Universidade do Minho
Escola de Ciências

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Study of antifungal activity and mechanisms of action of plant extracts with potential application in sustainable agricultural practices

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Diana Machado

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Work under the supervision of:
Professor Doutor Rui Pedro Soares de Oliveira

December 2021

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(Diana Campos de Azevedo Ferraz Machado)

Estudo da atividade antifúngica e mecanismos de ação de extratos de plantas com potencial aplicação em práticas agrícolas sustentáveis

Resumo

O grande impacto económico proveniente de danos nas culturas agrícolas causados por fungos fitopatogénicos leva, por vezes, ao uso exacerbado e continuado de fungicidas sintéticos na agricultura convencional. Alguns destes fungicidas, caracterizados pela alta especificidade do seu mecanismo de ação, resultaram no surgimento de estirpes resistentes e também efeitos prejudiciais para o ambiente. Estudos recentes abordam os extratos de plantas como potenciais antifúngicos naturais a serem usados na agricultura orgânica, pela sua complexidade e biodegradabilidade intrínseca, contudo ainda é necessário descobrir novos extratos com atividade antifúngica e de que forma é que estes atuam. Assim, com este trabalho pretendeu-se estudar a atividade antifúngica e possíveis mecanismos de ação de extratos de plantas com potencial aplicação em práticas agrícolas sustentáveis. Através de ensaios de viabilidade foi demostrado que, dos quatro extratos etanólicos testados, *Callistemon citrinus* (CC), *Cistus ladanifer* (CL), *Fraxinus angustifolia* (FA) e *Pistacia terebinthus* (PT), *Saccharomyces cerevisiae* é sensível ao extrato CL em todos os tempos avaliados, em particular, após 2 h de tratamento com 400 µg/ml de CL, a percentagem de viabilidade da celular foi 71,20 % ± 4,16. Além disso, foi determinada a percentagem de inibição do crescimento do micélio (%ICM) após 9 dias de incubação com o extrato CL em fungos fitopatogénicos. Concretamente, a %ICM de *Colletotrichum acutatum, C. gloeosporioides* e *Diplodia corticola* foi 38,09 % ± 4,00; 30,32 % ± 4,00 e 37,4 2% ± 8,76, respetivamente, após 9 dias de incubação com o extrato CL em fungos fitopatogénicos. O estudo de possíveis mecanismos de toxicidade envolvidos nas vias de biossíntese de ergosterol, integridade da parede celular e no processo de apoptose foi realizado através de ensaios de viabilidade com as estirpes mutantes de *S. cerevisiae erg2*, *bck1*, *mkk1/mkk2* e *yca1* e através da análise do potencial de membrana interna mitocondrial por citometria de fluxo. As mutantes *erg2*, *bck1* e *mkk1/mkk2* foram mais sensíveis e a mutante *yca1* mais resistente ao extrato CL, comparativamente à estirpe selvagem. Segundo os resultados preliminares de citometria, CL desencadeia a despolarização da membrana mitocondrial. Assim, os resultados sugerem que o extrato CL apresenta atividade antifúngica e afeta estas vias. Contudo, novos estudos, com outras mutantes, por exemplo, devem ser realizados para confirmar os resultados obtidos, sobretudo o efeito do extrato no processo de apoptose, uma vez que os resultados de citometria sugerem despolarização da membrana mitocondrial após a exposição de *S. cerevisiae* ao extrato CL.

Palavras-chave: fungos fitopatogénicos; biofungicida; extratos de plantas; mecanismo de ação; *Cistus ladanifer*

Study of antifungal activity and mechanisms of action of plant extracts with potential application in sustainable agricultural practices

Abstract

The great economic impact arising from damage to agricultural crops caused by phytopathogenic fungi sometimes leads to the exacerbated and continued use of synthetic fungicides in conventional agriculture. Some of these fungicides, characterized by the high specificity of their mechanism of action, have resulted in the emergence of resistant strains and harmful effects to the environment. Recent studies approach plant extracts as potential natural antifungals to be used in organic agriculture, due to their complexity and intrinsic biodegradability, however it is still necessary to discover new extracts with antifungal activity and how they act. Thus, with this work it is intended to study the antifungal activity and possible mechanisms of action of plant extracts with potential application in sustainable agricultural practices. Through viability assays it was demonstrated that, of the four ethanolic extracts tested, *Callistemon citrinus* (CC), *Cistus ladanifer* (CL), *Fraxinus angustifolia* (FA) and *Pistacia terebinthus* (PT), *Saccharomyces cerevisiae* was sensitive to the extract CL in all the evaluated times, in particular, after 2h of treatment with 400 µg/ml CL (71.20 % ± 4.16 viability). Furthermore, the percentage of mycelium growth inhibition (%ICM) after 9 days of incubation with the CL extract on phytopathogenic fungi was determined. Specifically, the %ICM of *Colletotrichum acutatum*, *C. gloeosporioides* and *Diplodia corticola* were 38.09 % ± 4.00; 30.32 % ± 4.00 e 37.42 % ± 8.76, respectively, after 9 days of incubation with 500 µg/ml CL extract. The study of possible toxicity mechanisms involved in ergosterol biosynthesis pathways, cell wall integrity and apoptosis process was performed by viability assays with the *erg2*, *bck1*, *mkk1/mkk2* and *yca1* mutant *S. cerevisiae* strains and by analysis of mitochondrial inner membrane potential by flow cytometry. The *erg2*, *bck1* and *mkk1/mkk2* mutants were more sensitive and the *yca1* mutant more resistant to the CL extract compared to the wild type strain. Additionally, according to preliminary cytometry results, CL triggers mitochondrial membrane depolarization. Thus, the results suggest that CL extract exhibits antifungal activity and affects these pathways. However, further studies with other mutants, for example, should be performed to confirm the results obtained, especially the effect of the extract on the apoptosis process, since cytometry results suggest mitochondrial membrane depolarization after exposure of *S. cerevisiae* to CL extract.

Keywords: phytopathogenic fungi; biofungicide; plant extracts; mechanism of action; *Cistus ladanifer*

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Abbreviations List

BHA – Butylated hydroxyanisole

CAZymes – Carbohydrate active enzymes

CC – *Callistemon citrinus*

CFUs – Colony forming units

CL – *Cistus ladanifer*

Cyt C – Cytochrome C

DiOC6(3) – 3,3'-dihexyloxacarbocyanine iodide

DMSO – Dimethyl sulfoxide

FA – *Fraxinus angustifolia*

FRAC – Fungicide Resistance Action Committee

HPLC – High performed liquid chromatography

IC_{50} – Half maximal inhibitory concentration

ns – not significant

PDA – Potato-Dextrose-Agar

PI – Propidium iodide

PKC1 – Protein kinase C

PT – *Pistacia terebinthus*

Qo – Quinone-outside

ROS – Reactive oxygen species

SD – Standard deviation

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1. Introduction

1.1. Phytopathogenic fungi

Fungi perform activities relevant to the development, nutrition and health of plants, including crops. Saprobic fungi make minerals available in the soil by the decomposition of dead organic matter and the symbionts form mutually favourable associations with the roots of higher plants (mycorrhizae). However, there are fungi that establish non mutualistic relationships with plants and that can lead to plant disease and death, when the plant's defence mechanisms are not sufficient to overcome the virulence of fungi (Klein & Paschke, 2004). According to Raaijmakers *et al.* (2009) and Yang *et al.* (2019) 70 to 80 % of total plant damage are caused by phytopathogenic fungi.

The reproduction process of fungi (Fig. 1) occurs mainly through spores – structures composed of one or a few cells, resistant to extreme conditions (e.g. cold and lack of water) – which may result from sexual or asexual reproduction (Griffin, 1993; Watling, 2003; Agrios, 2009). In sexual reproduction cycle, when a haploid mycelium meets a mycelium from another fungal organism and join, the cytoplasm from each is fused, although the nuclei remain separate – dikaryotic hyphae. The dikaryotic nuclei are then fused into one and the cell undergoes meiosis to form sexual haploid spores. The spores are released, and the germination process can occur, resulting in the formation of a new filamentous fungus. In an asexual reproduction cycle, the haploid mycelium is fractured to produce spores for subsequent segregated development via germination. Note that sexual and asexual reproduction may happen depending on the species and the conditions (Griffin, 1993; Watling, 2003). The basis of fungal reproduction process is similar among fungi, however special details were not specified here. The reproduction cycles of phytopathogenic fungi can be related with host plant changes – disease cycle. Briefly, the disease cycle begins when the fungus enters the plant, through natural openings or by penetrating the exterior of host, and interacting with susceptible cells to start the infection. At this phase symptoms may be detectable or there may be a latency period. The fungus will grow and actively spread through host cells causing local or systemic infections. Thousands of spores are created during reproduction, these can be spread by air, water, insects, and people, where they germinate and infect another host. (Kendrick, 2001; Sinclair & Lyons, 1987). Because phytopathogenic fungi use diverse strategies to

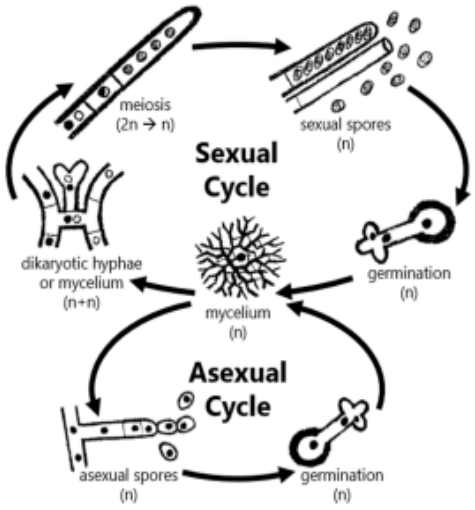

Figure 1 – Diagram of fungal life cycle: sexual and asexual cycles. Adapted from Roehl (2021).

infect and colonize plants, they have varied lifestyles, such as biotrophs, necrotrophs, and hemibiotrophs. Biotrophs, such as *Cladosporium fluvum*, grow and reproduce in living plant tissue, whereas necrotrophs like *Botrytis cinerea* destroy host tissue, colonize it, and then feed on dead plant cells (Zhang *et al.*, 2018). Hemibiotrophs first form a biotrophic relationship with the host, but as the infection progresses, the host cells die, for example, *Colletotrichum acutatum* and *Diplodia corticola* (Horbach *et al.*, 2011; Ureña-Padilla *et al.*, 2002; van den Berg *et al.*, 2021).

