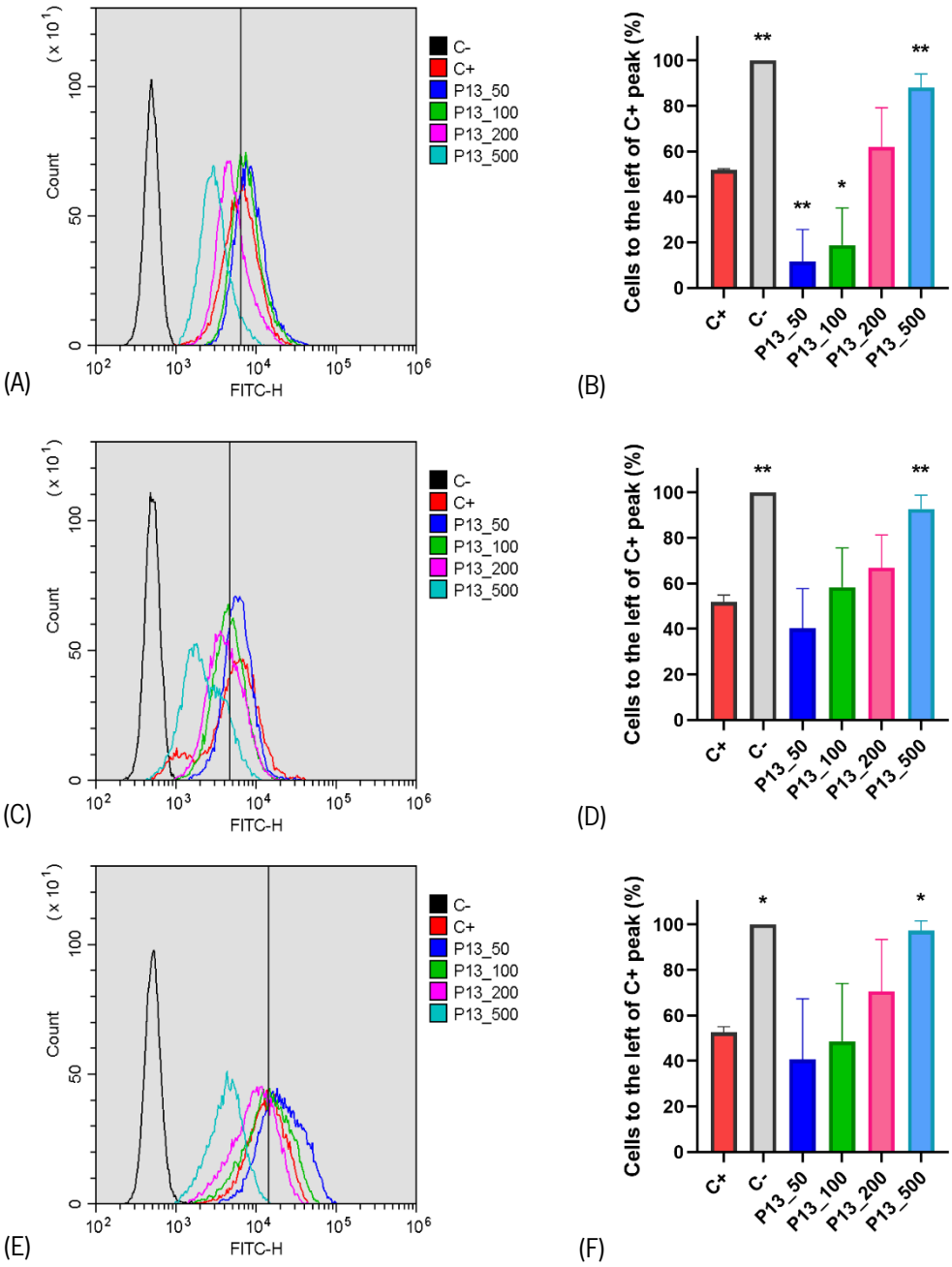

Figure 6. **P13.EE decreases intracellular oxidation of cells from *S. cerevisiae* mutant strains *Δyap1*, *Δsod1*, and *Δsod2*.** H_2DCFDA-loaded cells from the *S. cerevisiae* mutant strains *Δyap1* (A-B), *Δsod1* (C-D), and *Δsod2* (E-F), were treated with P13.EE at 50 (P13_50), 100 (P13_100), 200 (P13_200), or 500 µg/mL (P13_500) and with 5 mM H_2O_2, and incubated at 30 °C for 20 min. For the negative control (C-) cells were treated with absolute EtOH, at the same volume as for 500 µg/mL P13.EE, and for the positive control (C+) cells were treated with 5 mM H_2O_2. (A, C, E) Histograms of one representative experiment out of three independent replicates. (B, D, F) Percentage of cells with decreased intracellular oxidation. A gating was drawn at the peak of C+ to obtain the percentage of cells exhibiting less fluorescence emission (cells to the left of the gating). These values represent the mean ± SD of n = 3 independent replicates. One-way ANOVA was performed, followed by Dunnett's test for multiple comparisons. Asterisks represent significant differences between treatments and C+ (* $p < 0.05$, ** $p < 0.01$).

Similarly to what was observed in the parental strain (Figure 5), cells of C- displayed very low levels of fluorescence, while cells exposed to H_2O_2 (C+) exhibited higher fluorescence, suggesting the presence of oxidative stress. Again, all of the mutant cells treated with P13.EE at the concentrations of 50 and 100 μg/mL did not exhibit significantly different fluorescence emission when compared to the C+ cells (Figures 6B, 6D and 6F), except for the *Δyap1* mutant, where the percentage of cells to the left of the C+ peak was significantly lower than the percentage of C+ cells itself, suggesting that, in this strain, the lower concentrations of P13.EE not only did not decrease intracellular oxidation, but they increased it. Reports of this effect were not found in the literature, but there is a possibility of a synergism occurring between the extract and H_2O_2, which appears to cease when the extract is at a concentration of 200 and 500 μg/mL. Further studies are required in order to confirm and interpret these results, possibly with isolated compounds of P13.EE. Contrastingly to what was observed in the parent strain, cells treated with the 200 μg/mL P13.EE did not exhibit a significant decrease in fluorescence emission when compared to the C+, however, P13.EE at 500 μg/mL still caused a significantly lower fluorescence emission on all of the tested strains, with a percentage of cells to the left of the C+ peak of 89.26 ± 5.41 % for *Δyap1*, 92.72 ± 6.06 % for *Δsod1*, and 97.28 ± 4.22 % for *Δsod2*, suggesting that it is causing a decrease in the intracellular oxidation of all of the mutants tested.

The difference in the results from the parent strain and the mutants suggest that the antioxidant activity of P13.EE may be involved in the direct scavenging of free radicals, since it still is able to decrease intracellular oxidation when genes involved in the oxidative stress response are deleted, but they also suggest that the extract may additionally interact and activate the yeast antioxidant mechanisms that these genes are involved in, since without them, the extract is not able to have the same antioxidant effect. Additionally, since the yeast's oxidative stress response involves several proteins, like the mentioned Sod1, Sod2 and Yap1, but also Ctt1, a catalase (Petrova *et al.*, 2004), the studied mutants are not entirely incapable of responding to oxidative stress, as they only lack a single specific protein and the rest remain active.

3.3. Chemical characterization of phenolic content from P13.EE

Since P13.EE had not yet been chemically characterized, an UPLC-DAD-ESI/MS analysis was carried out in order to assess its chemical profile of phenolic compounds. This type of analysis allows for the identification of these compounds, by the determination of their chromatographic behavior, UV spectra, and mass spectrometry. Figure 7 represents the chromatographic profile of P13.EE at 280 nm, where 21 fractions were identified. These fractions were further analyzed by electrospray ionization/mass

spectrometry (ESI/MS), allowing for the identification of the probable compounds each fraction represents (Table 2), by comparison with data published in the literature.

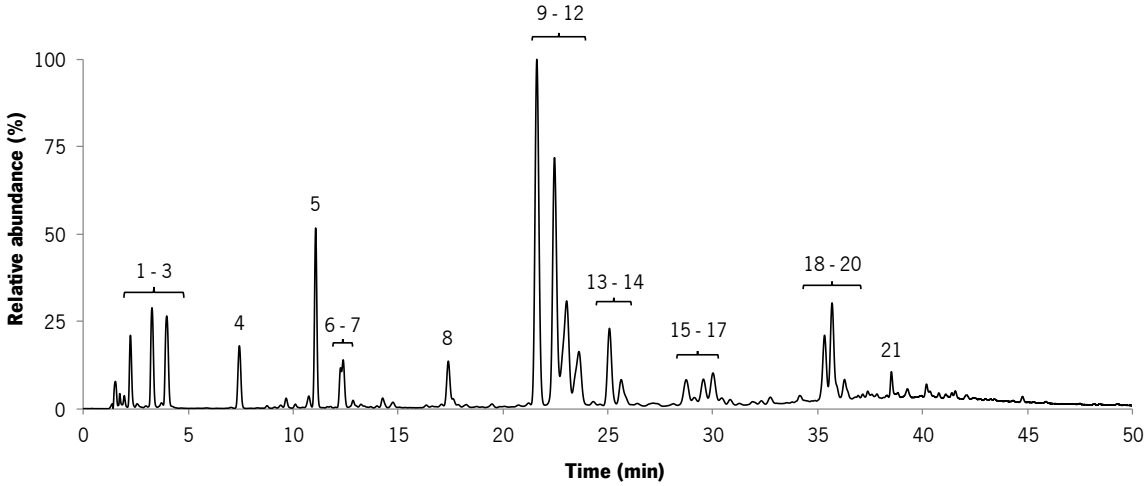

Figure 7. Chromatographic profile of P13.EE at 280 nm obtained by UPLC-DAD-ESI/MS. Numbers in the figure represent the fractions that were further analyzed by ESI/MS.

From the ESI/MS analysis, it was possible to suggest several phenolic compounds present in the P13.EE extract (Table 2). Although the most abundant compound in the extract is caffeic acid isoprenyl ester (peaks 9 and 10), several other compounds were identified, such as caffeic acid, *p*-coumaric acid and derivatives, ferulic acid, apigenin, pinocembrin, pinobanksin and derivatives, galangin, and derivatives of caffeic acid, like CAPE, along with some compounds which had not been referred in the literature but were also present in the ethanol extract of a propolis sample harvested in 2010 from the same apiary (peaks 18 and 19; Silva-Carvalho *et al.*, 2014). These compounds are commonly present in propolis samples and are associated with antioxidant activity (Russo *et al.*, 2002; Kowalczyk *et al.*, 2017; Okińczyc *et al.*, 2021; Kurek-Górecka *et al.*, 2022; Xu *et al.*, 2022). Furthermore, these results support the observed anti and pro-oxidant dose-dependent antagonistic effects of P13.EE, as the presence of CAPE along with antioxidants like pinobanksin and pinocembrin have been linked to this effect by Cruz *et al.* (2016).

Table 2. Chemical characterization of phenolic content of P13.EE obtained by UPLC-DAD-ESI/MS.

Peak	t_R (min)	λmax (nm)	$[M - H]^-$	Probable compound
1	2.2	216, 231sh, 296sh, 322	179	Caffeic acid
2	3.2	208, 224, 296sh, 309	163	*p*-Coumaric acid
3	4.0	216, 239sh, 297sh, 323	193	Ferulic acid
4	7.4	216, 236, 295sh, 321	207	3,4-Dimethyl-caffeic acid (DMCA)
5	11.1	226, 308	n.d.	*p*-Coumaric acid derivative

6	12.2	196, 209, 267, 294sh, 336	269	Apigenin
7	12.4	212, 226sh, 291	271	Pinobanksin
8	17.4	236, 311	313	Unknown
9	21.6	211, 245, 270, 297sh, 325	247	Caffeic acid isoprenyl ester
10	22.4	215, 236, 297sh, 325	247	Caffeic acid isoprenyl ester
11	23.0	210, 226sh, 289, 326	255	Pinocembrin
12	23.6	231, 266, 290sh, 355	269	Galangin
13	25.1	211, 227sh, 293	313	Pinobanksin-3-*O*-acetate
14	25.6	243, 297sh, 325	283	Caffeic acid phenylethyl ester
15	28.7	234, 311	231	*p*-Coumaric acid isoprenyl ester
16	29.5	235, 311	231	*p*-Coumaric acid isoprenyl ester
17	30.0	238, 291sh, 325	231	*p*-Coumaric acid isoprenyl ester
18	35.3	211, 268, 309	387	Unknown
			621	Unknown
19	35.7	211, 268, 292, 357	279	Unknown
			387	Unknown
20	36.3	239, 268, 292, 357	341	Pinobanksin-3-*O*-butyrate or isobutyrate
			621	Unknown
21	38.5	236, 269, 396sh, 344	565	*p*-Coumaric acid-4-hydroxyphenylethyl

The chromatographic profile of the extract from the 2010 sample (P10.EE) was also analyzed in the same conditions as for the P13.EE (Figure A1, Appendix 1), for the purpose of comparing the two. Both extracts display very similar chromatographic profiles, suggesting that their differences in biological activities, which will be exhibited in the next chapter, are probably not related to their phenolic content, at least not in a qualitative manner. Additionally, total flavonoid and phenolics contents have been estimated for both extracts, with P13.EE having a total phenolic content (TPC) of 217.6 ± 6.6 mg gallic acid equivalent/g extract and a total flavonoid content (TFC) of 38 ± 2.0 mg quercetin equivalent/g extract (Peixoto *et al.*, 2022), and P10.EE with a TPC of 252.4 ± 13.4 mg gallic acid equivalent/g extract and TFC of 51.3 ± 2.4 mg quercetin/g extract (Silva-Carvalho *et al.*, 2014). Although comparison of TPC and TFC of both extracts should be cautious, since different compounds contribute to these chemical classes, both extracts present values in the same range, further supporting the conclusion that the biological activity differences might be due to other chemical families.

4. Conclusions

Antioxidant activity in natural products is usually related to the presence of polyphenolic compounds (Balasundam at al., 2006). Polyphenols have exhibited many beneficial effects on human health due to their antioxidant activity (Pandey & Rizvi, 2009), therefore natural products with this property are of great interest to the pharmaceutical and cosmetic industries. Propolis is well known for its strong antioxidant activity, due to its high polyphenolic content, making it a great contender for these industries.

From the work presented in this chapter, it can be concluded, through viability assays, that P13.EE was able to maintain its low cytotoxic effect on *S. cerevisiae* over the years of storage (Figure 4A), and that effect appears dose-dependent, exhibiting a slight cytotoxic effect at higher concentrations. This observation was corroborated with the results from viability assays with the *Δyap1*, *Δsod1,* and *Δsod2* mutants (Figures 4B, 4C and 4D), which suggested that P13.EE could be having a pro-oxidant effect at the concentration of 750 μg/mL. These effects could be related to the presence of CAPE, and antioxidants like pinobanksin and pinocembrin in the extract (Table 2), as reported by Cruz *et al.* (2016)

Results from intracellular oxidation assays with *S. cerevisiae* BY4741 strain (Figure 5) are in accordance with the antioxidant activity reports previously made by Marques (2015) and Barroso (2017), as P13.EE was shown to reduce H_2O_2-induced intracellular oxidation at the concentrations of 200 and 500 μg/mL. However, the extract was less effective in the *Δyap1*, *Δsod1,* and *Δsod2* mutants (Figure 6), suggesting that although the extract is able to directly scavenge free radicals, it may also interact with the genes *YAP1*, *SOD1* and *SOD2* and/or with the proteins encoded by these genes for its antioxidant activity, possibly aiding in the activation of the yeast's antioxidant mechanisms, as these genes are involved in the yeast's oxidative stress response and each of the studied strains only lacked one specific gene, meaning that the rest are active and the cells are not entirely incapable of responding to oxidative stress.

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CHAPTER 3. Cytotoxic activity of Pereiro propolis

1. Introduction

1.1. Cell death in yeast

According to the Nomenclature Committee on Cell Death (NCCD), a cell can only be considered as dead upon non-reversible loss of plasma membrane integrity or complete cellular fragmentation (Galluzzi *et al.*, 2014). Cell death can be divided into different types: accidental and regulated. Regulated cell death (RCD) refers to cell death that occurs with the activation or inactivation of signaling pathways and can be at least partially avoided with genetic or pharmacological intervention – when RCD occurs during normal physiological processes that allow the preservation of homeostasis, such as cell turnover, it is referred to as programmed cell death (PCD) (Carmona-Gutierrez *et al.*, 2018). On the contrary, accidental cell death (ACD) refers to cells that die uncontrollably when exposed to extreme conditions, being unavoidable (Galluzzi *et al.*, 2014, 2018).

There are three established cell death pathways: apoptosis, necrosis, and autophagy. Apoptosis is a form of RCD and, much like in mammals, it is suggested to work as an organism protecting process in yeast (Farrugia & Balzan, 2012; Falcone & Mazzoni, 2016). Even though yeast is a unicellular organism, and cell death means organism death, it is proposed that this process discards old and abnormal cells so that protection of the whole yeast colony is possible, increasing the availability of nutrients and therefore promoting the survival of the more adapted clones (Huettenbrenner *et al.*, 2003; Farrugia & Balzan, 2012; Iranzo *et al.*, 2014; Vanchurin *et al.*, 2022). Apoptosis is an energy-dependent process that is very similar in many organisms, displaying several molecular and biochemical markers, such as nuclear fragmentation and chromatin condensation, phosphatidylserine (PS) externalization, accumulation of ROS, mitochondrial cytochrome *c* release and cell shrinkage (Kroemer *et al.*, 2009; Carmona-Gutierrez *et al.*, 2010; Grosfeld *et al.*, 2021). It can be induced by external triggers, such as exposure to low doses of oxidants like H_2O_2 and acetic acid, but also by internal mechanisms – various yeast orthologs of major mammalian apoptotic regulators, such as yeast caspase protein Yca1, apoptosis inducing factor Aifp1, inhibitor of apoptosis protein Birp1 and mitochondrial endonuclease G Nuc1p have been discovered (Madeo *et al.*, 2002; Wissing *et al.*, 2004; Walter *et al.*, 2006; Büttner *et al.*, 2007).

Necrosis, although firstly thought to only occur accidentally, is now also considered a form of RCD, since it can be regulated by the involvement of catabolic and signaling proteins (Baines, 2010; Galluzzi *et al.*, 2011). It is suggested that the regulated form of this cell death allows the sending of warning signals to the remaining cells of the colony, by releasing damage-associated molecular patterns (DAMPs). This theory has been corroborated by the observation of the nucleo-cytosolic release of the non-histone protein 6A (Nhp6A) in yeast necrotic cells, which is a yeast homologue of the mammalian danger

signal high-mobility group box 1 (HMGB1), however, it is still not confirmed that both have the same function (Carmona-Gutierrez *et al.*, 2010; Galluzzi *et al.*, 2011). Necrosis in yeast is characterized by bioenergetic impairment and several morphological features like oncosis (cell and organelle swelling), disruption of plasma membrane and consequent leakage of intracellular content, and the absence of typical apoptosis morphological features (Zong & Thompson, 2006; Eisenberg *et al.*, 2010; Carmona-Gutierrez *et al.*, 2018). Accidental necrosis can be induced by exposure to the same oxidants that cause apoptosis, although in much higher concentrations (Ludovico *et al.*, 2001; Farrugia & Balzan, 2012). Regulated necrosis occurs through programmed pathways and although they are still not completely understood, several yeast homologues of eukaryote proteins involved in mammalian cell necrosis have been discovered, like yeast cyclophilin D Cpr3p, yeast calpain Cp11p, yeast cathepsin Pep4p and yeast heat shock protein Hsp90 (Futai *et al.*, 1999; Mason *et al.*, 2005; Pereira *et al.*, 2007; Dudgeon *et al.*, 2008; Eisenberg *et al.*, 2010; Carmona-Gutierrez *et al.*, 2018). Vacuolar and cytoplasm acidification may also induce necrosis in yeast cells (Chaves *et al.*, 2021).

Finally, autophagy is a pro-survival cell process that occurs in response to various stresses such as accumulation of ROS and nutritional starvation, its main effects being to preserve homeostasis through degradation and recycling of cytoplasmic components. Autophagy occurs at basal levels in yeast cells and is morphologically characterized by the formation of the autophagosome – a double membrane vesicle where the cytoplasmic components are sequestered – which is then fused with the vacuole, the components are degraded and the products released back into the cytosol for reuse (Cebollero & Reggiori, 2009; Falcone & Mazzoni, 2016). It can be induced by nutritional changes and even pharmacological signals, being regulated by the target of rapamycin complex 1 (TORC1), that coordinates the autophagy related genes (*Atg*), which are involved in the autophagosome formation – most of these *Atg* are conserved in higher eukaryotes (Falcone & Mazzoni, 2016; Noda, 2017; Rangarajan *et al.*, 2020). Although not yet completely understood, it is thought that autophagy may also be involved and/or accelerate yeast cell death when there is too much stress (Farrugia & Balzan, 2012): rapamycin-induced autophagy in yeast resulted in decreased cell viability, accumulation of ROS and mitochondrial lipid peroxidation (Kiššová *et al.*, 2004, 2006); deletion of the autophagic protein Atg8 resulted in a delay in yeast cell death upon leucine starvation (Dziedzic & Caplan, 2012). Additionally, numerous autophagic bodies have been observed in cells going through RCD (Tsujimoto & Shimizu, 2005; Kourtis & Tavernarakis, 2008).

To simplify cell death interpretation and assessment, Carmona-Gutierrez *et al.* (2018) made an extensive review and outlined an approach to correctly identify the different types of cell death. Firstly, cell membrane integrity should be assessed – this can be done by staining with propidium iodide (PI),

and the percentage of stained cells represent the cells with a disrupted membrane; viability assays are an important complement, as a decrease in viability should accompany an increase in PI positive cells. Secondly, morphological and biochemical features should be analyzed and compared to the characteristic markers of apoptosis, necrosis and autophagy (summarized in Figure 8). Lastly, the authors suggest a genetic analysis to determine the regulatory pathway. Since a common biochemical denominator of necrotic cell death in yeast is not known, a combination of methods is recommended to determine this specific type of death: besides the already mentioned assessment of cell membrane integrity and complementary viability assay, the confirmation of the Nhp6A protein release from the nucleus to the cytosol is a strong indicator of necrosis and, finally, the confirmation of a lack of apoptotic markers – altogether, these assays will define necrotic cell death in yeast (Eisenberg *et al.*, 2010; Carmona-Gutierrez *et al.*, 2018).

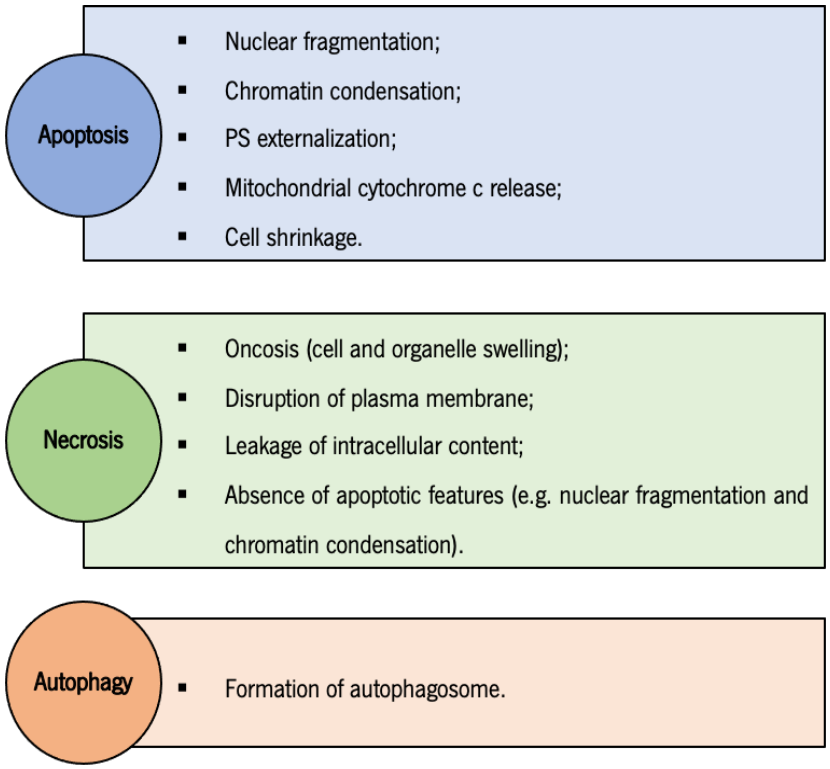

Figure 8. Summary of the morphological and biochemical characteristics of the yeast RCD types apoptosis, necrosis and autophagy.

1.2. Cytotoxic activity of Pereiro propolis

In this chapter, the focus will be on Pereiro propolis harvested in 2010 (P10). An ethanol extract of this sample (P10.EE) has been studied in previous works and was shown to be very cytotoxic towards *S. cerevisiae* while presenting little to no antioxidant activity, which was unusual, since Portuguese propolis is known for its high antioxidant activity and ethanol extracts of Pereiro propolis samples from

different years were much less cytotoxic and presented high antioxidant activity (Marques, 2015; Barroso, 2017; Peixoto *et al.*, 2022). Although not a novelty, as differences in propolis samples harvested from the same apiary have been reported (Miguel *et al.*, 2010; Peixoto *et al.*, 2022), this difference in samples from Pereiro is intriguing and led to the question of what could possibly be behind it. Previous work has been made regarding the mechanism of action of P10.EE in the past years, which will be discussed below.

As mentioned, P10.EE was shown to negatively affect the viability of the yeast *S. cerevisiae*, presenting high cytotoxicity in the concentrations of 500 and 750 μg/mL – these effects were tested by Marques (2015) and their constancy was confirmed by Barroso (2017). As an attempt to better understand the pathway of this cytotoxicity, Marques (2015) assessed the viability effects of the extract in several mutant strains of *S. cerevisiae* (*Δsod1*, *Δsod2*, *Δyap1*, *Δvph1*, *Δvps45*, *Δndi1*, *Δatg1*, and *Δyca1*) and, through the analysis of their differences in viability, the author observed that P10.EE toxic action was not linked to an oxidative stress pathway, but instead could be related to vacuolar biogenesis and acidification, autophagy, and even apoptotic cell death.

To assess P10.EE genotoxicity, Marques (2015) measured the *S. cerevisiae* DNA damage through a comet assay both in the parent strain and on the *yca1* mutant, a strain lacking the gene *YCA1*, which is the yeast orthologue to mammalian metacaspase (involved in the trigger of apoptosis) (Madeo *et al.*, 2002). P10.EE caused significant DNA damage in the parent strain, in a dose-dependent manner, starting at a concentration of 25 μg/mL. Consistently with the results from the viability assay, P10.EE caused no significant DNA damage in the mutant strain at the concentrations tested (10, 25, 50, 100 and 200 μg/mL). These results suggest that the DNA damage caused by P10.EE could originate from apoptosis – since the mutant strain is not able to start an apoptotic process through this pathway, there is no visible DNA damage.