1.1.1. *Botrytis cinerea*

Botrytis cinerea (associated with gray mold disease) infects several economically important crops, such as tomato (*Solanum lycopersicum*), ornamental flowers (*Petunia* spp.) and grapevine (*Vitis vinifera*; Fig. 2A; Legard *et al.*, 2000; Walker *et al.*, 2013). Particularly, grapevine is regarded as one of the world's major fruit crops, both in terms of cultivated land and economic value (Petronilho, Coimbra & Rocha, 2014). Various host organs, including fruits, flowers, leaves, and shoots, with mature or senescent tissues (Williamson *et al.*, 2007), may be damaged, generating distinct indications of infection as bloom blights and fruit rots. One of the plant's defense mechanisms against fungal attack is localized lesions generated by an oxidative burst, which results in hypersensitive cell death of the host - hypersensitive response (Govrin & Levine, 2000). However, being a necrotrophic fungus with reactive oxygen species (ROS) tolerance (Choquer *et al.*, 2007; Walker *et al.*, 2013), *B. cinerea* continues to expand the infection zone. Other factors associated with *B. cinerea* virulence include (1) the production of organic acids (e.g., oxalic acid), which creates a low pH environment and, as a result, cell wall-degrading enzymes (i.e., pectinases) are enhanced, plant-protection enzymes are inhibited, and stomatal closure is deregulated (Amselem *et al.*, 2011); and (2) the production of sclerotia, since this structure can detach itself from the fungus and remain dormant in extreme environmental conditions until favourable growth conditions return (Tulasne, 1853; Zhang *et al.*, 2018).

1.1.2. *Colletotrichum* spp.

Colletotrichum spp. (associated with anthracnose disease) was classified as one of ten genera of economic and scientific importance with a broad host range (Dean *et al.*, 2012), including infections in chestnut and strawberries by *C. gloeosporioides* (Fig. 2B) and *C. acutatum* (Fig. 2C; Damm *et al.*, 2012; Miller, 2017), respectively. The economic impact is significant, particularly for fruit, vegetable, and ornamental crops. The majority of *Colletotrichum* species establish their infection during a short biotrophic phase, related to the occurrence of large intracellular primary hyphae, however when the fungus switches to necrotrophic phase, secondary hyphae can be seen ramifying throughout the host

tissue. Moreover, the first phase is characterized by a higher expression of secondary metabolism-associated genes and carbohydrate active enzymes (CAZymes; Gan *et al.*, 2012; Zhang *et al.*, 2018; Villa-Rivera, 2017), while in the second phase normally leads to a completely distinct expression pattern with a higher expression of genes encoding hydrolases, proteases and transporters (Alkan *et al.*, 2015; Gan *et al.*, 2012; Zhang *et al.*, 2018).

1.1.3. *Diplodia corticola*

Diplodia corticola (associated with bot canker of oak disease) is known to infect grapevine and several oak species, including cork oak (*Quercus suber;* Fig. 2D; Alves *et al.*, 2004; Úrbez-Torres *et al.*, 2010). Cork oak is a forestry species protected by legislation in Portugal due to the safeguarding of agricultural areas, as the bark of the cork oak is extremely resistant to fire, its environmental value, associated for example with soil conservation, water quality and biodiversity, and also its economic interest, with Portugal producing over 50 % of the world's cork (reviewed by Pintor *et al.*, 2012). Various host organs, including oak twigs, branches, and stems, as well as host cambial tissues, may be affected. When *D. corticola* degrades the cambial tissue - layer that provides partially undifferentiated cells for plant growth – it can produce wilting and dieback symptoms. The Botryosphaeriaceae – the *D. corticola* family –, live within plants and then switch to a pathogenic phase, triggered by plant physiologic disturbances as a result of environmental stresses (e.g. insect damage, water stress, pruning and transplanting; Félix *et al.*, 2017), and, ultimately, often culminates in the death of the host. Factors such as the synthesis of specific proteins, which has been linked to host tissue destruction via redox processes, and aminobutyric acid metabolization, which may be involved in the shift from latent to pathogenic phase, may be linked to *D. corticola* virulence (Fernandes, 2015).

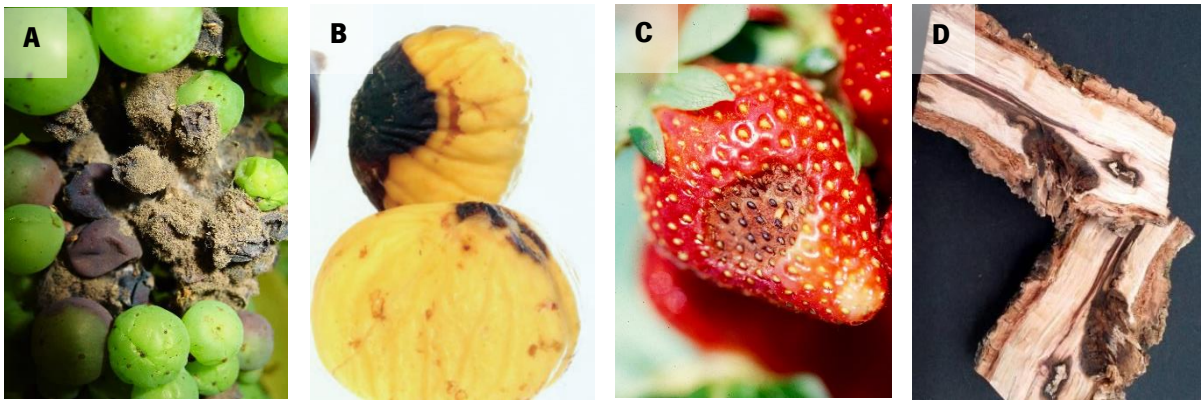

Figure 2 – Plant organs infected by phytopathogenic fungi. (**A**) Grapes with grey mold, *Botrytis cinerea*; (**B**) Chestnut exhibiting blossom end rot, *Colletotrichum gloeosporioides*; (**C**) Strawberry with anthracnose, *Colletotrichum acutatum*; (**D**) Cork oak with extensive branch dieback, *Diplodia corticola* (among other species). Adapted from Holmes (1997), Miller (2017), Smahi *et al.* (2017) and Taylor (2021).

1.2. Synthetic fungicides

1.2.1. Mechanisms of action

Fungicides are compounds of synthetic or natural origin that allow to kill or inhibit fungi or fungal spore germination, through several different modes of action. The modes of action of fungicides can be divided into three broad groups based in fungicide targets: (1) the cell membrane synthesis and integrity, (2) the mitochondrial respiration and (3) the nucleic acid, enzyme and protein synthesis. The first group includes the disruption of fungi membrane (fungicide example: polyenes) and the inhibition of ergosterol synthesis (fungicide examples: imidazoles and alylamine), which can lead to apoptosis. The second group targets mitochondrial respiration and includes quinone-outside (Qo) inhibitors (fungicide example: napthoquinone) that interact with the Qo site of cytochrome b, as well as fungal energy deficiency, affecting spore germination process and zoospore motility (Fernndez-ortuo & Prez-garc, 2010). The third group includes inhibition of DNA and RNA synthesis (fungicide example: flucytosine), and the disruption of microtubule (fungicide example: griseofulvin) causing problems in the cell cycle, as well as the inhibition of $\beta(1\rightarrow3)$ glucans (fungicide example: echinocandids) fragilizing the cell wall, one of the physical protective barriers of fungi (Fig. 3; reviewed by Lumen, 2021).

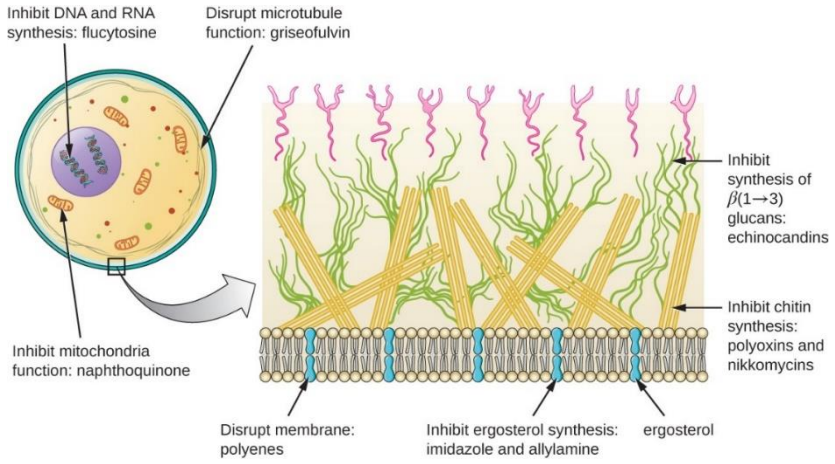

Figure 3 – Antifungal drugs target a variety of cell structures. From Lumen, 2021

1.2.2. Resistance and environmental impact

In a 2016 study published by Nature Plants (reviewed by Reganold & Wachter, 2016), the performance of conventional and organic agriculture was examined taking into account four main areas of sustainability: productivity, environmental impact, economic viability, and social well-being. Organic agriculture systems are more profitable, environmentally friendly, and provide equally or more nutritious food that contains less or no pesticide residues, when compared to conventional agriculture. Furthermore, preliminary research suggests that organic agricultural systems provide greater ecosystem services and social benefits. However, there is a significant downside to this system, which is its poor yield in comparison to conventional agriculture. In part, the high yield of the conventional system is due to the

effective combat of fungal diseases, one of the biggest threats to crop production (Fisher *et al.*, 2012). One of the ways that conventional agriculture uses to combat phytopathogenic fungi is the use of fungicides with highly specific targets. Azoles, the main antifungal class used for crops, are an example, these fungicides interfere with lanosterol 14α-demethylase perturbing the conversion of lanosterol to ergosterol (Lucas, Hawkins & Fraaije, 2015). Consequently, the widespread use of these fungicides resulted in (1) high environmental accumulation, for instance azole fungicides are resistant to degradation by microbes and are therefore frequently detected in surface water and sediment (Chen & Ying, 2015); (2) ecotoxicity, fungicides can reach aquatic habitats through wastewater treatment plants following agricultural usage (Kahle *et al.*, 2008), or through water runoff from agricultural fields (Bereswill *et al.*, 2012), which can be toxic to a wide spectrum of non-target species (Köhler *et al.*, 2017); and (3) an increasing number of target pathogens developing resistance, *Venturia inaequalis*, *Penicillium digitatum*, *Cercospora beticola*, *Monilinia fructicola*, and *Blumeriella jaapii* are species associated with azole-resistance (Chauhan *et al.*, 2019; Carvalho, 2017; Lucas *et al.*, 2015). Not only azole resistance but also other new emerging antifungal agents, the resistance to antifungal agents that are used to control phytopathogenic fungi is a well-documented problem. Since both plants and their fungal pathogens are eukaryotic organisms, there are few possible drug targets that are unique and vital to the fungus but not to the host (Anderson, 2005). The evolution of antifungal resistance starts with single or multiple mutations in the pathogen that produce a fungicide-resistant phenotype, which then develops through natural selection. Important mechanisms of resistance include (1) overproduction of the fungicide target, due to gene upregulation; (2) detoxification of the fungicide by metabolic enzymes; (3) loss of enzyme activity preventing the accumulation of a toxic product in the presence of the drug; (4) alteration in a few specific amino acids of the target, avoiding the fungicide-target bond or the allosteric inactivation of the target due to point mutations; (5) decrease in drug effective concentration, caused by overexpression of ATP-binding cassette transporters or major facilitator proteins in the cell membrane due to mutations in transcriptional regulators (Lucas *et al.*, 2015; Lupetti *et al.*, 2002; Sanglard, 2016; Sanglard & Odds, 2002; Scorzoni *et al.*, 2017). The spread of a fungicide-resistant fungal pathogen depends on some biological factors such as a short life cycle, abundant sporulation, and long-distance spore dispersal (Brent *et al.,* 1990).