Contrastingly to the previous approach, Alves (2018) assessed the yeast nucleo-cytosolic release of the Nhp6A protein, which is a necrotic cell death hallmark, by fusing green fluorescent protein (GFP) to the target protein and observing its cellular localization through fluorescence microscopy – the author observed that P10.EE, at the concentration of 500 μg/mL, was able to trigger the translocation of this protein from the nucleus to the cytoplasm, suggesting that P10.EE cytotoxicity could be involved in a necrotic cell death pathway. To complement these results, Alves (2018) assessed the effect of P10.EE on the yeast plasma membrane integrity, with PI staining, observing that a high percentage of the cells treated with 500 μg/mL of P10.EE presented loss of plasma membrane integrity, hence corroborating the previous results.

The combination of these results is extremely intriguing, raising more questions than answers. Could P10.EE be involved in apoptosis, necrosis, or even both? And what could be causing this propolis extract activity? The aim of this chapter is to investigate further into P10.EE mechanism of action, using the biological model *S. cerevisiae*, with the intention of reaching closer to the answers for these questions.

2. Materials and methods

2.1. Propolis samples and ethanol extract preparation

The propolis sample used in this work was obtained from the Pereiro apiary located at Beira Alta, Pinhel city, Guarda district (N 40° 44'57.135", W 7° 0'59.403", elevation: 672.6 m) – and was collected in the year 2010 (P10), from *A. melifera* beehives by conventional scraping. An EE was then prepared from this sample, as described for P13.EE in Chapter 2, section 2.1., obtaining P10.EE (Valença, 2011).

2.2. *Saccharomyces cerevisiae* strains and culture growth

The effects of P10.EE were evaluated using the *Saccharomyces cerevisiae* BY4741 strain (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), obtained from Euroscarf. Cultures of the selected *S. cerevisiae* strain were prepared as described in Chapter 2, section 2.2..

2.3. Evaluation of cellular viability

Evaluation of cellular viability was performed with P10.EE as described in Chapter 2, section 2.3. for P13.EE.

2.4. Evaluation of apoptotic characteristics with 4',6-diamidino-2-phenylindole staining

A *S. cerevisiae* BY4741 YPD-culture was prepared as described in Chapter 2, section 2.2. and, after OD_{600} confirmation, aliquots of the cell suspension were placed in microtubes. P10.EE was added to one of the microtubes, at the final concentration of 500 μg/mL. A negative control was prepared with EtOH (same volume as for the P.EE) and an apoptosis and a necrosis control were prepared with 10 and 180 mM H_2O_2, respectively. The cells were then incubated at 30 °C and 200 rpm for 90 min and at time-points 0, 30, 60, and 90 min, aliquots of 10 μL of each sample were placed on a microscope slide and the cells were heat fixed. A drop of 2 μg/mL 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) was added to the fixed cells, the coverslip was placed on top, and the slides were incubated at room temperature for 5 min in the dark. The cells were observed using a Leica DM-5000B fluorescence microscope (Leica Microsystems), with the blue channel filter and a 100x oil-immersion objective. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

2.5. Evaluation of plasma membrane integrity with propidium iodide staining

Aliquots of a *S. cerevisiae* BY4741 YPD-culture prepared as described above and with OD_{600} = 0.4 were placed in microtubes and P10.EE was added to one of the microtubes, at the final concentration of 500 μg/mL. A negative control as well as apoptosis and necrosis controls were prepared as described (section 2.4. of the present chapter). The cells were then incubated at 30 °C and 200 rpm for 90 min and at time points 0, 30, 60, and 90 min, aliquots of 100 μL of each sample were stained with propidium iodide (PI; Sigma-Aldrich) at a final concentration of 2 μg/mL and incubated at room temperature for 10 min in the dark. The cells were observed using a Leica DM-5000B fluorescence microscope (Leica Microsystems), with the red channel filter and a 100x oil-immersion objective. Images were acquired with a Leica DCF350FX digital camera and processed with LAS AF Leica Microsystems software.

2.6. Analysis of cell cycle progression

A *S. cerevisiae* BY4741 YPD-culture was prepared as described and, after OD_{600} confirmation, the yeast cell cycle was synchronized with the addition of hydroxyurea (HU; Sigma-Aldrich) at a final concentration of 200 mM, causing an arrest in early S phase (Rosebrock, 2017b). The cells were then incubated at 30 °C and 200 rpm for 2.5 h. To release the cells from the arrest, the culture was centrifuged at 4 °C and 4,400 *g*, for 3 min (Eppendorf Centrifuge 5804 R), the pellet washed two times with ultra-pure H_2O (upH_2O) and resuspended in fresh YPD medium. Aliquots of 500 μL were collected before and after synchronization, for controls.

The synchronized cell suspension was divided into Falcon® tubes and P10.EE was added to one of the tubes, at the final concentration of 200 μg/mL. A negative control was prepared with EtOH (same volume as for the P.EE). The cells were then incubated at 30 °C and 200 rpm for 180 min and at time points 0, 30, 60, 90, 120, and 180 min, aliquots of 500 μL were collected and centrifuged at 2,500 *g* for 4 min (Eppendorf 5453 MiniSpin Plus Centifuge). The supernatant was discarded and the pelleted cells were fixed with 70 % EtOH (added drop by drop, with frequent vortexing) and incubated at -20 °C overnight (Rosebrock, 2017a).

After fixation, the samples were centrifuged at 10,000 *g* for 20 min (Eppendorf 5453 MiniSpin Plus Centifuge), and the pellets were resuspended in 500 μL of 50 mM sodium citrate buffer (50 mM Trisodium citrate dihydrate (PanReac AppliChem; pH 7.2) and incubated for 10 min at room temperature. The cells were centrifuged at 5,000 *g* for 5 min (Eppendorf 5453 MiniSpin Plus Centifuge), the supernatant discarded, and the washing was repeated. The resulting pellet was then resuspended in in

500 µL of 50 mM sodium citrate buffer (pH 7.2) with 20 µg/mL RNase A (PanReac AppliChem) and 2.5 µM SYTOX™ Green Nucleic Acid Stain (Invitrogen) and the cells were incubated at 37 °C for 60 min. Afterwards, 10 µL of 20 mg/mL proteinase K (Alfa Aesar) was added to the samples and incubated at 55 °C for 60 min followed by incubation at 4 °C overnight (Rosebrock, 2017a). Finally, the samples were sonicated in an ultrasonic bath, for 30 s at the highest intensity, and then placed on a flat bottom 96-well plate and analyzed in a flow cytometer (Beckman Coulter CytoFLEX V0-B4-R2), using a blue laser for excitation at 488 nm and the FITC filter. Data analysis was done with the program CytExpert 2.5.

To confirm that the cells used for the cell cycle progression analysis were alive, a viability assay was conducted. At each time point, aliquots of 100 µL were collected and serially diluted from 10^{-1} to 10^{-4} in sterilized distilled H_2O (dH_2O) and 3 drops of 40 µL from the 10^{-4} dilution were placed on YPDA medium plates. After incubation at 30 °C for 48 h, in the dark, the colonies were counted to assay the viability of the culture over time. Viability of each time-point was calculated as the percentage of colony-forming units (CFU), taking time 0 min as 100 %.

2.7. Statistical analysis

Unless otherwise stated, the experiments were done in at least three independent replicas, and the results are either presented as one representative experiment or as the mean ± SD. For all of the statistical tests, a 95 % confidence interval was assumed. Data was analyzed with Two-way ANOVA, followed by *post hoc* tests for multiple comparisons (Tukey or Sidak). The significance of differences between the mean values is represented by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Data was analyzed and graphs were created using GraphPad Prism 8.0.

3. Results and discussion

3.1. Evaluation of cellular viability – P10.EE maintains its cytotoxicity against *Saccharomyces cerevisiae*

As mentioned in this chapter, besides exhibiting low antioxidant activity, P10.EE stands out for its cytotoxic activity, when compared to EEs of propolis samples from the same apiary but from different years. Marques (2015) assessed the cytotoxic effect of this extract through viability assays, treating a YPD-culture of *S. cerevisiae* BY4741 cells with P.EE, collecting aliquots after 0, 30, 60 and 90 min of treatment and plating 10^{-1} to 10^{-4} dilutions of each aliquot on YPDA plates. This sample was collected in 2010, and its EE has been stored in a Falcon® tube at -20 °C since it was prepared, in 2011. In order to confirm if P10.EE maintained its effect on the viability of *S. cerevisiae*, after a prolonged storage, this assay was replicated in the present study (Figure 9). A cytotoxic effect of P10.EE can be observed by a decrease in number of colonies when compared to the C- (cells treated with absolute EtOH at the same volume as for the P10.EE), which is very clear at 60 and 90 min incubation with 500 μg/mL and at 30, 60 and 90 min with 750 μg/mL.

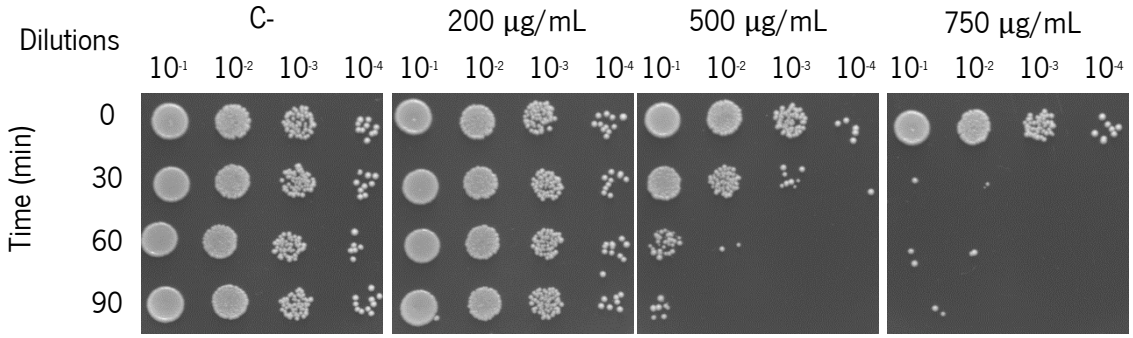

Figure 9. Effects of P10.EE on the viability of *S. cerevisiae*. Cells from the *S. cerevisiae* BY4741 strain were treated with P10.EE at the concentrations of 200, 500, or 750 μg/mL. For the negative control (C-) cells were treated with absolute EtOH, at the same volume as for the P10.EE. Aliquots were collected after 0, 30, 60, and 90 min, and serially diluted from 10^{-1} to 10^{-4}, and 5 μL drops of each dilution were placed on YPDA plates and incubated at 30 °C for 48 h. Cytotoxicity is observed by a decrease in number of colonies when compared to C-.

As observed in Figure 9, P10.EE shows a strong cytotoxic effect at the concentrations of 500 and 750 μg/mL, visibly decreasing the viability of *S. cerevisiae* after 60 min of incubation for the concentration of 500 μg/mL and after 30 min for 750 μg/mL. These results are similar to the ones observed by Marques (2015), confirming that P10.EE was able to maintain its activity over the many years of storage at -20 °C.

3.2. Evaluation of apoptotic characteristics with 4',6-diamidino-2-phenylindole staining – P10.EE does not seem to induce apoptosis in *Saccharomyces cerevisiae*

Previous results from Marques (2015) and Alves (2018) suggested that the cytotoxic activity exhibited by P10.EE is involved in a cell death inducing mechanism, but they were not conclusive on the type of cell death, as viability and DNA damage assays with mutants treated with the extract suggested apoptotic cell death (Marques, 2015) and the nucleo-cytosolic release of the Nhp6A protein on cells treated with P10.EE, observed by Alves (2018), suggested necrotic cell death.

Yeast apoptotic characteristics, such as chromatin condensation and fragmentation, can be observed through fluorescence microscopy with the use of DNA fluorescent dyes, like DAPI (Carmona-Gutierrez at al., 2018). DAPI is a blue fluorescent dye that binds to A-T rich regions in DNA, forming a fluorescent complex. However, DAPI does not pass efficiently through the cell membrane, therefore cells need to be fixed before staining (Wloch-Salamon & Bem, 2013). In order to analyze the effect of P10.EE on yeast cell nuclear morphology, *S. cerevisiae* BY4741 cells were treated with P10.EE at the concentration of 500 μg/mL and incubated for 90 min. Aliquots were collected at 0, 30, 60 and 90 min, and the cells were heat-fixed and stained with DAPI. For this assay, three controls were prepared: the negative control, with cells treated with absolute EtOH at the same volume as for the P10.EE; the apoptosis control, with cells treated with H_2O_2 at 10 mM (Ribeiro *et al.*, 2006); and the necrosis control, with cells treated with H_2O_2 at 180 mM (Madeo *et al.*, 1999). For each time-point and treatment, a total of 100 cells were randomly counted, and out of those, cells presenting chromatin condensation and/or fragmentation were counted. Graphical representation of these cells is shown in Figure 10.

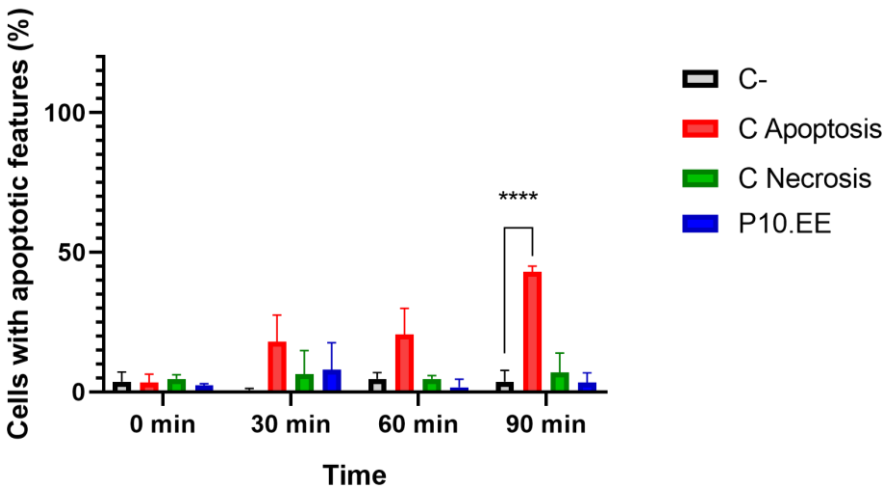

Figure 10. **P10.EE-treated *S. cerevisiae* cells do not present apoptotic features**. Percentage of *S. cerevisiae* BY4741 cells presenting apoptotic features, such as chromatin condensation and/or fragmentation, after 0, 30, 60, and 90 min of incubation with 500 μg/mL P10.EE. At each time-point, aliquots were collected and the cells were heat-fixed and stained with DAPI. For the negative control (C-) cells were treated with absolute EtOH, at the same volume as for the P10.EE, for the apoptosis control (C Apoptosis) cells were treated with H_2O_2 at 10 mM, and for the necrosis control (C Necrosis) cells were treated with H_2O_2 at 180 mM. These values represent the mean ± SD of n = 3 independent replicates. Two-way ANOVA was performed, followed by Tukey's test for multiple comparisons. Asterisks represent significant differences between treatments and C- (**** $p < 0.0001$).

As expected, the necrosis control showed no significant differences to the C- for the percentage of cells presenting apoptotic features, at all the tested time-points, contrarily to what was observed for the apoptosis control, that presented 43.00 ± 2.00 % of cells with apoptotic features at 90 min of treatment, a value significantly higher when compared to the C- at the same time-point. Similarly to the C- and the necrosis control, cells treated with P10.EE did not exhibit apoptotic features at any of the tested time-points, showing no significant differences to the C- at all of the time-points. . Images of a representative experiment obtained from bright filed (BF) and fluorescence microscopy of the yeast cells at 90 min of treatment can be observed in Figure 11, where chromatin condensation and fragmentation are clearly visible. Images of all the treatments from the time-points 0, 30, and 60 min of this representative experiment can be observed in Figures A2-4 (Appendix 2). These results suggest that P10.EE is not inducing cell death in *S. cerevisiae* through an apoptotic mechanism.

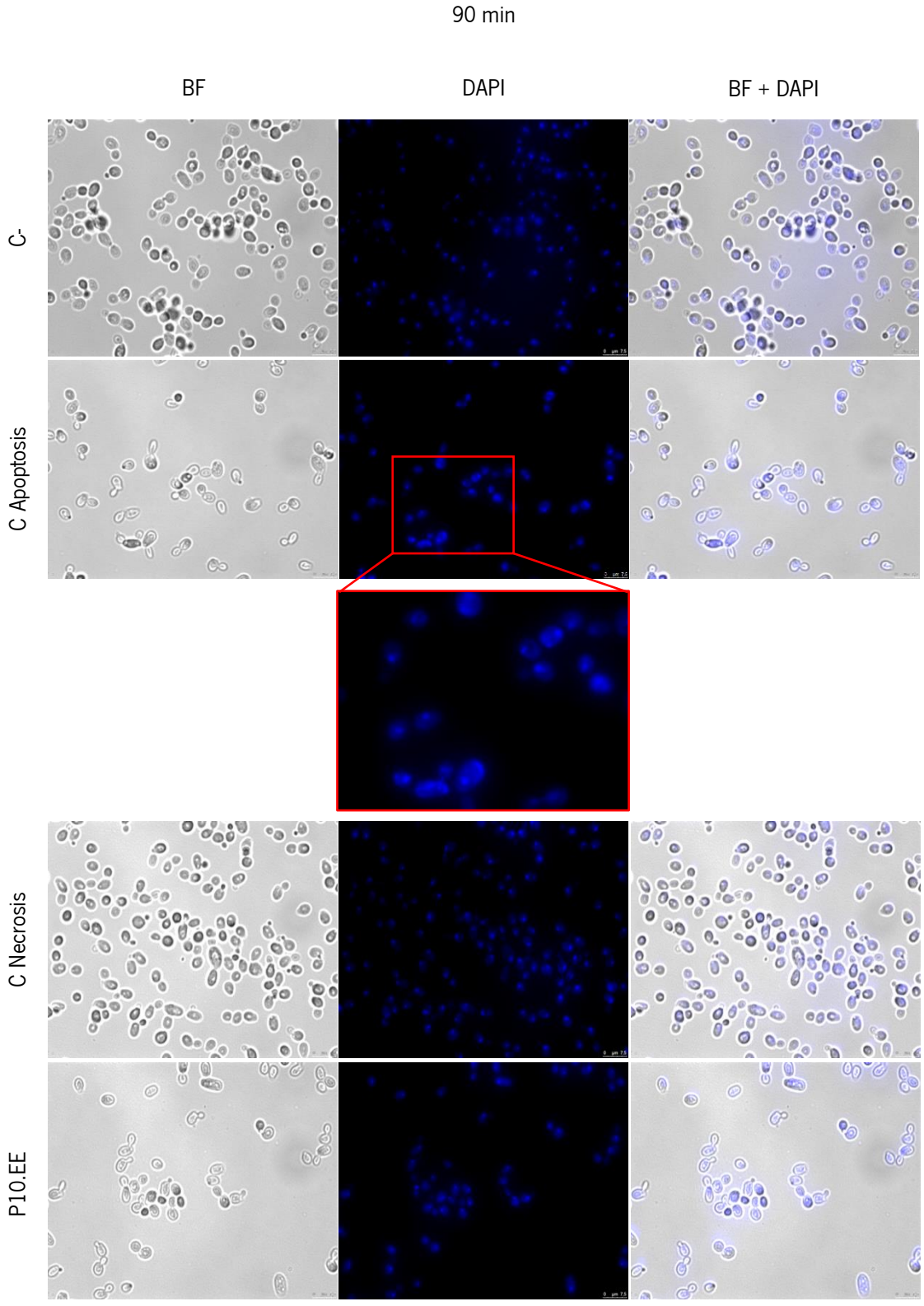

Figure 11. Analysis of chromatin condensation and nuclei fragmentation through fluorescence microscopy. *S. cerevisiae* BY4741 cells treated with absolute EtOH (C-), 10 mM H_2O_2 (C Apoptosis), 180 mM H_2O_2 (C Necrosis) and 500 µg/mL P10.EE, after 90 min of incubation, were heat-fixed and stained with DAPI. Images were obtained through BF and fluorescence microscopy (magnification of 100x) and are from one representative experiment, out of three independent replicates.

3.3. Evaluation of plasma membrane integrity with propidium iodide staining – P10.EE leads to loss of plasma membrane integrity in *Saccharomyces cerevisiae*

Since P10.EE does not appear to be causing apoptotic cell death in *S. cerevisiae*, its effect in the cell membrane integrity was assessed, as loss in membrane integrity is a marker associated with necrotic cell death. For this assay, cells were stained with PI and analyzed through fluorescence microscopy, as PI is a red fluorescent dye that binds to DNA and it is commonly used to detect dead cells, because it is cell-impermeant and can only enter the cell when the plasma membrane has lost its integrity (Büttner *et al.*, 2007). *Saccharomyces cerevisiae* BY4741 cells were treated with P10.EE at the concentration of 500 µg/mL and incubated for 90 min. Aliquots were collected at 0, 30, 60 and 90 min, and the cells were stained with PI. Negative, apoptosis and necrosis control treatments were as described for the previous assay. For each time-point and treatment, a total of 100 cells were randomly counted, and out of those, cells emitting red fluorescence (PI positive cells) were counted. Graphical representation of these cells is shown in Figure 12.

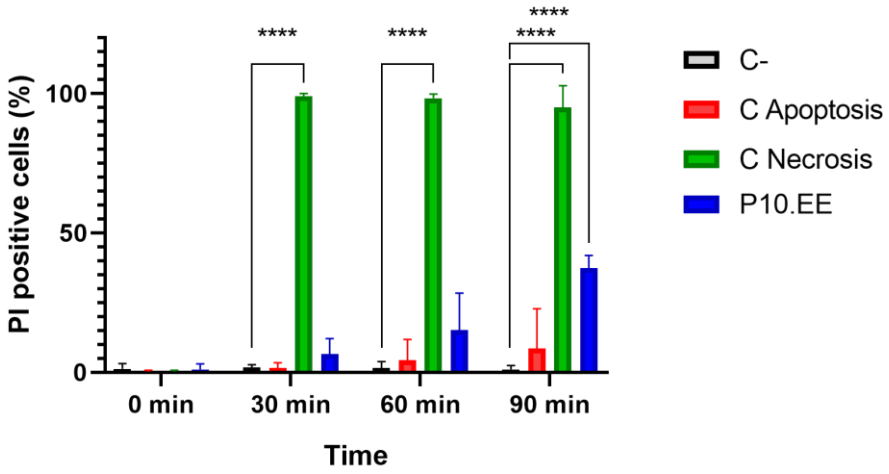

Figure 12. **P10.EE-treated *S. cerevisiae* cells exhibit loss of membrane integrity**. Percentage of *S. cerevisiae* BY4741 PI positive cells, after 0, 30, 60, and 90 min of incubation with 500 µg/mL P10.EE. At each time-point, aliquots were collected and the cells were stained with PI. For the negative control (C-) cells were treated with absolute EtOH, at the same volume as for the P10.EE, for the apoptosis control (C Apoptosis) cells were treated with 10 mM H_2O_2, and for the necrosis control (C Necrosis) cells were treated with 180 mM H_2O_2. These values represent the mean ± SD of n = 4 independent replicates. Two-way ANOVA was performed, followed by Tukey's test for multiple comparisons. Asterisks represent significant differences between treatments and C- (**** $p < 0.0001$).

As expected, the apoptosis control showed no significant differences to the C- for the percentage of PI positive cells, at all the tested time-points, contrarily to what was observed for the necrosis control, that presented almost 100 % of PI positive cells at 30, 60, and 90 min of treatment, which are values significantly higher when compared to the C- at the same time-point. Cells treated with P10.EE exhibited

a gradual increase in percentage of PI positive cells over the tested time-points, reaching a percentage of 37.33 ± 4.61 % at 90 min of treatment, which is a value significantly higher when compared to the C- at that time-point. Images of a representative experiment obtained from bright filed (BF) and fluorescence microscopy of the yeast cells at 90 min of treatment can be observed in Figure 13, where PI positive cells are clearly visible in the necrosis control and P10.EE treated cells. Images from the time-points 0, 30, and 60 min of this representative experiment can be observed in Figures A5-7 (Appendix 2). These results, together with the results from the previous assays and the ones reported by Alves (2018), suggest that P10.EE could be inducing cell death in *S. cerevisiae* through a necrotic mechanism. Yeast necrotic cell death induced by propolis has already been observed by Castro *et al.* (2011) in a Brazilian green propolis ethanol extract, that although initially induced apoptosis, an increased exposure to propolis caused an increase in necrotic cells. The author suggested that these effects were partially mediated by *YCA1* metacaspase, as a mutant with a deletion of this gene proved to be more resistant to propolis, which goes accordingly to the results reported by Marques (2015).

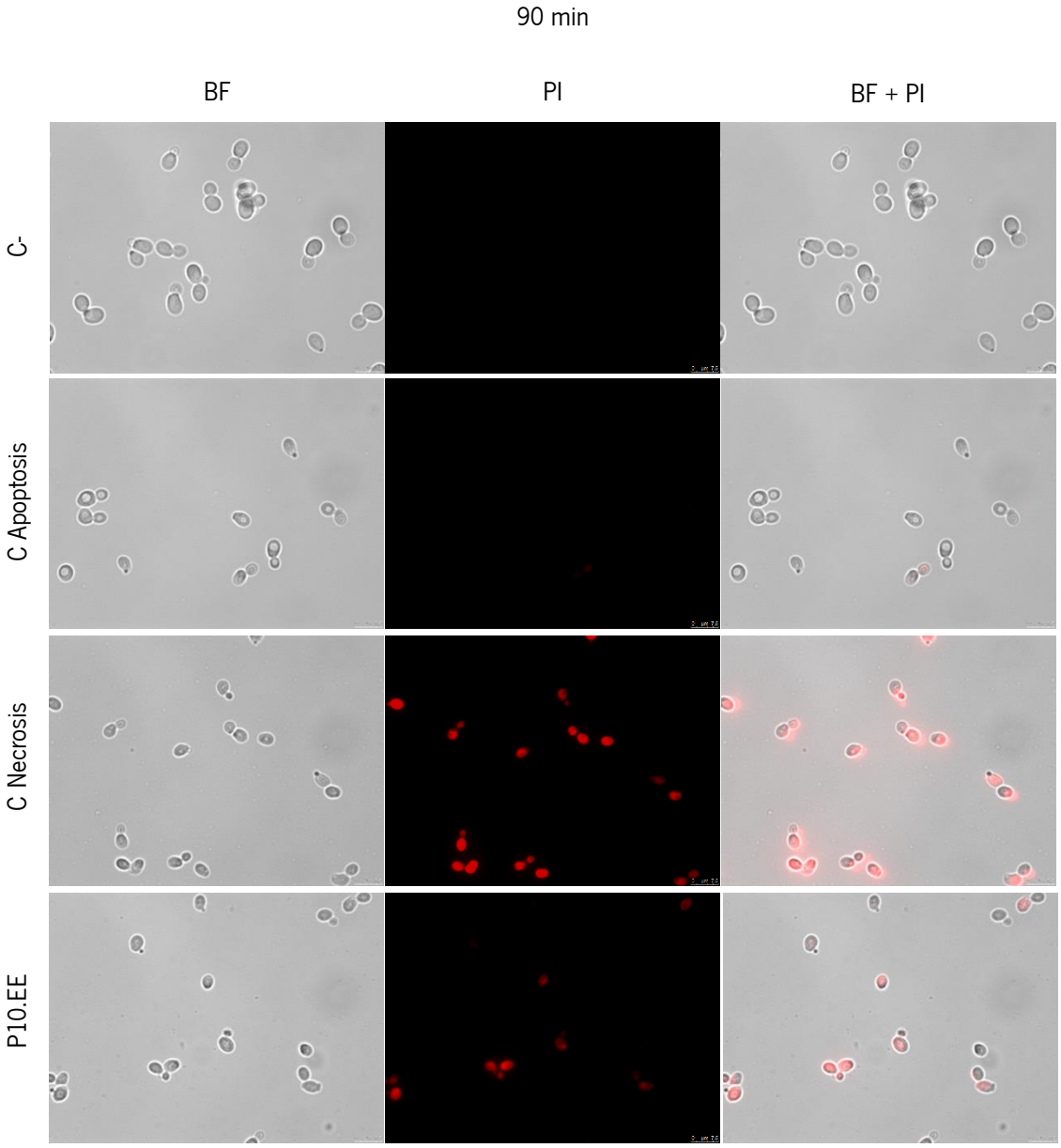

Figure 13. **Analysis of plasma membrane integrity through fluorescence microscopy**. *S. cerevisiae* BY4741 cells treated with absolute EtOH (C-), 10 mM H_2O_2 (C Apoptosis), 180 mM H_2O_2 (C Necrosis) and 500 µg/mL P10.EE, after 90 min of incubation, were stained with PI. Images were obtained through BF and fluorescence microscopy (magnification of 100x) and are from one representative experiment, out of four independent replicates.