1.3. Natural fungicides

1.3.1. Plant secondary metabolites

The development of new fungicides has been challenging due to increased resistance to fungal pathogens and public demand for environmentally sustainable antifungal agents. New antifungal

treatment strategies include the formulation of natural fungicides based on plant extracts (Brauer *et al.*, 2019). Several extracts have been described, through many *in vitro* assays and some *in vivo* assays, as antifungal agents against different phytopathogenic fungi (Wahab *et al.*, 2021) and approaches to develop safe, stable and efficient delivery systems have been investigated (Liao *et al.*, 2021; Muzzalupo *et al.*, 2020). Plant contains a diverse range of molecules synthesized to provide a selective advantage by increasing its survivability or pollination in response to environmental stimuli – secondary metabolites. Secondary metabolites are associated to a variety of biological activities, making them an attractive alternative to synthetic fungicides (Li *et al.*, 2020). They include phenolic compounds, flavonoids, coumarins, saponins and terpenes (Kumar & Mina, 2013).

Phenolic compounds, the most abundant secondary metabolites in plants (Ayad & Akkal, 2019), are compounds with hydroxylated aromatic rings. These compounds can combine with mono- and polysaccharides or can occur as derivatives, such as ester or methyl esters. Flavonoids have a 15-carbon skeleton that is formed by two phenyl rings and a heterocyclic ring (Farias, Costa & Martins, 2021). Flavonoids are the most important plant pigments for attracting pollinators and are also involved in UV filtration and symbiotic nitrogen fixation (Ferreyra, Rius & Casati, 2012). Coumarins are natural benzopyrone derivatives present in plants both free and as glycosides, with phototoxic and carcinogenic effects and a variety of pharmacological properties including antitumor, antihypertension, antiseptic, and analgesic properties (Wu *et al.*, 2013). Saponins are associated to the plant's defence system against pathogens (Manach *et al.*, 2004; Morrissey & Osbourn, 1999). The chemical structure contains a hydrophobic aglycone, consisting of either a sterol or a triterpenoid, and hydrophilic sugar residues (Moghimipour & Handali, 2015). Terpenes are a large group of unsaturated hydrocarbons produced, predominantly, by plants (Davis & Rodney, 2000). Short-chain terpenes are frequently found in conjunction with sesquiterpenes and diterpenes, playing a variety of ecological roles, such as pollinator attractant and antimicrobial properties (Tian *et al.*, 2012).

1.3.2. Mechanisms of action

Secondary metabolites have been shown to be effective against phytopathogenic fungi, however specific data on their mechanism of action is still lacking. For decades, phenolic compounds have been extensively studied for their wide range of antioxidants and beneficial effects on the human body. However, various active compounds that are active against *C. albicans* have been identified. Compounds with antifungal activity include phenols such as gallic acid, thymol, and flavonoids (particularly catechin), as well as polyphenols such as tannins, terpenoids and saponins (reviwed by Hsu, Sheth & Veses, 2020). The antifungal mechanism of gallic acid present in *Cochlospermum regium* plant has been demonstrated.

Gallic acid binds to ergosterol on the cell membrane, resulting in pore formation, or by distrusting the enzymes responsible for the synthesized ergosterol, resulting in membrane damage (Carvalho *et al.*, 2018). Saponins are found primarily in peas, soybeans, and herbs. Several studies have discovered that saponins have antifungal properties against *C. albicans.* Previous research has shown that saponins can interfere with sterols, inhibiting yeast-hyphal transition and biofilm formation (Chevalier, Medioni & Prêcheur, 2012; Njateng *et al.*, 2015; Pereira *et al.*, 2016). Catechins were discovered to be able to trigger the generation of reactive oxygen species (ROS). ROS have been linked to the disruption of molecular mechanisms such as angiogenesis and extracellular matrix degradation, as well as cell apoptosis (Bernatoniene & Kopustinskiene, 2018). Terpenoids have a fungistatic effect on *Candida* by modulating specific signalling pathways (TOR pathway or calcium signalling), rather than by causing nonspecific membrane lesions, according to the proposed mechanism of action. As a result, gene transcription is altered and stasis occurs (Luiz *et al.*, 2015; Rao *et al.*, 2010; Suurbaar, Mosobil & Donkor, 2017).

Despite some disadvantages of using plant extracts as natural fungicides, such as rapid degradation after treatment and low efficiency when compared to synthetic fungicides, they are more environmentally friendly due to their inherent biodegradability and may act through multiple mechanisms of action, reducing the likelihood of the emergence of new resistant strains (Scognamiglio *et al.*, 2019). In addition, further study of the modes of action allows for the acquisition of new knowledge, which can then be used to improve the bioavailability and efficacy of natural antifungal products.

1.4. Biological activities of plant extracts

Plants have been used over centuries for beneficial applications to humans, which is in part the result of the antimicrobial properties of their natural extracts. The emerging interest for a natural antifungal, characterized by high biodegradability (Scognamiglio *et al.*, 2019), appears as an alternative to synthetic antifungal since the use of these compounds may represent potential risks to environment sustainability. Blad fungicides are an example of natural fungicides recognised by the Fungicide Resistance Action Committee (FRAC; Ferreira *et al.*, 2006; Carreira *et al.*, 2011a, b, c). They contain 250 g of Blad active ingredient per litre and other inert ingredients. Blad is the commercial name given to a polypeptide that constitutes the main subunit of a glyco-oligomer (Melo *et al.*, 1994; Ferreira *et al.*, 1999). This glyco-oligomer results from the catabolism of β-conglutin and is extracted from the cotyledons of the species *Lupinus albus* (plant sweet lupin), as it substantially accumulates in these organs after 4 to 12 days from the beginning of germination (Ramos *et al.*, 1997). Research indicates that Blad has multiple mechanisms of action. Blad is suggested to degrade chitin by catalysing and successfully removing the *N*-acetyl-*D*-

glucosamine terminal monomers, thereby disrupting the fungal cell wall's stability (Monteiro *et al.*, 2015). It was also indicated with chelating agent activity on several divalent ions, disturbing the fungal homeostasis (Pinheiro *et al.*, 2017). Finally, results demonstrated the destabilization of *C. albicans* cell membrane when treated with Blad-containing oligomer (Pinheiro *et al.*, 2016).

The extracts of four common plant species in Portugal – *Callistemon citrinus*, *Pistacia terebinthus*, *Cistus ladanifer* and *Fraxinus angustifolia* (Fig. 4) – are products of natural origin with potential antifungal activity due to the composition and/or antifungal activity of these plants or plants belonging to the same genus. Although antifungal activity has been studied, research on this activity is still limited, with most studies focusing on activity against clinical fungi rather than phytopathogenic fungi. Thus, it is of interest to study these properties in fungal plant pathogens with high incidence in Portugal. This investigation can contribute to new potential sustainable agricultural practices.

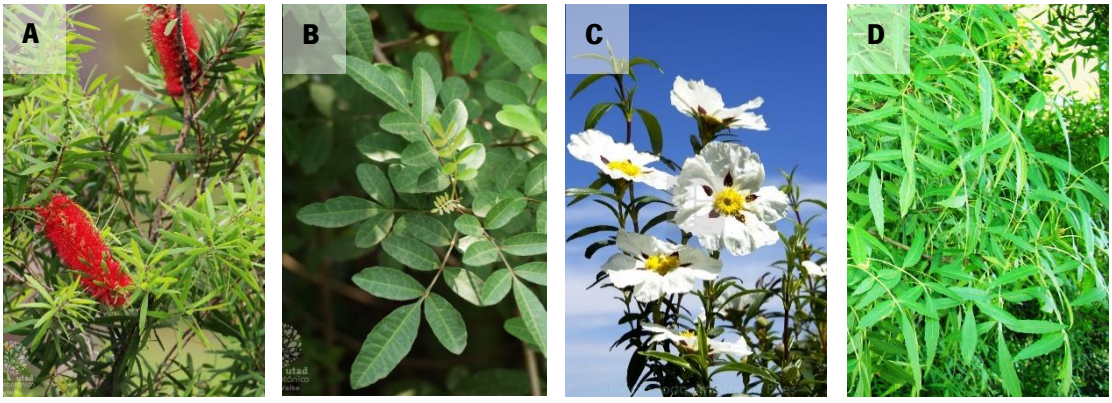

Figure 4 – Images of the plant species investigated in the present study. (**A**) *Callistemon citrinus* aerial parts, (**B**) *Pistacia terebinthus* leaves, (**C**) *Cistus ladanifer* aerial parts, (**D**) *Fraxinus angustifolia* leaves. Adapted from Bertinetto (2007), Cabral (2010), Ng (2017), Pires (2016) and Valke (2017).

1.4.1. *Callistemon citrinus*

Callistemon citrinus (Curtis) Skeels [also known as *Callistemon lanceolatus* (Sweet) DC] is an Australian perennial shrub that belongs to the family Myrtaceae and it is commonly referred to as crimson bottlebrush. Currently, it is widely distributed all over the world as an ornamental plant and its leaves have been used as a tea substitute due to its refreshing flavor (Goyal, *et al.*, 2012; Oyedeji *et al.*, 2009). In traditional medicine, *Callistemon* spp. have been used in the treatment of cough, bronchitis, microbial infections (Goyal *et al.*, 2012), however these properties are not fully understood and the analysis of the chemical composition of *C. citrinus* is still very limited. Secondary metabolites already reported in *C. citrinus* leaves include alkaloids, saponins, steroids, and triterpenoids, fat and oils, flavonoids, phenols, and tannins (Mabhiza *et al.*, 2016). Analysis of n-hexane, ethyl acetate and n-butanol extracts from a plant species (leaves) of the same genus, *Callistemon viminalis*, indicates the presence of saponins (3-*O*-

α-L-arabinopyranoside hederagenin and hederagenin 3-*O*-β-glucopyranosyl-(1→2)-β-*D*-xylopyranoside) with antifungal activity (Tshibangu, 2010). The antifungal activity of *C. citrinus* aqueous extract was tested on fungi isolated from old books, *Chaetomium spiralis*, *Alternaria alternata* and *Aspergillus niger*, resulting in mycelial growth inhibition of 24.7 %, 24.4 % and 23.8 %, respectively (Kalbende & Dalal, 2019). Reports also indicate other activities of *C. citrinus*, such as antibacterial (Seyydnejad *et al.*, 2010; Tshibangu, 2010), anti-inflammatory (Kumar *et al.*, 2011), antioxidant, anticholinesterase (Gupta *et al.*, n.d.), antidiabetic, hypolipidemic, antidiabetic, (Kumar *et al.*, 2011), cardioprotective (Firoz *et al.*, 2011), hepatoprotective (Jain *et al.*, 2007) activities.