3.4. Analysis of cell cycle progression – P10.EE provokes an arrest in the cell cycle of *Saccharomyces cerevisiae*

The yeast cell cycle, like in other eukaryotes, is a tightly controlled process divided into four different phases: G1, where cells prepare for duplication; S, where cells begin to duplicate genetic information; G2, where cells get ready to be divided; and M, where cells are divided into two "daughter"

cells (Jiménez *et al.*, 2015). During the normal progression of the cell cycle, there are various checkpoints that can cause an arrest or a delay in the cell cycle if DNA damage is detected (Demeter *et al.*, 2000). Marques (2015) has previously reported that P10.EE causes significant DNA damage, and to test if this damage affects the progression of the yeast cell cycle, *S. cerevisiae* BY4741 cells treated with P10.EE were analyzed through flow cytometry.

In this assay, the tested concentration of extract was 200 μg/mL, as it does not affect the yeast viability and cells should be viable for a reliable cell cycle analysis. A viability test was conducted alongside every independent replicate of this assay, to confirm the viability of the treated cells (Figure A8, Appendix 2), and showed that cells were alive during the whole assay meaning that an arrest in the cell cycle would not be due to cell death, but instead to repair damage allowing for the cell survival. Firstly, the yeast cell cycle was synchronized with HU at a concentration of 200 mM – HU inhibits ribonucleotide reductase (RNR), causing an arrest in early S phase/G1 phase (Juanes, 2017). After the synchronization, cells were released from the arrest and treated with P10.EE. At different time-points, aliquots were collected and prepared as described for staining with SYTOX™ Green Nucleic Acid Stain (Invitrogen) and flow cytometry analysis. SYTOX™ Green Nucleic Acid Stain (Invitrogen) is a green fluorescent dye that binds to nucleic acids and allows for the identification of cell cycle phases through flow cytometry, as the amount of fluorescence is directly proportional to the amount of DNA and number of cells. For the C-, cells were treated with absolute EtOH at the same volume as for the P10.EE. From the histograms obtained (Figure A9, Appendix 2), and with the CytExpert 2.5 software, three gates were drawn to define the percentage of cells in the phases G1, S and G2/M, based on the amount of fluorescence and number of cells on the unsynchronized cells – a first peak with less fluorescence represents G1 phase, a second peak with higher amount of fluorescence represents G2/M phases, and the cells in between the two peaks represent S phase (Figure 14A). Graphical representation of this distribution for all of the tested time-points is shown in Figure 14B. Percentage of P10.EE cells in a certain cell cycle phase was compared to the percentage of C- cells in the same phase.

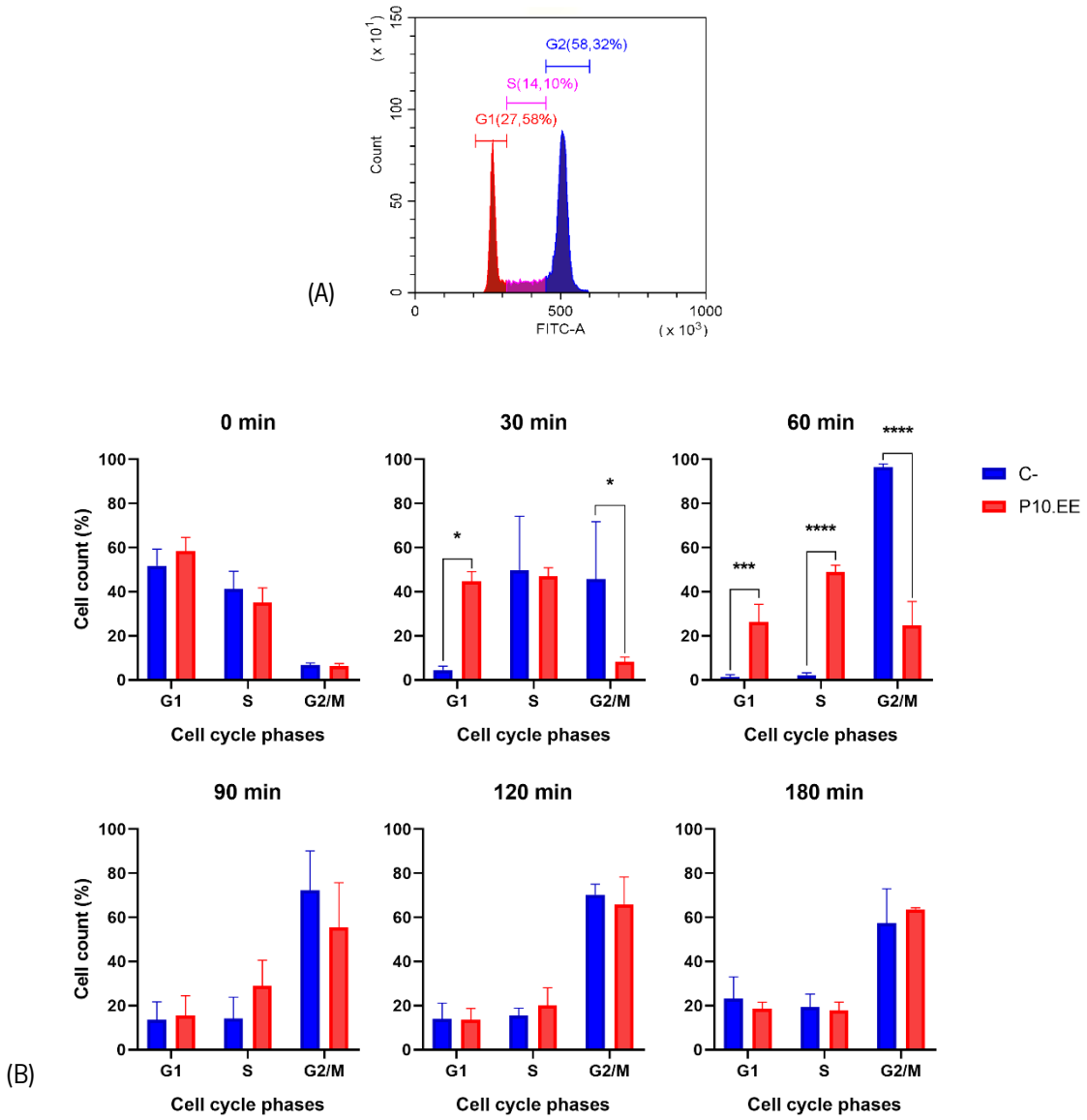

Figure 14. **P10.EE affects *S. cerevisiae* cell cycle progression**. (A) Using the CytExpert 2.5 software, three gates were drawn to define the percentage of *S. cerevisiae* BY4741 cells, prior to HU synchronization, in the cell cycle phases G1, S and G2/M, based on the amount of fluorescence and number of cells – a first peak with less fluorescence represented G1 phase, a second peak with higher amount of fluorescence represents G2/M phases, and the cells in between the two peaks represent S phase. The histogram presented is of one representative experiment out of three independent replicates. (B) Percentage of *S. cerevisiae* BY4741 cells in the cell cycle phases G1, S, and G2/M, after 0, 30, 60, 90, 120, and 180 min of treatment with 200 µg/mL P10.EE. Aliquots were collected at each time-point and cells were prepared for flow cytometry analysis and stained with SYTOX™ Green Nucleic Acid Stain (Invitrogen). For the negative control (C-) cells were treated with absolute EtOH, at the same volume as for the P10.EE. These values represent the mean ± SD of n = 3 independent replicates. Two-way ANOVA was performed, followed by Sidak's test for multiple comparisons. Asterisks represent significant differences between treatments and C- (* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$).

At the 0 min time-point, both the C- and the P10.EE treated cells presented a similar distribution over G1 and S phases, which was expected, as cells had just been released from the cell cycle arrest caused by HU – the results at this time-point are identical to the ones observed for the synchronization control (Figure A10, Appendix 2). After 30 min of treatment, for the C-, it is clear that the cell cycle has

progressed, presenting a similar distribution of cells in S and G2/M phases, while for the P10.EE-treated cells, the cell cycle distribution is very similar to the observed at 0 min, presenting a significantly higher percentage of cells in G1 phase and a significantly lower percentage of cells in G2/M phase, when compared to the C-. After 60 min of treatment, for the negative control, almost all the cells have progressed into G2/M phase, with a percentage of 96.41 ± 1.48 %, while for the P10.EE treated cells, the majority of the cells remained in G1 (26.36 ± 7.91 %) and S (48.98 ± 3.05 %) phases, and only 24.65 ± 10.88 % of the cells have progressed into G2/M phase, all of these values presenting significant differences when compared to the C-. After 90, 120, and 180 min, P10.EE treated cells started to progress in the cell cycle, not showing any significant differences when compared to the C- cells.

These results suggest that P10.EE causes an arrest in G1/S phases of the yeast cell cycle, which is probably related to the DNA damage it has been reported to cause (Marques, 2015). However, since the cell cycle was able to progress and reestablish itself after 90 min of incubation, the damage caused by the extract, at 200 μg/mL, seems to be reversible. These results go accordingly with the results from the viability assays for this concentration (Figures 9 & A8, Appendix 2), as it did not affect the yeast viability.

4. Conclusions

Natural products, due to their chemical diversity and abundance of bioactive compounds with therapeutic potentials, have been of major importance in cancer therapy research (Huang *et al.*, 2021). Propolis is one of these natural products, as several types of propolis have been shown to exhibit anticancer properties, and it has been under research for its potential use as a chemotherapeutic and chemopreventive product for the past years (Chiu *et al.*, 2020; Forma & Bryś, 2021).

From the work presented in this chapter, it can be concluded that P10.EE was able to maintain its cytotoxic activity (Figure 9), after being stored in a Falcon® tube at -20 °C since 2015, when Marques (2015) first tested its effect on *S. cerevisiae* viability, meaning that this method of storage does not affect the extract's activity. The fluorescence microscopy assays showed that P10.EE does not cause chromatin condensation or fragmentation on *S. cerevisiae* cells (Figure 10), suggesting that it does not induce apoptotic cell death in yeast, however, it was shown to cause the loss of plasma membrane integrity, at the same concentration (Figures 12 and 13), which is a characteristic feature of necrotic cell death in yeast. It was also observed that P10.EE induces a reversible cell cycle arrest in *S. cerevisiae*, in the phases G1/S (Figure 14B). Following the recommendations of Carmona-Gutierrez *et al.* (2018) and Eisenberg *et al.* (2010) to identify the different types of RCD, these results, together with the ones previously reported, suggest that the mechanism of action of P10.EE in yeast comprises a necrotic programmed cell death pathway, as there is loss of plasma membrane integrity (Figures 12 and 13), loss of viability (Figure 9), lack of apoptotic morphological features (Figure 10) and the nucleo-cytosolic release of Nhp6A, as reported by Alves (2018).

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CHAPTER 4. Final remarks and future perspectives

1. Final remarks and future perspectives

Propolis is a natural product produced by honeybees that has been recognized worldwide for its many biological properties, such as antioxidant, cytotoxic, and antimicrobial activities, yet depending on a very diverse chemical composition. Because of this plethora of biological activities, the demand for propolis research for potential pharmaceutical, medical, cosmetic, and food applications has been growing. Despite this increasing demand, Portuguese propolis research has been stagnant for many years, only starting to slowly gain ground recently. However, it is still an undervalued product in Portugal, creating a necessity to further investigate this Portuguese beehive product and its mechanism of action, in order to contribute to its national and international recognition and economic valorization.

The aims of this work were to further study the intriguing differences previously observed in the ethanol extracts of two Portuguese propolis samples from the Pereiro apiary, P10.EE and P13.EE, by Marques (2015), Barroso (2017) and Alves (2018), diving deeper into P13.EE antioxidant activity and P10.EE cytotoxic activity, in an attempt to better understand their distinct mechanisms of action and what are the causes for these differences in samples from the same apiary. The present work was able to successfully confirm these activities and infer on their modes of action, although it is still not clear what the differences in the extracts' chemical compositions are. As it is proposed in this work that such difference is not related to their phenolic content, a gas chromatography-mass spectrometry (GC-MS) analysis could be performed on both P13.EE and P10.EE, allowing for a more complete chemical characterization of the extracts, namely of different types of compounds like terpenes, which have already been associated with propolis antimicrobial (e.g. isocupressic acid, diphenylheptanoids) and anticancer (e.g. clerodane diterpenes) activities (reviewed by Aminimoghadamfarouj & Nematollahi, 2017).

Although the conjunction of the results from this and the previous works allow for a much clearer view on the different extracts' mechanisms of action, other approaches can be followed to further study and clarify these mechanisms. For the P13.EE, an intracellular oxidation assay could be done on an *S. cerevisiae* mutant with a combined deletion of the *YAP1*, *SOD1* and *SOD2* genes, to observe how the P13.EE ability to decrease the yeast intracellular oxidation would be affected. For the P10.EE, an Annexin V/PI flow cytometry assay could be done, in order to confidently confirm the occurrence of necrotic cell death.

Nonetheless, taken all together, the results observed for the assays with P13.EE suggest promising antioxidant properties, that could be further explored for possible pharmaceutical, cosmetic, and food applications. P10.EE cytotoxic activity, possibly through a necrotic pathway, could be of great interest for antitumoral applications or the development of anticancer drugs, as it has already been

reported by Silva-Carvalho *et al.* (2014) to decrease cell viability of the tumor cell lines MDA-MB-231 and DU145 while being less cytotoxic to non-tumoral cells, and especially since apoptosis resistance is becoming a problem during cancer treatment, as the conventional therapeutic pathway is pro-apoptotic (Galluzzi *et al.*, 2011; Gong *et al.*, 2019). Indeed, the development of a non-apoptotic treatment would be of an enormous scientific and medical significance. However, P10.EE effect needs to be further investigated in different cellular models, such as mammalian cell lines and cancer cell lines, to really comprehend how the extract works in more advanced organisms. Furthermore, since P10.EE exhibits cytotoxicity against *S. cerevisiae*, an application in biopesticides could be considered, after further studies on its antifungal activity.

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APPENDIX 1

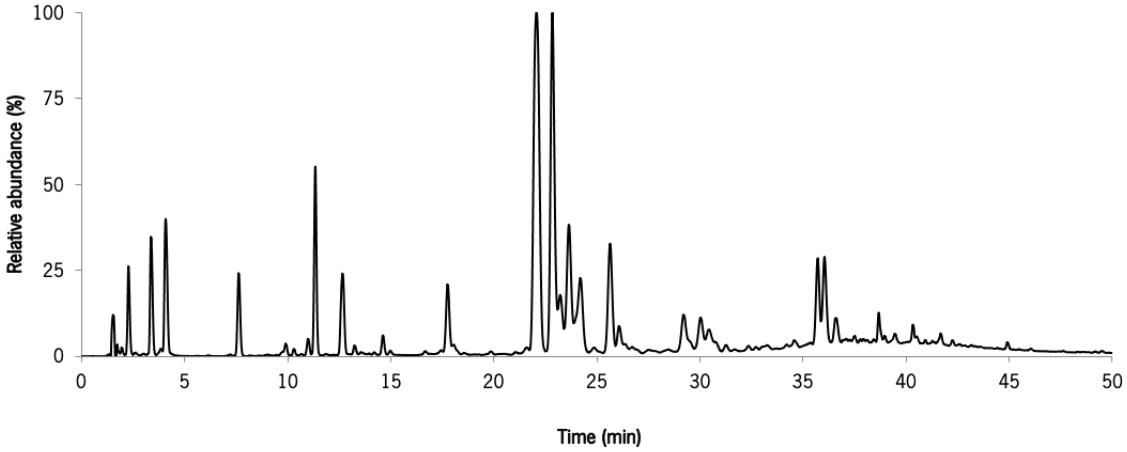


Figure A1. Chromatographic profile of P10.EE at 280 nm obtained by UPLC-DAD-ESI/MS.

APPENDIX 2

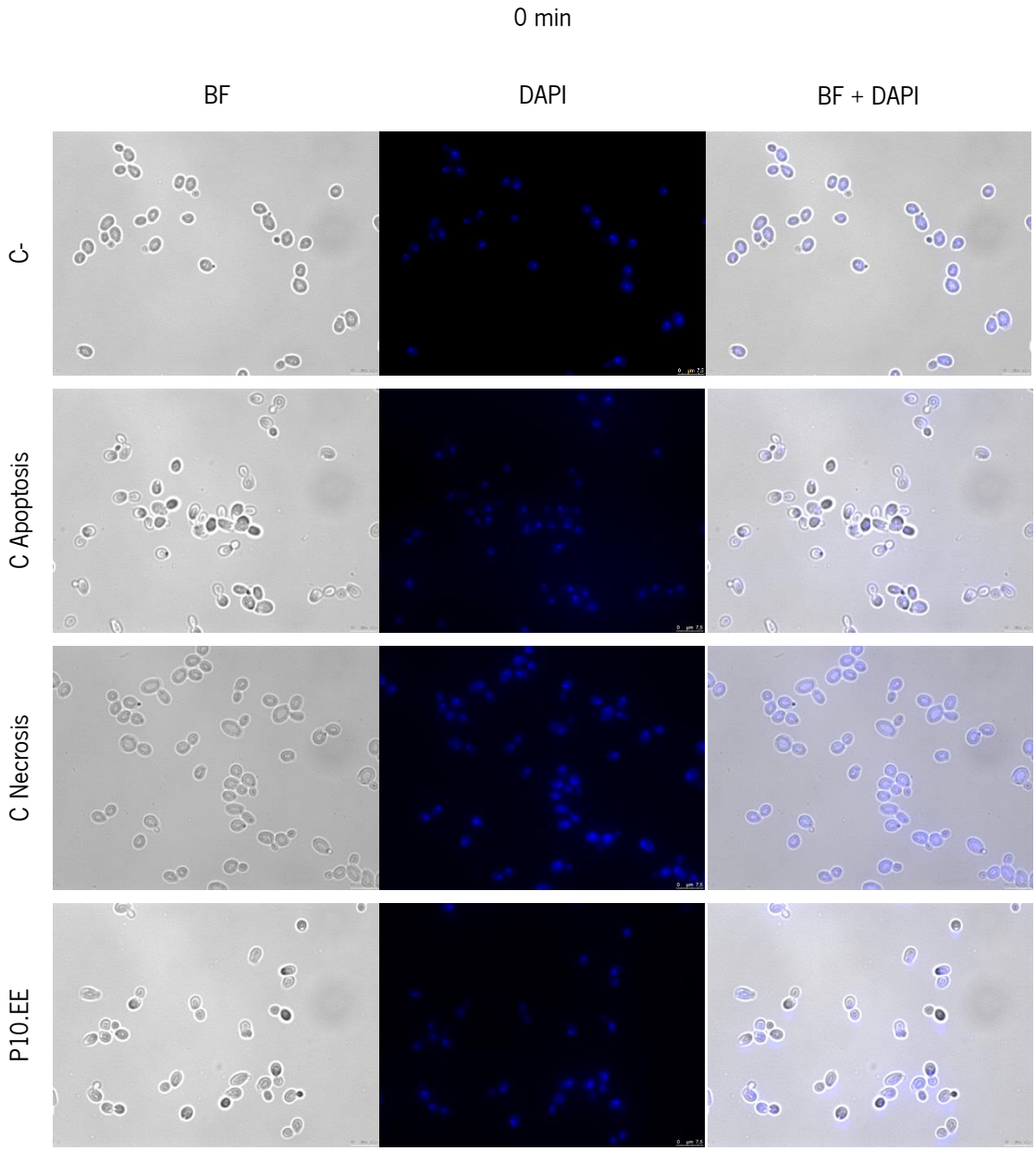


Figure A2. **Analysis of chromatin condensation and nuclei fragmentation through fluorescence microscopy**. *S. cerevisiae* BY4741 cells treated with absolute EtOH (C-), 10 mM H_2O_2 (C Apoptosis), 180 mM H_2O_2 (C Necrosis) and 500 μg/mL P10.EE, after 0 min of incubation, were heat-fixed and stained with DAPI. Images were obtained through BF and fluorescence microscopy (magnification of 100x) and are from one representative experiment, out of three independent replicates.

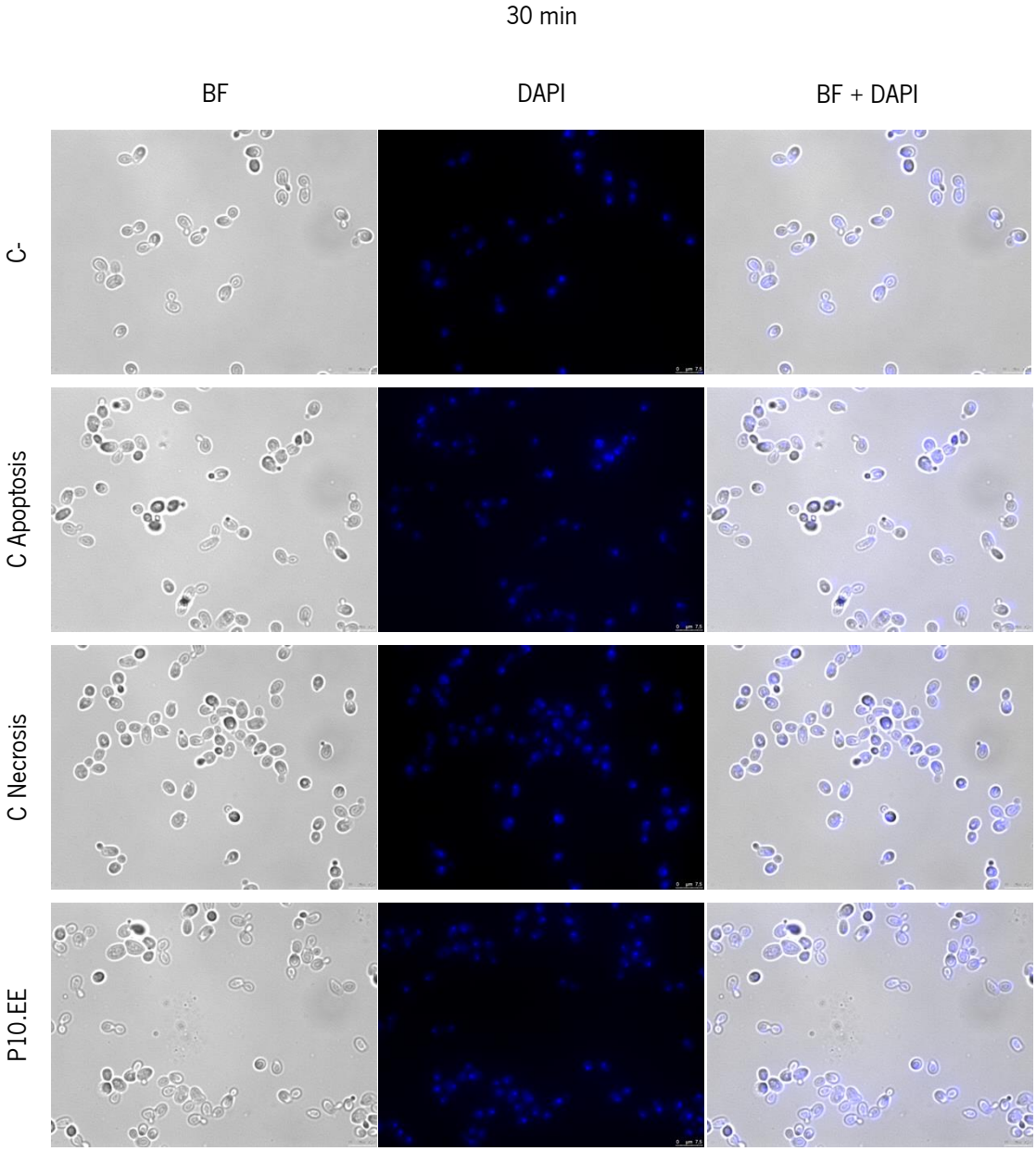


Figure A3. **Analysis of chromatin condensation and nuclei fragmentation through fluorescence microscopy**. *S. cerevisiae* BY4741 cells treated with absolute EtOH (C-), 10 mM H_2O_2 (C Apoptosis), 180 mM H_2O_2 (C Necrosis) and 500 μg/mL P10.EE, after 30 min of incubation, were heat-fixed and stained with DAPI. Images were obtained through BF and fluorescence microscopy (magnification of 100x) and are from one representative experiment, out of three independent replicates.