Despite the fact that aqueous and methanolic extracts of *C. citrinus* have been described to have antifungal activity (Kalbende & Dalal, 2019), the mechanisms are not fully understood and to our knowledge there is limited evidence on its activity against phytophatogenic fungi. Considering that aqueous and methanolic extracts of *C. citrinus* exhibited antifungal activity (Kalbende & Dalal, 2019), the analysis of n-hexane, ethyl acetate and n-butanol extracts of *C. viminalis*, indicates the presence of saponins (amphiphilic molecules) with antifungal activity (Tshibangu, 2010) and the fact that saponins have been associated to antifungal properties (Sparg *et al.*, 2004), it is likely that the antifungal activity of *C. citrinus* may be due to the action of saponins. One of the saponins present in *C. viminalis*, 3-*O*-α-L-arabinopyranoside hederagenin, is known to interact with cholesterol (structurally analogous to ergosterol), consequently disrupting membranes and leading to cell death (Lorent *et al.*, 2013; Tshibangu, 2010). Since *C. citrinus* and *C. viminalis* are related, some compounds may be common, and consequently biological activities may be similar as well.

1.4.2. *Pistacia terebinthus*

Pistacia terebinthus L. is a Mediterranean deciduous tree (Portugal native) that belongs to the Anacardiaceae family and is commonly called terebinth. In traditional medicine, the leaves of this species have been used as diuretic, antihypertensive, antidiabetic, treatment of jaundice, astringent, treatment of diarrhoea, treatment of stomach ache and treatment of mycosis (reviewed by Bozorgi *et al.*, 2013), however these properties are not fully understood and, as far as we know, the chemical composition has not been published. The antifungal activity of ethanolic extract of the leaves of this species as tested on some fungi and it significantly inhibited the mycelial growth of *Pythium ultimum* (Kordali *et al.*, 2003). From the publications found so far, the essential oils showed potential antifungal (Piras *et al.,* 2017), antioxidant (Durmaz & Gökmen, 2011), antidiabetic (Uyar & Abdulrahman, 2020) and antibacterial (Pulaj *et al.*, 2016) activities.

Despite the fact that *P. terebinthus* extract has the ability to inhibit the mycelial growth of an

oomycete, *Pythium ultimum,* (Kordali *et al.*, 2003), the study of hydrophilic extracts activities remains scarce and their composition is not known yet. Since one of the differences between oomycete and true fungi is the absence of ergosterol in the membrane, it would be interesting to investigate if this activity can also occur in true fungi and the potential metabolites involved in the antifungal mechanisms.

1.4.3. *Cistus ladanifer*

Cistus ladanifer subesp. *ladanifer* L. [also known as *Cistus ladaniferus* L.] is a Mediterranean perennial shrub (Portugal native) that belongs to the family Cistaceae and is commonly called gum rockrose among other names. *Cistus ladanifer* presents mechanisms that allows adaptation to abiotic stresses, such as low water availability and high solar exposition conditions, persistence in poor and contaminated soils. In addition, it is well known for producing labdanum, an aromatic resin with a highly appreciated odour (Andrade *et al.*, 2009; Raimundo *et al.*, 2018) and high content in potential bioactive compounds (diterpenes and flavonoids; Alías *et al.*, 2006; Oller-López *et al.,* 2005; Masa *et al.*, 2016), and therefore used in the industry of perfumes, cosmetics, soaps, detergents, and deodorants (Oyaizu, 1986). The secondary metabolites reported so far in *C. ladanifer* include terpenes, alkaloids, polyacetylenes, steroids and phenols (Andrade *et al.*, 2009; Chaves *et al.*, 2001; Dias & Moreira, 2002). The phenolic profile of *C. ladanifer* hydromethanolic extract from leaves is composed of phenolic acids and derivatives (*e.g.* galloyl glucose), flavonoids (*e.g.* catechins) and ellagic acid derivatives (*e.g.* punicalagin gallate), the latter group being the most abundant (Barros *et al.,* 2013). The rich phenolic composition is a possible explanation for the significant antioxidant (Gaweł-Bęben *et al.,* 2020). In addition to antioxidant activity, strong antifungal activity was also indicated against: human fungal pathogens (*Candida albicans*, *C. glabrata* and *C. parapsilosis*), in which *C. ladanifer* leaves were previously extracted with methanol:water and then dissolved in water (Barros *et al.*, 2013) and a plant fungal pathogen (*Geotrichum candidum*), in which the *C. ladanifer* stem and leaves were previously extracted and dissolved in water (Karim *et al.,* 2017). Reports also indicate other activities to *C. ladanifer*, such as antibacterial, anti-carcinogenic, anti-inflammatory and anti-nociceptive and insecticidal activities (Deforce, 2006; Demetzos *et al.*, 2002; Dimas *et al.*, 1998).

Although antifungal activity of the *C. ladanifer* extract has already been described, the reported activity in fungi with human and plant pathogenicity is limited, having been assessed, to the best of our knowledge, against genus *Candida* (Barros *et al.*, 2013) and *G. candidum* (Karim *et al.*, 2017), respectively. The ability to inhibit the mycelial growth of some fungi may be due to the action of ellagic acid derivates, since ellagic acid was indicated to inhibit the ergosterol biosynthesis, leading to the induction of apoptosis (Li *et al.*, 2020).

1.4.4. *Fraxinus angustifolia*

Fraxinus angustifolia Vahl subesp. *angustifolia* is a Mediterranean deciduous tree (Portugal native) that belongs to the family Oleaceae and is commonly called narrow-leafed ash. In traditional medicine, *Fraxinus* has been used against inflammatory diseases, infections, constipation, and as diuretic and hepatoprotective agent (Kumar & Kashyap, 2015), however these properties are not fully understood. Several studies have reported that the chemical composition of ethanolic extracts of *F. angustifolia* were indicated as rich in polyphenols (Ayouni *et al.*, 2016; Moulaoui *et al.*, 2015). Fractions of leaf extracts present secoiridoids (*e.g.* oleuropein and ligstroside), flavonoids (kaempferol 3-*O*-rutinoside and quercetin 3-*O*-glucoside), and verbascoside (Ayouni *et al.*, 2016). Phytochemical compounds from a plant species of the same genus, *Fraxinus hupehensis*, were tested against three plant pathogenic fungi, *Bipolaris maydis, Sclerotium rolfsii* and *Alternaria solani*. Fraxetin (*O*-methylated coumarin), one of the compounds present in the hydromethanolic *F. hupehensis* extract, exhibited significant inhibitory when compared to the positive control, carbendazim (Zhao *et al.*, 2020). Reports also indicate that *F. angustifoli*a extracts exhibit other potential activities, such as antidiabetic, hepatoprotective (Medjahed *et al.*, 2016), antimutagenic (Bouguellid *et al.*, 2020), antiproliferative (Bouguellid *et al.*, 2020; Restivo *et al.*, 2020) and anti-inflammatory (Attanzio *et al.*, 2019) activities.

Despite the presence of fraxetin as an antifungal compound in hydromethanolic *F. hupehensis* extract and the phenol-rich composition of *F. angustifolia*, the ethanolic extract of *F. angustifolia* has not been tested against any fungus, according to what we could find. Therefore, given these indications and that *F. angustifolia* is a Portuguese autochthonous plant species, it would be interesting to investigate this activity.

1.5. Eukaryotic cell model

1.5.1. *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a unicellular fungal yeast that divides asymmetrically by budding (Morgan, 2007), its genome, composed by 6275 genes compacted on 16 chromosomes, was completely sequenced in 1996 (Goffeau *et al.*, 1996). Although the *S. cerevisiae* and the phytopathogenic fungi – *B. cinerea*, *Colletotrichum* spp., and *D. corticola* – differ in form, reproduction, virulence, environmental adaptation and nutrients acquisition, all three still belong to the same fungi division (Ascomycetes), as *S. cerevisiae*. The degree of homology between the *S. cerevisiae* proteins involved in (1) ergosterol synthesis, *e.g.* Erg2, Idi 1, Erg25; (2) ergosterol regulation, *e.g.* Ecm22, Upc2, Hem1; and (3) mitochondrial metabolism, *e.g.* Mdl22, Isu1, Nfs1, and their respective orthologues in two pathogenic ascomycetes (*Candida albicans* and *C. glabrata*) was already compared. The study found that the degree of homology

is rather high, particularly for ergosterol-associated proteins, when compared to proteins engaged in other processes (Skrzypek *et al.*, 2017). In addition, these genes and processes are common targets for antifungal agents, indicating that it is appropriate to utilize *S. cerevisiae* as an organism model in the present work. Thus, *S. cerevisiae* may be used as a screening platform in chemogenomics — the study of cellular chemical-biological interactions (*e.g.* elucidation of the mode of action of chemical compounds; Giaever *et al.*, 2004). To help the study of chemogenomics, deletion collections of this organism are easily available (Dujon, 1996). Additionally, the use of *S. cerevisiae* as a model organism facilitates the manipulation and experimentation, also the large amount of available data can help in a better understanding of experimental results.

1.6. Biological problem and objective

The damage caused by phytopathogenic fungi in conventional agriculture is usually prevented by using synthetic fungicides. These fungicides have some disadvantages, such as potentiating the emergence of resistant strains and unbalancing different ecosystems. Thus, plant extracts appear as potential fungicides for organic agriculture, since they may present different mechanisms of action and intrinsic biodegradability, avoiding the consequences of some widely used synthetic fungicides. With this work we intend to study the antifungal activity and mechanisms of action of plant extracts with potential application in sustainable agricultural practices. To achieve this objective, it is intended:

- to evaluate the antifungal activity of four extracts of common plants in the Portuguese flora (*C. citrinus*, *C. ladanifer*, *F. angustifolia* and *P. terebinthus*), through viability tests with *S. cerevisiae*, used as an experimental model of fungi;
- to assess whether the extracts, having demonstrated antifungal activity in the previous test, inhibit the mycelium growth of four fungi known to cause damage to important crops in Portugal (*B. cinerea*, *C. acutatum, C. gloeosporioides* and *D. corticola*), through viability assays with these filamentous fungi;
- to understand which mechanisms of action might be involved in the antifungal activity of the extract, through viability assays with mutants affected in ergosterol biosynthesis, cell wall integrity pathway and apoptosis process and, also, flow cytometry assays to understand the effect of the extracts on the mitochondrial inner membrane potential.