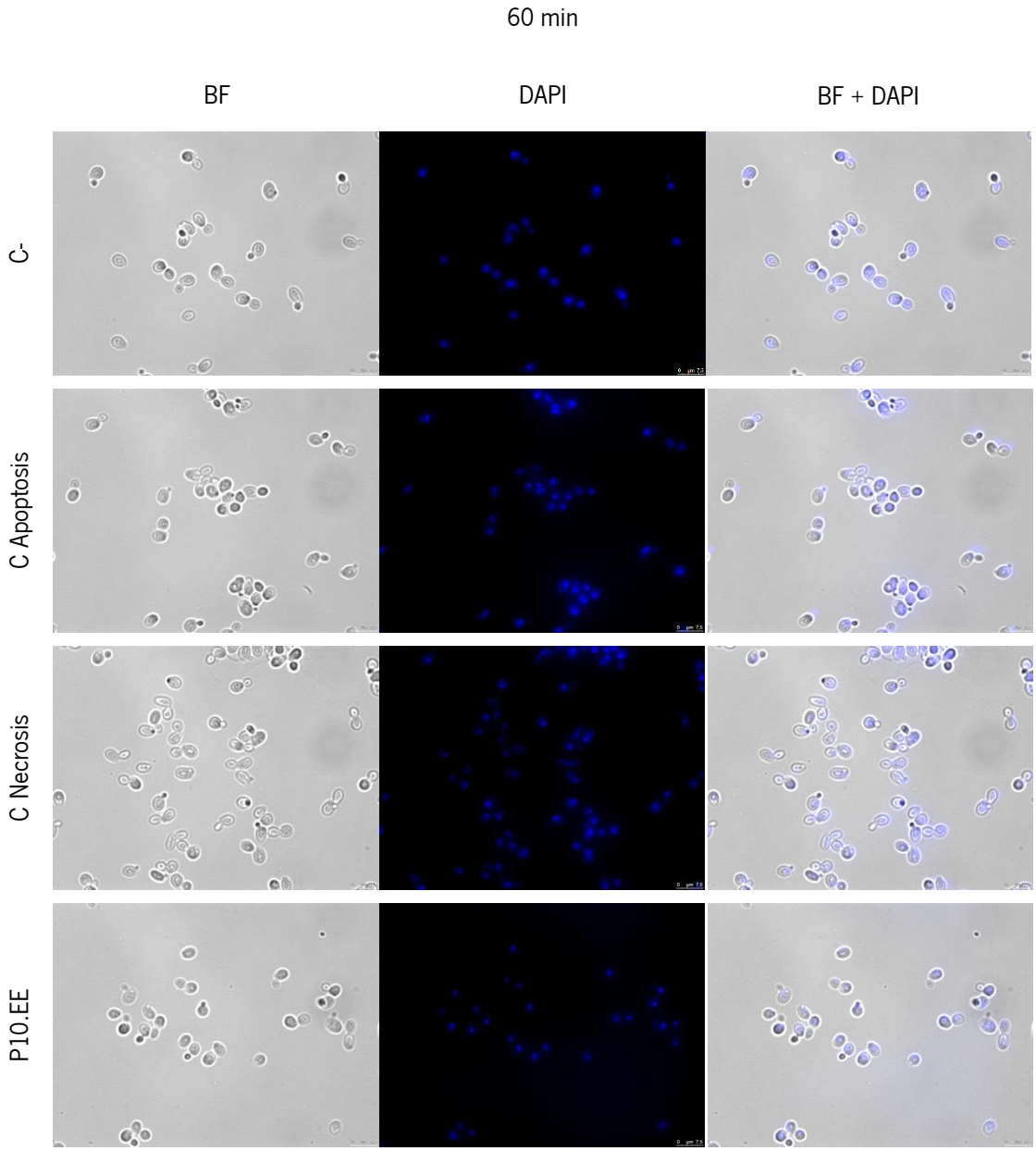


Figure A4. **Analysis of chromatin condensation and nuclei fragmentation through fluorescence microscopy**. *S. cerevisiae* BY4741 cells treated with absolute EtOH (C-), 10 mM H_2O_2 (C Apoptosis), 180 mM H_2O_2 (C Necrosis) and 500 μg/mL P10.EE, after 60 min of incubation, were heat-fixed and stained with DAPI. Images were obtained through BF and fluorescence microscopy (magnification of 100x) and are from one representative experiment, out of three independent replicates.

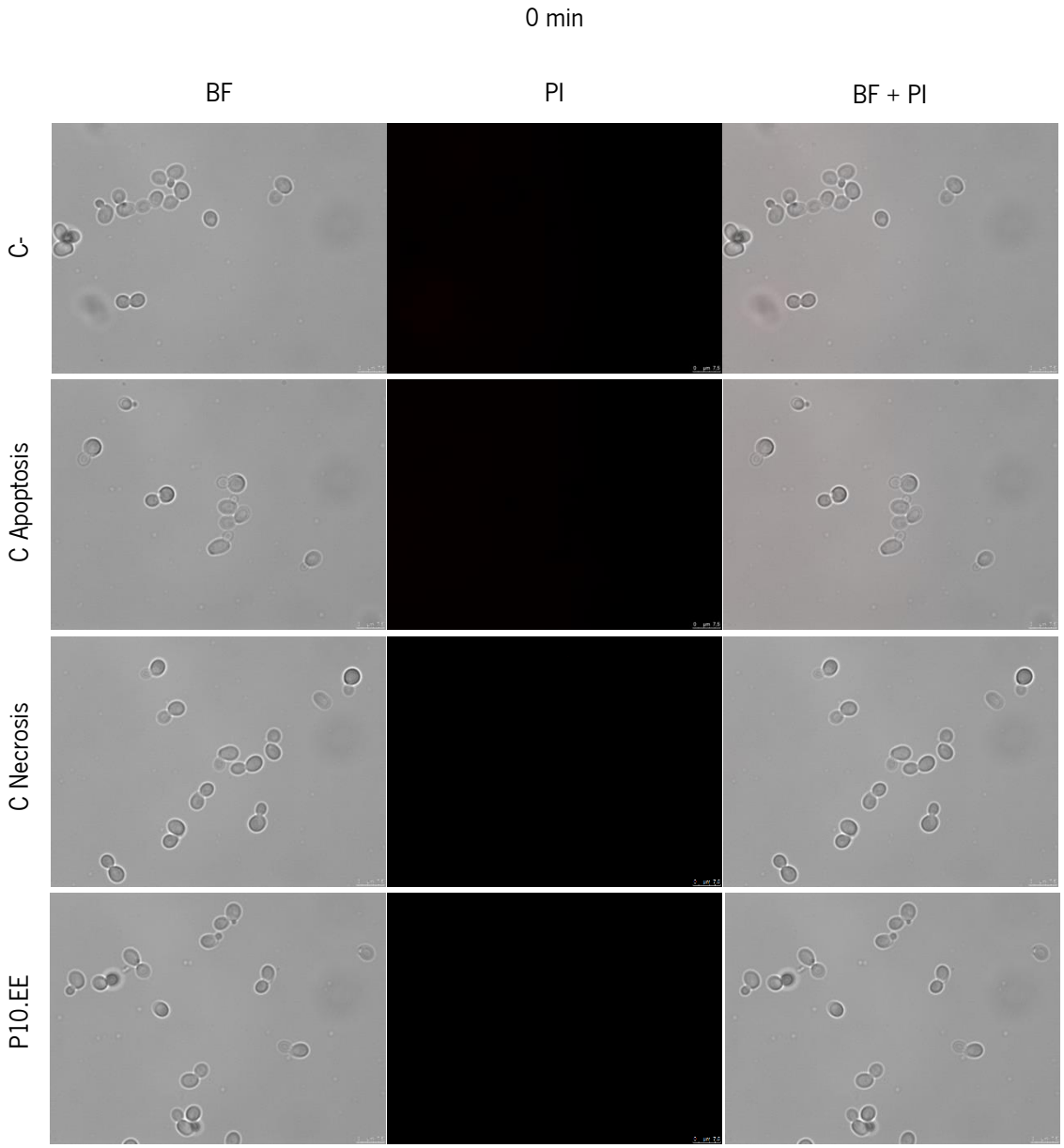


Figure A5. Analysis of plasma membrane integrity through fluorescence microscopy. *S. cerevisiae* BY4741 cells treated with absolute EtOH (C-), 10 mM H_2O_2 (C Apoptosis), 180 mM H_2O_2 (C Necrosis) and 500 µg/mL P10.EE, after 0 min of incubation, were stained with PI. Images were obtained through BF and fluorescence microscopy (magnification of 100x) and are from one representative experiment, out of four independent replicates.

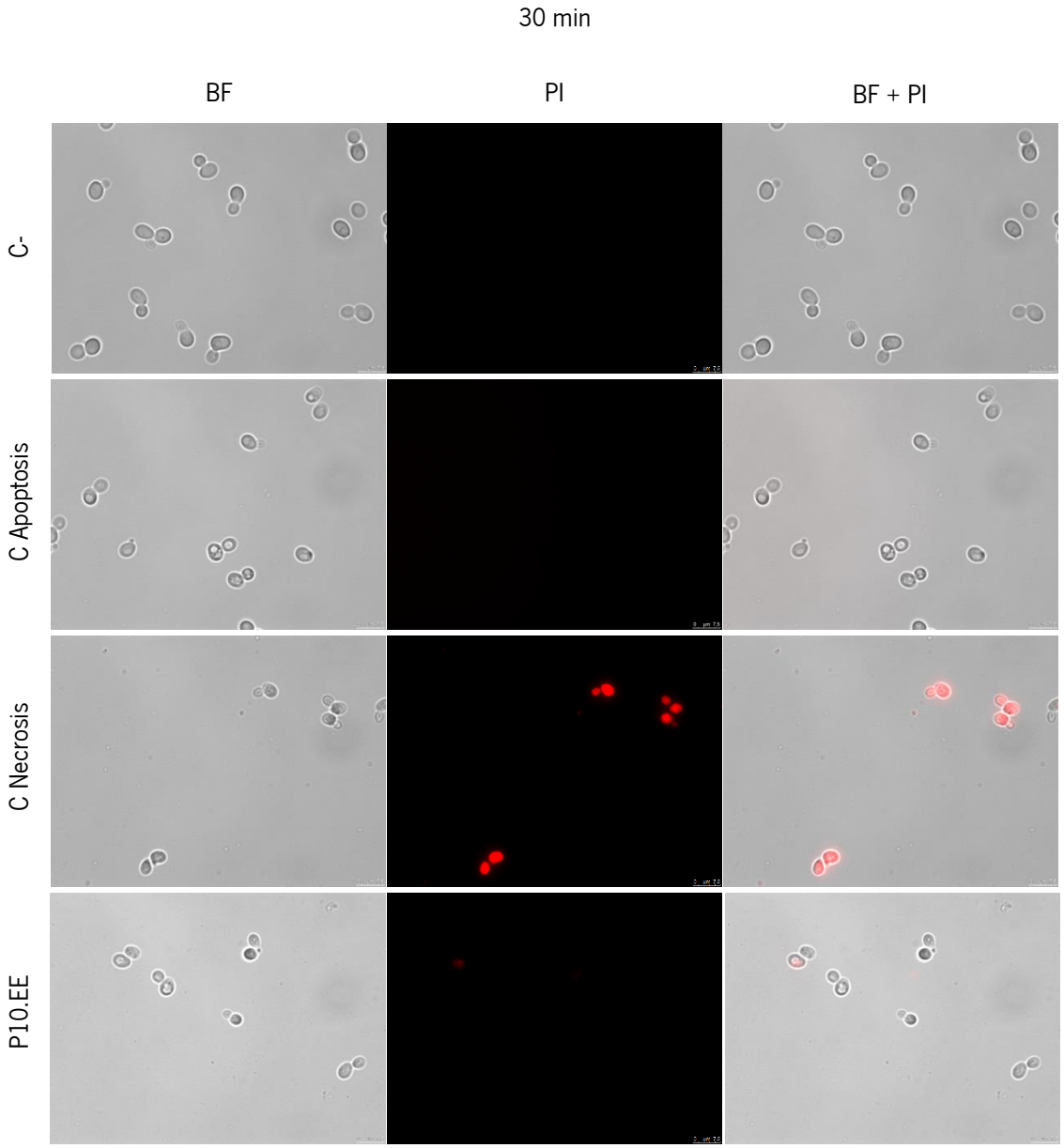


Figure A6. Analysis of plasma membrane integrity through fluorescence microscopy. *S. cerevisiae* BY4741 cells treated with absolute EtOH (C-), 10 mM H_2O_2 (C Apoptosis), 180 mM H_2O_2 (C Necrosis) and 500 µg/mL P10.EE, after 30 min of incubation, were stained with PI. Images were obtained through BF and fluorescence microscopy (magnification of 100x) and are from one representative experiment, out of four independent replicates.

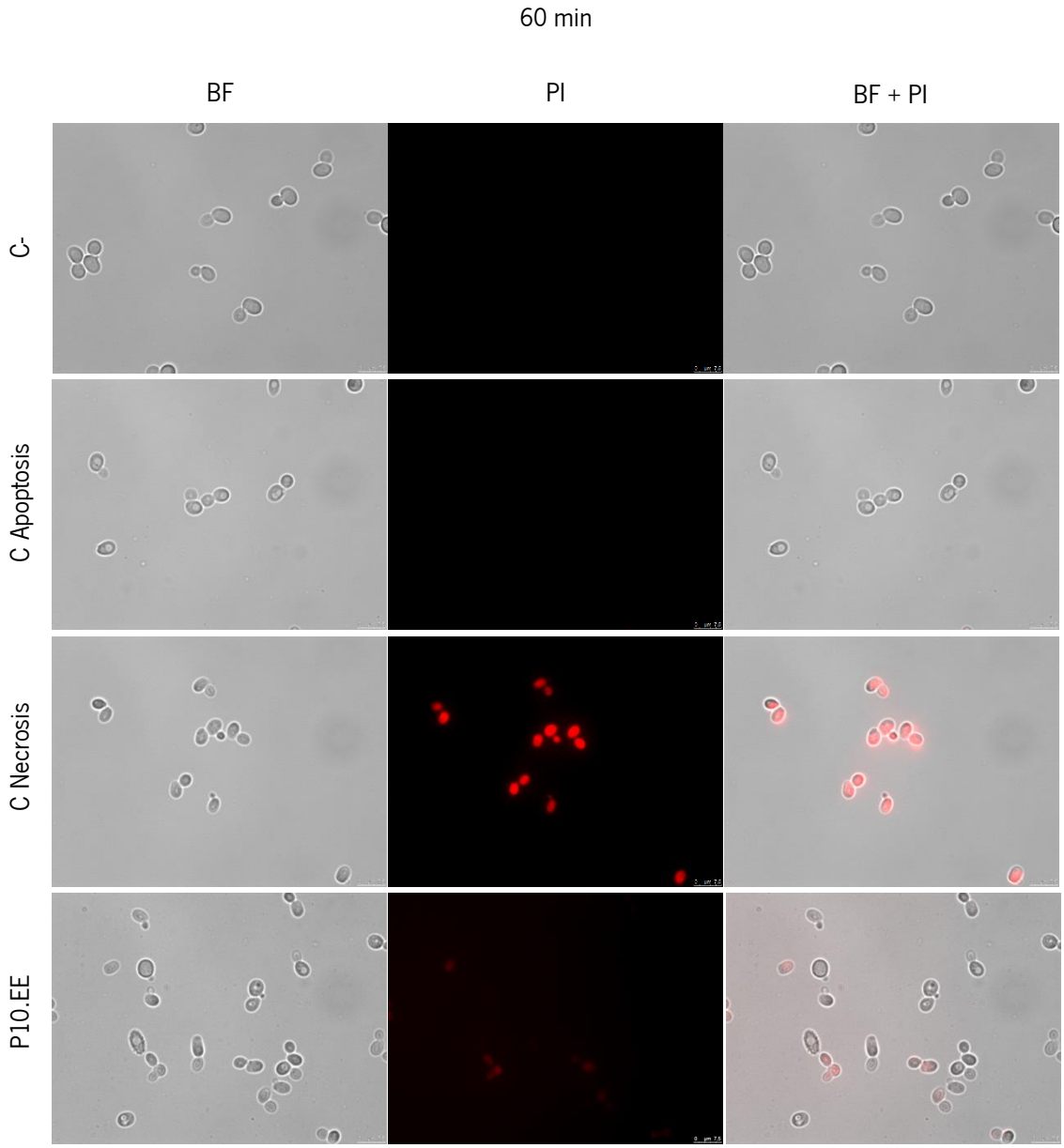


Figure A7. Analysis of plasma membrane integrity through fluorescence microscopy. *S. cerevisiae* BY4741 cells treated with absolute EtOH (C-), 10 mM H_2O_2 (C Apoptosis), 180 mM H_2O_2 (C Necrosis) and 500 µg/mL P10.EE, after 60 min of incubation, were stained with PI. Images were obtained through BF and fluorescence microscopy (magnification of 100x) and are from one representative experiment, out of four independent replicates.