2. Materials and Methods

2.1. Preparation of plant extracts

Aerial parts of four plant species were used to prepare the ethanolic extracts analysed in this work. To prepare the four extracts, *C. citrinus* (leaves), *C. ladanifer* (leaves and branches), *F. angustifolia* (leaves) and *P. terebinthus* (leaves), the respective aerial parts were collected from the Botanical Garden of the University of Trás-os-Montes e Alto Douro in June 2020. The leaves and branches were dried in the dark, at room temperature for three weeks. All extracts were prepared with plant ground material in absolute ethanol, using the mass/solvent proportions: 1:10 (w/v) for *C. citrinus* (CC) and *C. ladanifer* (CL), 1:4 (w/v) for *F. angustifolia* (FA) and 1:2 (w/v) for *P. terebinthus* (PT). The extractions were carried out for seven days at room temperature with daily manual shaking. After the extraction of each plant, gravity filtration was performed with Whatman® paper No. 1 and the ethanol was evaporated in a Rotavapor® at 40 °C and 47 rpm. Deionized water was added to transfer the dried residues and the samples were frozen at - 80 °C. Finally, following lyophilization, stock solutions were prepared using ethanol as solvent: 50 mg/ml (stock used in assays with *S. cerevisiae* yeast) and 150 mg/ml (stock used in assays with phytopathogenic fungi).

2.2. Eukaryotic cell models, media and growth conditions

The organisms used in this study are listed in Table 1 and 2. For *S. cerevisiae* strains, each week, the stock culture was prepared in YPDA medium (1 % w/v yeast extract BD Bacto™, 2 % w/v peptone, 2 % w/v glucose and 2 % w/v agar) with 48 h incubation at 30 °C and finally stored at 4 °C. The stock cultures of the filamentous fungi *B. cinerea*, *Colletotrichum* spp. and *D. corticola* were prepared on plates with Potato-Dextrose-Agar medium (PDA; BioLife®) with an 8 mm disc mycelium in the center and then incubated in the dark at 25 °C. For the flow cytometry assay, the culture medium used was YPEtOH (1 % w/v yeast extract BD Bacto™, 2 % w/v peptone and 2 % w/v ethanol). Cell proliferation was monitored by optical density at 600 nm (OD_{600}).

Table 1 – Yeast strains used in this work and their genotypes

Yeast strain		Genotype
Description	Name	
Wild type	BY4741	Mat *a*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*
Derived BY4741 mutants	*bck1*	Mat *a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YJL095w::kanMX4*
	egr2	Mat *a*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0 erg2Δ::kanMX*
	mkk1/mkk2	Mat *a; his31; leu20; met150; ura30; YPL140c::kanMX4; mkk1::LEU2*
Wild type	W303-1A	Mat *a, ade2-1, ura3-1, leu2- 3112, trp1-1, his3-1115 can1-100*
Derived W303-1A mutant	*yca1*	Mat *a, ade2-1, ura3-1, leu2-3112, trp1-1, his3-1115, can1-100 yca1::KanMX4*

Table 2 – Phytopathogenic fungi used in this work and their suppliers

Wild type isolate	Suppliers
Botrytis cinerea	António Teixeira, Hernâni Gerós and Richard Gonçalves (University of Minho)
Colletotrichum acutatum isolate PT227	Pedro Talhinhas (Higher School of Agronomy, University of Lisbon)
Colletotrichum gloeosporioides isolate 15-025	Pedro Talhinhas (Higher School of Agronomy, University of Lisbon)
Diplodia corticola isolate CAA500	Ana Cristina Esteves (Centre for Environmental and Marine Studies, University of Aveiro)

2.3. Evaluation of antifungal activity and study of mechanisms of action in yeast

Pre-inocula of BY4741 and derived mutant strains were prepared using an isolated colony from the stock culture and subsequently suspended in YPD medium in a Falcon tube and incubated overnight at 30 °C and 200 rpm. The pre-inoculum was diluted to obtain an DO_{600} = 0.1 and was incubated under the same conditions for 4 h to obtain a DO_{600} between 0.4-0.6 (in exponential phase of culture growth). The culture was distributed into different tubes, then the extract at different concentrations were added. The tested concentrations of CC, FA and PT extracts were 500, 1000 and 1500 μg/ml, while the tested concentrations of CL extract were 150, 300, 400 and 500 μg/ml. Controls were included with deionised water (negative control) or ethanol (solvent control) at the same volume of the highest extract concentration. Aliquots of 100 μl were taken at 0 (no extract), 30, 60, 90 and 120 min to perform serial dilution (dilutions: 10^{-1},10^{-2},10^{-3},10^{-4}). From the latter dilution, 40 μl were pipetted 3 times onto plates with YPDA. After 48 h of incubation at 30 °C, the number of colony-forming units (CFUs) was counted and the percentage of viability was calculated for each concentration of extract and timepoint tested, the mean value of CFUs at different times ($\overline{x_t}$) was divided by the mean value at time 0 ($\overline{x_0}$, equation 1).

Equation 1: $\% \text{ viability} = \frac{\overline{x_t}}{\overline{x_0}} \times 100$

2.4. Evaluation of antifungal activity in phytopathogenic fungi

The mycelium growth inhibition of phytopathogenic fungi – *B. cinerea*, *Colletotrichum* spp. and *D. corticola* – by *C. ladanifer* ethanolic extract was evaluated through viability assays. Before solidification, the PDA medium was divided among different tubes (20 ml/tube). Deionised water (negative control) and absolute ethanol (solvent controls) were added as well as the stock extract (150 mg/ml) to test the following concentrations: 250, 500 and 1000 µg/ml. Note that each extract treatment has its respective negative control, which ethanol and extract volumes are equivalents and inferior to 0.7% of total volume in the tube. Each mixture was immediately poured into a plate and after solidification one mycelium disc (8 mm of diameter) was placed in plate center. All inoculations were incubated in the dark under ventilation at 25 °C. After 9 days of incubation, images of the inoculations were taken and two perpendicular diameters of radial mycelium growth measured through *ImageJ* software. The mycelium growth of treatments was compared with the respective negative control. The mycelium growth inhibition percentage (MGI%) was calculated, the diameter average of control (dc) subtracted to the diameter average of correspondent treatment (dt) and then divided by same diameter average of control (Equation 2).

Equation 2: $\text{MGI\%} = \frac{dc - dt}{dc} \times 100$

2.5. Assessment of viability and mitochondrial inner membrane potential

Mitochondrial inner membrane potential was evaluated through flow cytometry in the wild type (wt) strain BY4741 of *S. cerevisiae*, using the mitochondrial membrane potential sensitive fluorochrome (DiOC6(3); Molecular Probes Eugene, OR) and propidium iodide (PI), which is used to quantify non-viable cells. To investigate the potential of mitochondrial membrane effects of *C. ladanifer* extract (CL), stock solutions were previously prepared: 30 mg/mL CL, 2 µM DiOC6(3) and 2 µg/mL PI using EtOH, DMSO and H_2O as solvents, respectively. Note that, for the assay, 0.5 % DMSO and 5 % EtOH of the total sample volume was never exceeded. For the assay, an inoculum of *S. cerevisiae* was prepared and incubated at 30 °C and 200 rpm, overnight in YPEtOH medium. In the next day, was to OD_{600} = 0.3 and incubated for further four hours under the same conditions. The OD_{600} was adjusted to 0.1 with fresh YPEtOH and divided in aliquots of 500 µL. The treatments were applied (100, 300 or 700 µg/ml extract), the controls were made with ethanol (extract solvent; solvent control) addition instead of extract and the negative control was made with H_2O addition instead of ethanol. The treatment and control samples were incubated

during 1 h at 30 °C and 200 rpm. An auto-fluorescence (without any fluorochrome) and two single labelling samples (DiOC6(3) or PI) were taken from the previously diluted culture, the PI simple labelling sample was centrifuged at 14 000 rcf for 2 min and resuspended in ethanol (dead cells sample) stored at 4 °C. The two simple labelling samples were used to identify and compensate a possible overlapping of the emission spectrum of DiOC6(3) with the one of PI. After treatment and control samples incubation, all samples (including the samples stored at 4 °C) were washed twice with MES buffer (0.1 mM $MgCl_2$, 2 % w/v glucose, pH 6.0). DiOC6(3) (20 nM) was added into each sample, except in the auto-fluorescence and dead cells samples and then incubated at 30 °C for 20 min in the dark. Further, PI (2 μg/mL) was added to all samples, except the simple labelling sample with living cells and autofluorescence, with incubation at 30 °C for 10 min in the dark. Afterwards, approximately 20,000 cells of each sample were immediately analysed by flow cytometry in a Cytoflex System B4-R2-V0 (BeckmanCoulter) flow cytometer with an argon-ion laser emitting a 488 nm beam at 15 mW. DiOC6(3) produces green fluorescence, which was collected through a 488 nm blocking filter, a 550 nm long-pass dichroic and a 525 nm band-pass filter, monitored in channel FL1. PI produces red fluorescence, which was collected through a 560 nm short-pass dichroic, 620 nm and 670 nm long-pass filters. The results were analysed using the CytExpert (version 2.4).

2.6. Statistical analysis

All results are presented as mean ± standard deviation (SD) from three independent assays. In the viability assays with the wt strain BY4741 of *S. cerevisiae* and filamentous fungi, the treatments were compared with the negative control. In the viability assays with *S. cerevisiae* mutants, three comparations were made: wt untreated with mutant untreated, mutant untreated with mutant treated and wt treated with mutant treated. The software GraphPad Prism v9.0 was used for the statistical analysis. The normality test was applied and, subsequently, One-way ANOVA and Dunnett tests were used for analysis of variance in viability assays with *S. cerevisiae* (wt). On the other hand, the t test was applied for the analysis of the results obtained with: mycelium growth assays and viability assays with *S. cerevisiae* and mutant strains. Of each test result a *p*-value that indicates the significance value of each tested sample. This significance is indicated in the figures with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) or **** ($p < 0.0001$).

3. Results and discussion

3.1. *S. cerevisiae* is sensitive to CL extract

The plant extracts are a mix of several components, some of them associated with toxic effects in fungal organisms, including human and plant fungal pathogens. The alcoholic extracts of *C. citrinus*, *C. ladanifer*, *F. angustifolia* and *P. terebinthus* were previously reported in the literature with potential antifungal activity due to the composition and/or antifungal activity of these plants or plants belonging to the same genus. In order to screen the ethanolic plant extracts, CC, CL, FA and PT, were prepared by doing ethanol extractions and tested in yeast, *S. cerevisiae* for assessment of viability as described in section 2.3. Ethanol was the extraction solvent and also solvent of the stock extract solutions, the same solvent was used to ensure that all components were dissolved. Furthermore, ethanol was chosen as it is less toxic than methanol regarding fungi. The solvent extract treatment, ethanol, did not present significant differences when compared with water treatment (Annex 1). The CC (Fig. 5A), FA (Fig. 5C) and PT (Fig. 5D) extracts did not promote loss of viability with any concentration used in the experimental times evaluated, since the effects observed were similar to the control. The same profile was observed for 150 and 300 µg/ml CL (Fig. 5B). However, for 400 and 500 µg/ml CL, viability decreased to around 60 % ($p < 0.05$ and $p < 0.01$) and 40 % ($p < 0.001$ and $p < 0.0001$), respectively, in all tested times. This suggests that CL caused toxicity to *S. cerevisiae*, revealing a dose-dependent effect, whereby the higher concentration tested was the most efficient. In addition, in the tested timepoints, the viability decrease was relatively constant, since CL's effect seemed to be maintained throughout the entire experiment. So, these experiments indicate that, in contrast to CC, PT and FA, CL extract has antifungal properties against yeast. The toxic effect of CL in *S. cerevisiae* yeast is in accordance with results previously reported by Li *et al.* (2015) and Barros *et al.* (2013). These investigators indicated that the *C. ladanifer* extract generated toxicity in human yeast pathogens, *C. albicans*, *C. tropicalis, C. glabrata* and *C. parapsilosis*. The antifungal activity of plant extracts can be associated to toxic effects caused by some components. Since ellagic acid derivatives are the most abundant components in the phenolic profile of *C. ladanifer* hydromethanolic extract, and Li *et al.* (2015) reported ellagic acid as having antifungal activity against two *Candida* species, the ellagic acid derivatives may be potential active agents of the extract. However, it is necessary to verify their presence in the ethanolic extract of CL by chemical analysis. Since *S. cerevisiae* was used as an experimental fungal model, the results suggest that the extract's activity may also be exerted against filamentous fungi, namely phytopathogenic fungi.

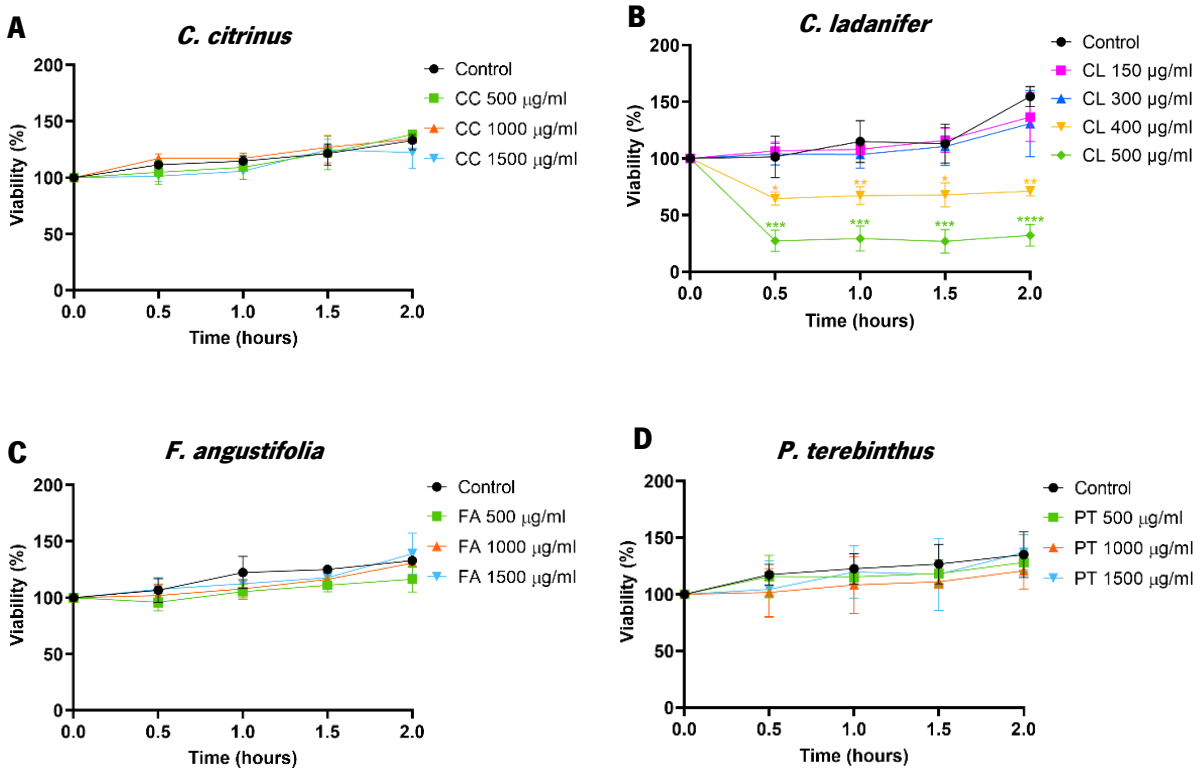

Figure 5 – Viability of *S. cerevisiae* is affected after treatment with of *C. ladanifer* but not with *C. citrinus, F. angustifolia* and *P. terebinthus* extracts. The cell suspension was incubated at 30 °C and 200 rpm to reach the OD_{600} between 0.4-0.6. The treatments were applied: 5 % EtOH, the solvent extract, (control); extract at different concentrations, 500, 1000 or 1500 µg/ml for *C. citrinus* (**A**), *F. angustifolia* (**C**) and *P. terebinthus* (**D**) and 150, 300, 400 or 500 µg/ml for *C. ladanifer* (**B**). The cell suspensions treated were incubated at different times: 0, 0.5, 1.0, 1.5 and 2 h and subsequently collected aliquots were serially diluted until 10^{-4}. Drops of the last dilution were placed in the plates and incubated for 48 h at 30 °C. The percentage of cell viability was calculated, the average value of CFUs was divided by the average value at 0 h. Data are presented as the average of three independent experiments ± SD. The variance analysis was performed using one-way ANOVA and Dunnett test for multiple comparisons: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. The significance is compared to the control in each time point.

3.2. CL extract inhibits the mycelium growth of *Colletotrichum acutatum*, *Colletotrichum gloeosporioides* and *Diplodia corticola*

To investigate the potential antifungal activity of CL in phytopathogenic fungi, *B. cinerea*, *C. acutatum*, *C. gloeosporioides* and *D. corticola* were challenged with different concentrations of the extract to study mycelium growth (Fig. 6) as described in section 2.4. The ethanol volume were inferior to 0.7 % of total volume in the tubes, while in the previous assay (viability assay with *S. cerevisiae*) were inferior to 5 %, since the filamentous fungi used in this work were more sensitive to ethanol in comparison to *S.cerevisiae*. Note that the diameter of the fungi colonies was not affected by the solvent of the extract, ethanol, as it did not show significant differences when compared to the negative control (Annex 2). The diameter of *B. cinerea* after 9 days of incubation with the extract tended to be smaller than the controls, but this difference was not significant (Fig. 6A), suggesting that CL was not toxic to this fungus. In contrast to *B. cinerea*, the diameters of *C. acutatum* with the 250 µg/ml ($p < 0.01$) or 500 µg/ml ($p < 0.05$) treatments were significantly lower when compared with the controls (Fig. 6B), with inhibition of 26.53 % and 38.09 %, respectively (Table 3), suggesting that CL is toxic to *C. acutatum* at these concentrations. In addition, the colour of the mycelium was lighter at higher concentrations (Fig. 6F). According to Langfelder *et al.* (2003) and Tsuji *et al.* (2003), melanin production by the fungus *Colletotrichum lagenarium* is an important factor for rush penetration into the host tissue, so CL extract in *C. acutatum* may be affecting melanin biosynthesis, decreasing virulence of this fungus. In a similar way to *C. acutatum*, the results with *C. gloeosporioides* suggest that CL is toxic to this fungus, since in the presence of 500 µg/ml ($p < 0.05$) or 1000 µg/ml ($p < 0.01$) CL extract, the mycelium diameter was significantly lower when compared with the controls (Fig. 6C), with inhibition of 30.32 % and 41.97 %, respectively (Table 3). Furthermore, a morphological difference was observed: in the controls, the mycelium appeared to have a cottony texture and in the presence of extract that texture was not so evident (Fig. 6G). Finally, the diameter of *D. corticola* was significantly smaller at all concentrations tested ($p < 0.01$) than in the controls (Fig. 6D). Particularly at the 250 µg/ml treatment, the %MGI, 31.56 %, was higher than all other fungi tested (Table 3). Therefore, the observations suggest an antifungal activity of CL, not only in yeast, as discussed previously (section 3.1), but also in phytopathogenic fungi (*C. acutatum*, *C. gloeosporioides* and *D. corticola*). The antifungal activity of CL ethanolic extract in phytopathogenic fungi is in accordance with Karim *et al.* (2017), in which aqueous extract of *C. ladanifer* is indicated as an inducer of toxicity to *Geotrichum candidum.* It is possible that one or more components of the aqueous extract are also present in the ethanolic extract or that the ethanolic extract presents different antifungal components from the aqueous extract. Additionally, the ellagic acid derivatives are major components in the phenolic profile of

C. ladanifer (Barros *et al.*, 2013) and ellagic acid presented antifungal activity against *Trichophyton* species, which are pathogenic to plants (Li *et al.*, 2015). However, as far as we know, this is the first time that CL extract is tested in fungi with relevance for crops of high economic importance in Portugal.