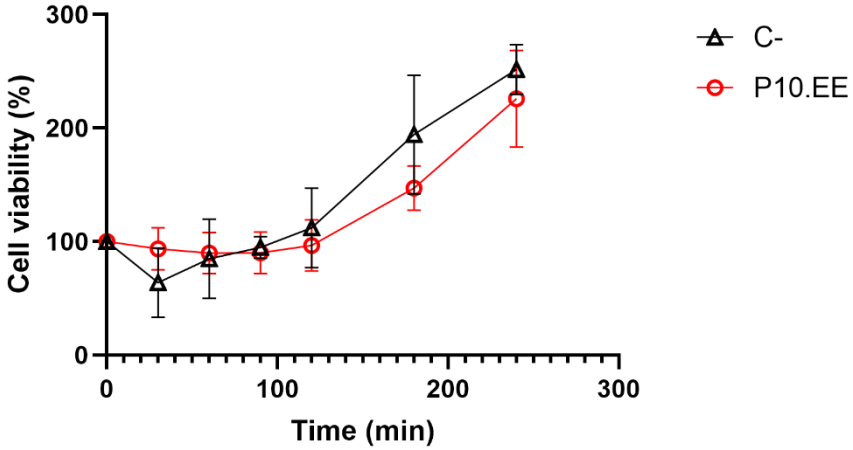
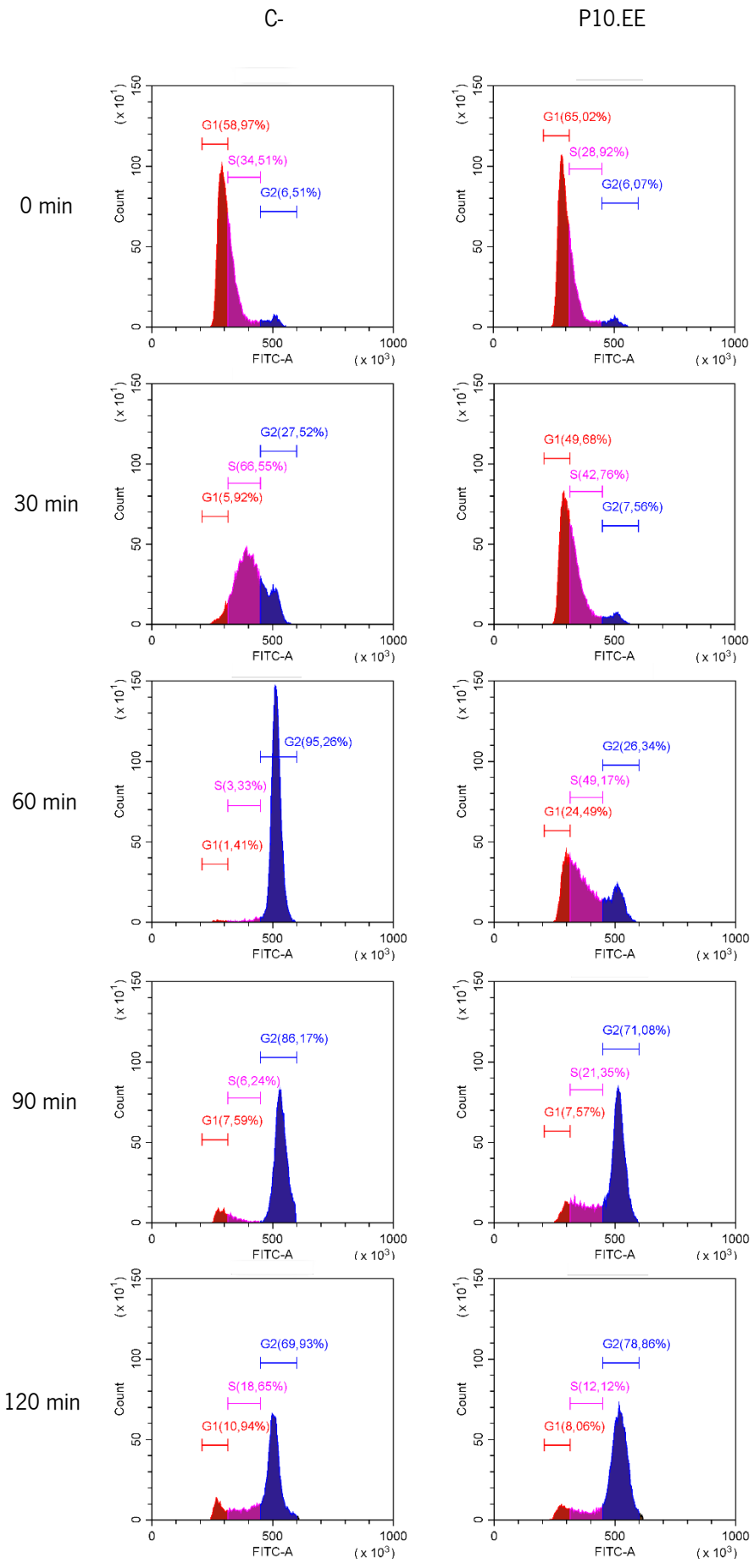


Figure A8. Analysis of P10.EE effect on the viability of *S. cerevisiae* cells. Percentage of *S. cerevisiae* BY4741 cell viability after 0, 30, 60, 90, 120, 180, and 240 min of treatment with 200 µg/mL P10.EE. Aliquots were collected at each timepoint, serially diluted from 10^{-1} to 10^{-4} and three 40 µL drops of the 10^{-4} dilution were placed on YPDA plates and incubated at 30 °C for 48 h. For the negative control (C-) cells were treated with absolute EtOH, at the same volume as for the P10.EE. Viability of each time-point was calculated as the percentage of colony-forming units, taking the 0 min time-point as 100 %. These values represent the mean ± SD of n = 3 independent replicates.


C-
P10.EE
0 min
30 min
60 min
90 min
120 min
G1(58,97%)
S(34,51%)
G2(6,51%)
G1(65,02%)
S(28,92%)
G2(6,07%)
G2(27,52%)
S(66,55%)
G1(5,92%)
G1(49,68%)
S(42,76%)
G2(7,56%)
G2(95,26%)
S(3,33%)
G1(1,41%)
G2(26,34%)
S(49,17%)
G1(24,49%)
G2(86,17%)
S(6,24%)
G1(7,59%)
G2(71,08%)
S(21,35%)
G1(7,57%)
G2(69,93%)
S(18,65%)
G1(10,94%)
G2(78,86%)
S(12,12%)
G1(8,06%)
Count
FITC-A
(x 10^1)
(x 10^3)

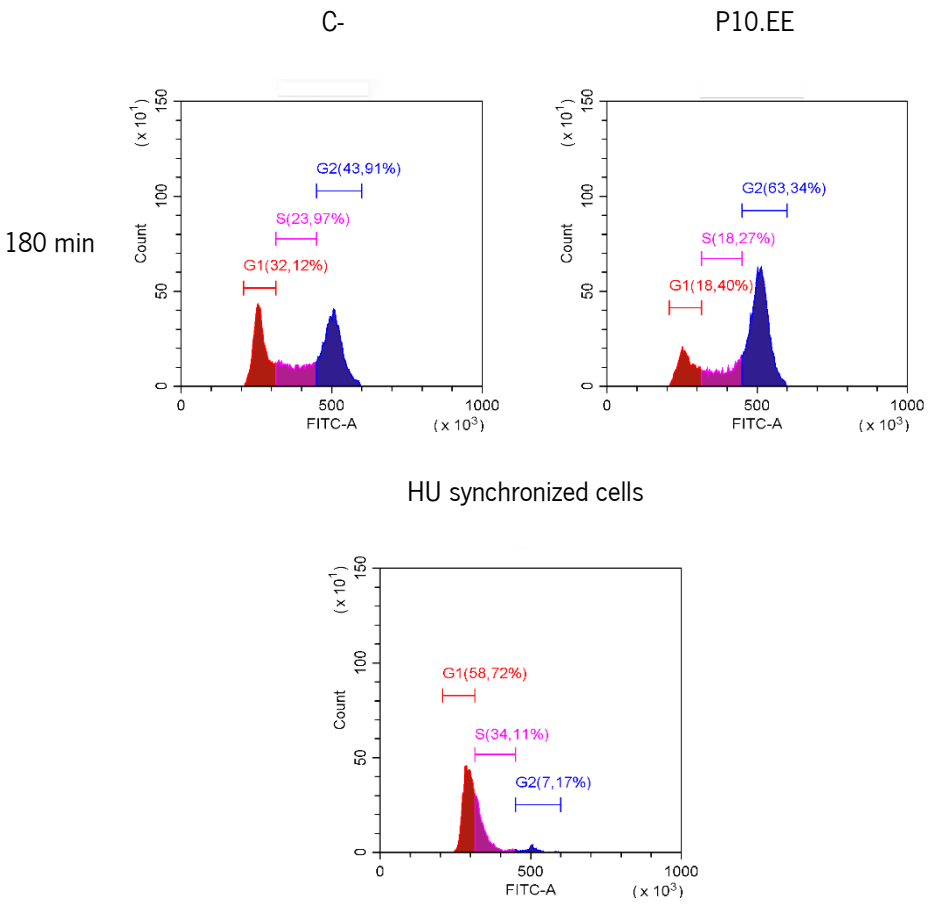


Figure A9. Analysis of P10.EE effect on *S. cerevisiae* cell cycle progression. HU synchronized *S. cerevisiae* BY4741 cells were treated with 200 µg/mL P10.EE for 180 min. After 0, 30, 60, 90, 120, and 180 min of incubation, aliquots were collected and cells were prepared for flow cytometry analysis and stained with SYTOX™ Green Nucleic Acid Stain (Invitrogen). For the negative control (C-), cells were treated with absolute EtOH, at the same volume as for the P10.EE. Histograms presented are of one representative experiment out of three independent replicates.

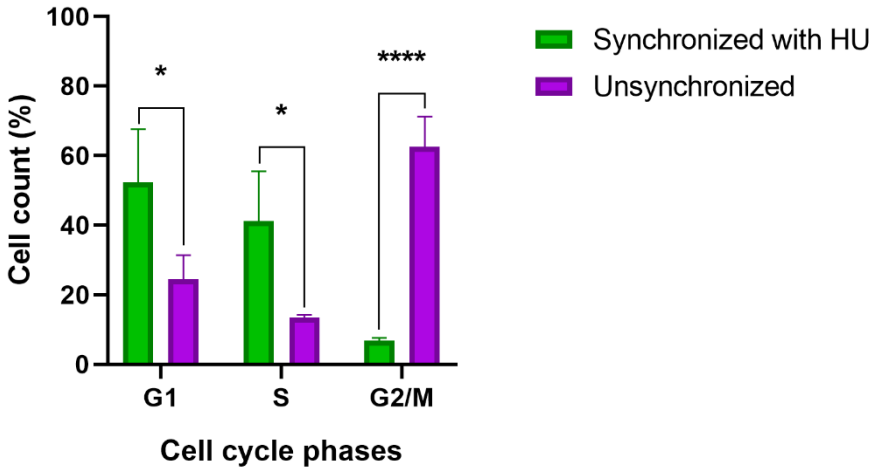


Figure A10. Synchronization of *S. cerevisiae* cell cycle. Percentage of *S. cerevisiae* BY4741 cells in the cell cycle phases G1, S, and G2/M, after synchronization of the cells with HU at 200 mM. Synchronized and unsynchronized cells were prepared for flow cytometry analysis and stained with SYTOX™ Green Nucleic Acid Stain (Invitrogen). These values represent the mean ± SD of n = 3 independent replicates. Two-way ANOVA was performed, followed by Sidak's test for multiple comparisons. Asterisks represent significant differences between synchronized and unsynchronized cells (* $p < 0.05$, **** $p < 0.0001$).