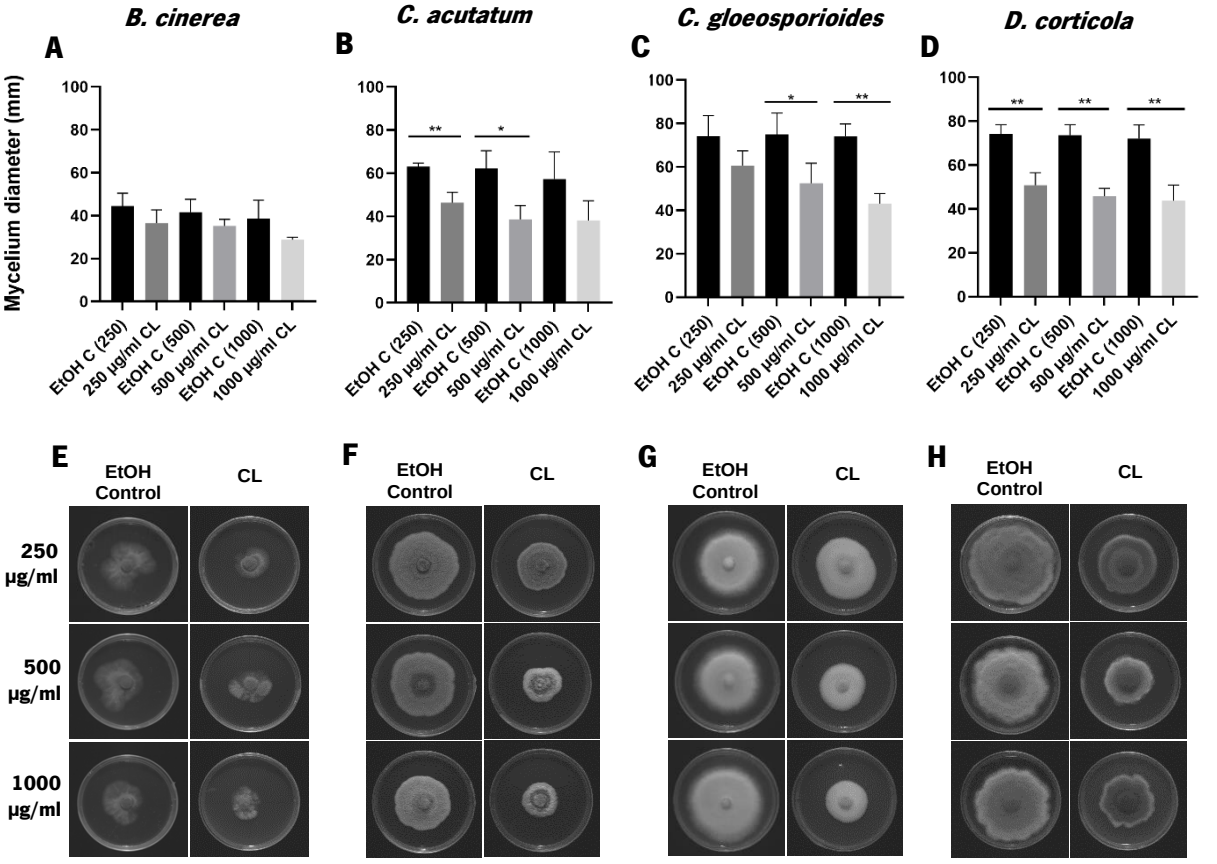

Figure 6 – Mycelium growth of *C. acutatum*, *C. gloeosporioides* and *D. corticola* is decreased in the presence of *C. ladanifer* (CL) extract but not with *B. cinerea*. The extract was incorporated in PDA medium: CL extract at different concentrations, 250, 500 or 1000 µg/ml, and controls (C), which ethanol has the same amount as the extract. In each plate a mycelium disk of fungus was placed in the center and incubated for 9 days at 25 °C in the dark. The mycelium diameter (**A-D**) and the mycelium images (**E-H**) of fungi, *B. cinerea* (**A and E**), *C. acutatum* (**B and F**), *C. gloeosporioides* (**C and G**) and *D. corticola* (**D and H**), were determined. Mycelium diameter data are presented as the average of three independent experiments ± SD. The variance analysis was performed using t tests for paired comparisons: $^{*}p < 0.05$ and $^{**}p < 0.01$. The significance is compared to the respective control. Mycelium images are representative of three experiments.

Table 3 – Mycelium growth inhibition (%) of *B. cinerea*, *C. acutatum*, *C. gloeosporioides* and *D. corticola*. The fungi were exposed to 250, 500 or 1000 µg/ml CL extract for 9 days of incubation at 25 °C. Data are presented as mean of three independent experiments ± SD.

CL extract (µg/ml)	Mycelium growth inhibition (%)			
	B. cinerea	*C. acutatum*	*C. gloeosporioides*	*D. corticola*
250	15.84 ± 25.70	26.53 ± 5.50	18.02 ± 5.12	31.56 ± 6.58
500	13.37 ± 20.94	38.09 ± 4.00	30.32 ± 4.00	37.42 ± 8.76
1000	22.58 ± 21.12	40.53 ± 11.25	41.97 ± 3.70	38.75 ± 12.96

3.3. *erg2*, *bck1*, *mkk1/mkk2* and *yca1* mutants present different susceptibilities to CL extract than wild type strains

To investigate the mechanisms of action of CL associated to the toxicity on fungi, five strains, BY4741 wt and four derived mutants, were challenged with CL. The mutants were selected in order to study mechanisms associated with (1) ergosterol biosynthesis, *erg2* mutant; (2) cell wall integrity pathway, *bck1* and *mkk1/mkk2* mutants (Jin *et al.*, 2015) and; (3) apoptotic process, *yca1* mutant (Khan *et al.*, 2005). Note that the mutant strains have deletion of one gene, except the *mkk1/mkk2* mutant where there is deletion of two genes, *MKK1* and *MKK2*. For these assays we used 400 µg/ml CL, since the percentage of viability for this treatment was, approximately, 70 % (Fig. 5), which allows us to measure viability values above and below this value. The viability of mutants was evaluated at different timepoints by viability assays as described in the section 2.3.

The biosynthesis of ergosterol is targeted by widely used fungicides (e.g. azoles), since it is the main sterol in the plasma membrane of fungi, in contrast to other eukaryotes (Hung *et al.*, 2016). In this pathway, fecosterol is converted to episterol by C-8 sterol isomerase, an enzyme located in the endoplasmic reticulum of *S. cerevisiae,* which results from the expression of *ERG2*. Moreover, *ERG2* is also down-regulated in transcription when ergosterol is in excess (Abe & Hiraki, 2009; Arthington *et al.*, 1991; Ashman *et al.*, 1991; Emter *et al.,* 2002; Kaur & Bachhawat, 1999; Silve *et al.*, 1996; Yuan & Ching, 2015). So, if CL interferes with ergosterol biosynthesis, the *erg2* mutant might be more sensitive to CL relative to the wt strain. The *erg2* mutant strain displayed similar viability as the wt strain, however, when in the presence of the extract, viability was less affected (Fig. 7A), with significant differences at 0.5, 1.5 and 2 h ($p > 0.01$; Annex 3). These results suggest that the mechanism of action of the extract requires the presence of the functional Erg2 protein. According to the literature (Kodedová & Sychrová, 2015), when treated with nystatin, a fungicide that binds to ergosterol within the plasma membrane to generate pores, the *erg2* mutant exhibited higher resistance when compared with the wt (BY4741). These authors hypothesise that this is due to a lower affinity of nystatin for fecosterol (substrate of sterol C-8 isomerase), which is accumulated in this mutant. Therefore, CL may have lower affinity for fecosterol and it is possible that at least one component of CL may interact with membrane ergosterol leading to antifungal activity.

Cell wall integrity is critical for cell viability, so destabilisation of the cell wall is a potential mechanism for a decrease of cell viability. Cell wall integrity is regulated by the protein kinase C (PKC1) signalling pathway. Upon activation of Pkc1 by phosphorylation, the signal is transduced in the cytoplasm through the following order: Bck1 protein (protein that results from *BCK1* expression), Mkk1 and Mkk2 redundant

proteins (proteins that result from *MKK1* and *MKK2* expression, respectively) and, finally, Slt2. This signal generates a response that will remodulate the cell wall (Byrne & Wolfe, 2005; Heinisch *et al.*, 1999; Irie *et al.*, 1993; Soler *et al.* , 1995). Additionally, it was reported that Slt2 is fully dependent on Bck1 activation (Arias et al., 2011). Therefore, we used the *bck1* mutant to test if the activity of the extract was still present when the PKC1 pathway was interrupted. So, if CL destabilises cell wall integrity, the *bck1* and *mkk1/mkk2* mutants would be more sensitive to CL in relation to the wt strain. The *bck1* mutant strain displayed similar growth as the wt strain, however, when in the presence of the extract, viability was less affected (Fig. 7B), with significant differences at 0.5 h ($p < 0.05$), 1.0 h ($p < 0.01$), 1.5 h ($p < 0.05$) and 2 h ($p < 0.0001$; Annex 3). These results suggest that the mechanism of action of the extract requires the presence of the functional Bck1 protein. To support the resistance of *bck1* mutant to CL, the *mkk1/mkk2* double mutant was used. The *mkk1/mkk2* mutant strain displayed similar growth as the wt strain, however, when in the presence of the extract, viability was less affected (Fig. 7C), with significant differences at 1.0 h and 2 h ($p < 0.05$; Annex 3). These results suggest that the mechanism of action of the extract requires the presence of the functional Mkk1/Mkk2 protein and support the *bck1* mutant resistance. If a CL compound acts, for example, as an allosteric modulator of the Bck1, Mkk1 or Mkk2 or if one of these enzymes acts in another pathway, then this may explain the higher resistance of these mutants to CL compared to the wt strain. Nevertheless, the resistance suggests that the cell wall integrity pathway is involved in the antifungal mechanism of action.

The apoptosis process is a form of programmed cell death in which several events occur, such as loss of cell volume, nuclear and DNA fragmentation among others. This highly regulated process, upon stress induction, has the metacaspase Yca1 as one of the positive regulators of apoptosis. Yca1 is an Ca^{2+}-dependent cysteine protease with endopeptidase activity localized in the cytosol and nucleus and resulting from *YCA1* gene expression (Moreno-Càceres & Fabregat, 2015; Severin *et al.*, 2008; Wong *et al.*, 2012). The *yca1* deletion increases the cellular resistance to stress by blocking the programmed cell death (Severin *et al.*, 2008). So, if CL induces apoptosis, the *yca1* mutant would be more resistant to CL than the wt strain. The *yca1* mutant strain displayed similar growth as the wt strain, however, when in the presence of the extract, viability was more affected (Fig. 7D), with significant differences at 0.5 h ($p < 0.05$), 1.0 h ($p < 0.05$), 1.5 h ($p < 0.01$) and 2 h ($p < 0.001$; Annex 3). These results suggest that the toxic activity of the extract requires the presence of the functional Yca1 protein. If a CL compound acts, for example, as a positive allosteric modulator of the metacaspase Yca1 or if this enzyme acts in another pathway, then this may explain that *yca1* mutant is more sensitive to CL than the wt strain. However, based on the observed results alone, the hypothesis that CL induces apoptosis cannot be ruled out, since,

according to Chin *et al.* (2014), the caspofungin fungicide seems to induce cell death without requiring the *YCA1* gene, but requiring the pro-apoptotic *AIF1* gene.

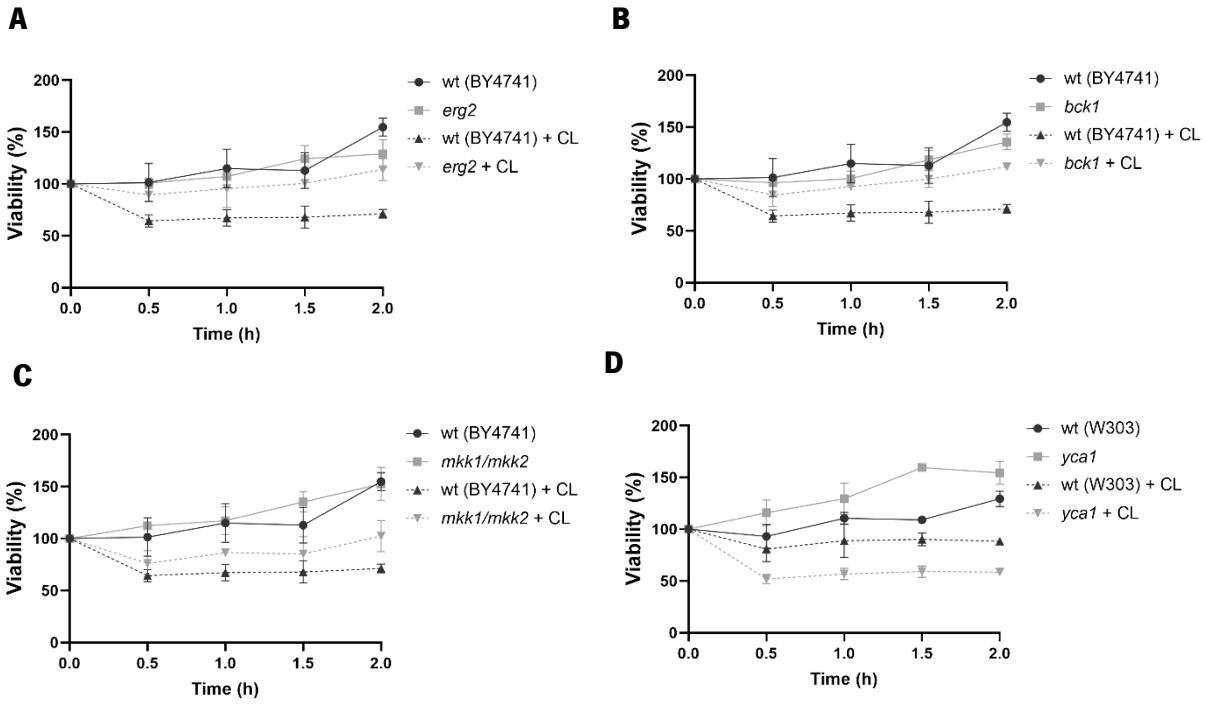

Figure 7 – *erg2, bck1* and *mkk1/mkk2* mutant strains appear to be more resistant and *yca1* mutant more sensitive to *C. ladanifer* (CL) extract than wild type (wt) strains. Cell suspensions were incubated at 30 °C and 200 rpm to reach the OD_{600} between 0.4-0.6. The treatments were applied in (**A**) *erg2*, (**B**) *bck1*, (**C**) *mkk1/mkk2* and (**D**) *yca1* mutant strains, as well as the respective wild type (wt), BY4741 and W303. The treatments were: 5 % EtOH, the solvent of the extract (controls, wt), and CL extract at 400 μg/ml. The cell suspensions were incubated at different times: 0, 0.5, 1.0, 1.5 and 2 h and subsequently collected aliquots were serially diluted until 10^{-4}. Drops of the last dilution were placed in the plates and incubated for 48 h at 30 °C. The percentage of cell viability was calculated, the average value of CFUs was divided by the average value at 0 h. Data are presented as the average of three independent experiments ± SD.

3.4. CL extract affects the inner mitochondrial membrane potential

Apoptotic stimuli can lead to a depolarization of the mitochondrial inner membrane potential and consequently cause structural changes in mitochondria (Kroemer & Reed, 2000). Therefore, the redistribution of cytochrome C (Cyt C) from the cristae of the inner membrane to the intermembrane space, makes Cyt C more susceptible to release. Cytosolic Cyt C, besides being indicated as a caspase cascade activator, was indicated to be involved in Yca1 metacaspase activation (Khan *et al.*, 2005). To investigate the effect of CL extract in potential of inner mitochondrial membrane, a flow cytometry assay was made with the fluorochrome DiOC6(3). In this assay, YPEtOH medium was used instead of YPD in order to prevent fermentation as the main pathway for energy production, so that cells would have to use ethanol as a respiring substrate as their sole carbon and energy source (Gasmi *et al.*, 2014). Thus, mitochondria would be more active and the DiOC6(3) staining would be ensured. DiOC6(3) is a lipophilic cationic fluorescent dye that crosses freely through plasma membrane and accumulates in mitochondria due to their large negative membrane potential, allowing the measurement of membrane potential state

(Chang, 2013). Thus, lower values of membrane potential of the inner mitochondria (depolarization), promote a lower accumulation of DiOC6(3), resulting in lower DiOC6(3) fluorescence. The results of the double labelling with DiOC6(3) and PI (Annex 4) were treated in order to select the cells negatively labelled with PI fluorescence (Annex 5) to obtain representative data of viable cells (Fig. 8). It was confirmed that the extract solvent did not affect the mitochondrial membrane potential (Annex 6). The three treatments with CL displayed less fluorescence relative to the control in both experiments, which indicates that CL causes depolarization of the inner mitochondrial membrane. The membrane depolarization correlates with an apoptosis process in the cells. With the 100 µg/mL and 300 µg/mL CL treatments, a dose-response effect was observed. However, results with 700 µg/ml might be correlated with a necrosis process because the population displayed a wide range of depolarization of the mitochondria, which would not be expected from a regulated process like apoptosis. If CL leads to depolarisation of the mitochondrial membrane, then CL may be involved in, at least, one process that induces this effect, including the process of apoptosis. Nevertheless, taking only into account the previous result with the *yca1* mutant (section 3.3), whose effect does not suggest apoptosis as a mechanism of antifungal action, CL might affect other processes that lead to membrane depolarization.

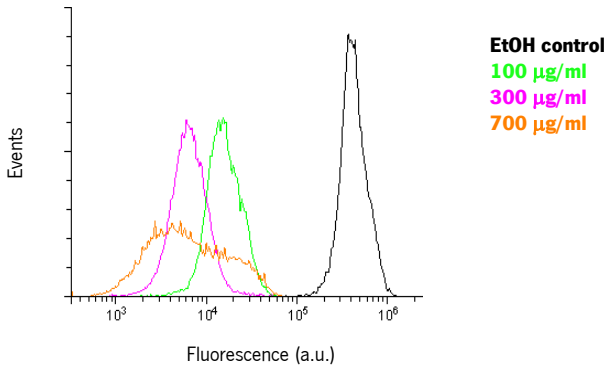

Figure 8 – CL extract triggers the depolarization of inner mitochondrial membrane in *S. cerevisiae.* The cell suspension was incubated in YPEtOH medium at 30 °C and 200 rpm to reach the OD_{600} between 0.4-0.6. The treatments were applied: 5 % EtOH (solvent control, black line) and CL extract at different concentrations, 100 (**green line**), 300 (**pink line**) and 700 (**orange line**) µg/ml. The cell suspensions treated were incubated for 1 h at 30 °C and 200 rpm. The fluorochromes were applied: DiOC6(3), for 30 min, and PI, for 10 min, at 30 °C and 200 rpm. The flow cytometry analysis was performed, whereby of the total events only PI negative events were selected to analyse the intensity of DiOC6(3) fluorescence. Data is representative of two independent experiments (a.u. means arbitrary units).

4. Conclusions and future perspectives

In this work we aimed to study plant extracts with potential antifungal activity and their possible mechanisms of action against relevant phytopathogenic fungi in Portugal for use in sustainable agricultural practices.

Based on the analysis of the viability of *S. cerevisiae* when exposed to *C. citrinus*, *C. ladanifer*, *F. angustifolia* or *P. terebinthus*, it was concluded that only *C. ladanifer* showed toxicity against the yeast. The toxic properties of CL extract, in part, may be contributed by ellagic acid derivates, since ellagic acids were described as having antifungal activity and were reported to be present in extracts of CL. However, to confirm this hypothesis and to discover other compounds that may be responsible for antifungal activity, chemical analysis of the extract must be performed. The identification of compounds and their respective quantification can be achieved by high performed liquid chromatography (HPLC) coupled to MS/MS. Subsequently, the compounds should be isolated, or purchased if commercially available, and used to test their toxic effects against fungi.

The CL extract was tested against phytopathogenic fungi and, after 9 days of exposure to 500 µg/ml CL, an inhibition of the growth of mycelium of *C. acutatum*, *C. gloeosporioides* and *D. corticola* of 38.09%, 30.32% and 37.42%, respectively, was observed. In the future, it would be interesting to see if the extract affects any of the proteins involved in melanin synthesis, since melanin production was indicated as an essential factor for penetration into the host (Langfelder *et al.*, 2003; Tsuji *et al.*, 2003) and the colour of the *C. acutatum* mycelium was lighter when exposed to the higher concentrations. Furthermore, the number of *B. cinerea* spores in suspension after incubation with the extract and the method of measuring the area of the mycelium could be experiments to be performed to further evaluate the antifungal activity against this fungus, since the growth of the mycelium were quite variable and, consequently, the values of standard deviations were high.

According to viability evaluation of the mutants, *erg2, bck1* and *mkk1/mkk2* were more resistant, when exposed to the extract, revealing that it may affects the ergosterol present in cell membrane and the cell wall integrity pathway. New experiments could be carried out with other mutants involved in these pathways, such as *pcl1, slt2, erg6* and *erg4*, to better understand the mode of action of the extract. Furthermore, it would be interesting to study if any of the compounds of the extract binds directly to membrane ergosterol, since the result obtained with *erg2* was similar to nystatin, a fungicide with this mode of action. Besides the mutants mentioned, the viability of the *yca1* mutant was also studied. This mutant revealed to be more sensitive when exposed to the extract, suggesting that the extract does not induce apoptosis, but affects this pathway. This study also indicated that CL leads to membrane

depolarisation, which happens in different cell processes, including apoptosis. However, the *yca1* mutant result should be confirmed using the *aif1* mutant, since the caspofungin fungicide seems to induce cell death without requiring the *YCA1* gene, but requiring the pro-apoptotic *AIF1* (Chin *et al.* (2014). If *aif1* is more resistant to the extract, then depolarisation of the mitochondrial membrane may be due to induction of apoptosis. If *aif1* is not affected or is more sensitive to the extract, then one of the mechanisms of action may be associated with other processes leading to membrane depolarisation. To complement the study of the effect of CL on apoptosis, cytometry assays with Annexin V or TUNEL staining can also be performed.

Since the objective is to formulate a natural fungicide derived from CL extract, further research is needed to determine its activity on plants infected with the fungi studied in this work, as already reported with other extracts on strawberry leaves (Ferreira, 2021). Encapsulation of this extract could also be carried out, as it has been done with olive leaf extract (Tavakoli *et al.* , 2018), to reduce the dosage of extract needed per cultivated area.

To conclude, this study found the extract of a Portuguese native plant, *C. ladanifer*, to be a potential antifungal against pathogenic fungi, which could prevent economic impact in strawberry, chestnut, cork and grapevines in a sustainable way. This study raised new questions about possible mechanisms of action that should help further research.

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6. Annexes

Annex 1

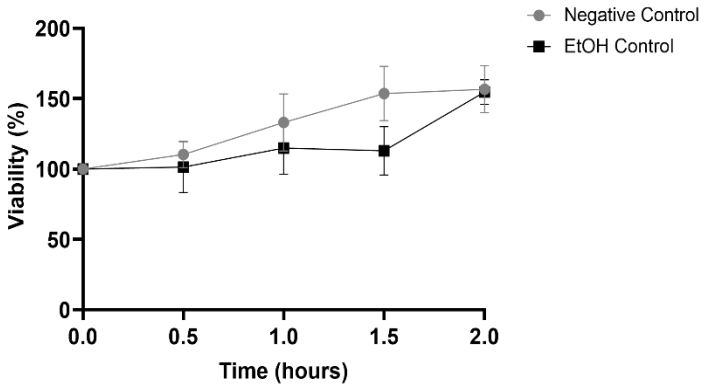

Figure 9 – Viability of *S. cerevisiae* is not affected by the solvent extracts (EtOH). The cell suspension was incubated at 30 °C and 200 rpm to reach the OD_{600} between 0.4-0.6. The treatments were applied: 5 % H_2O (negative control) or 5 % EtOH (solvent control). The cell suspensions treated were incubated at different times: 0, 0.5, 1.0, 1.5 and 2 h and subsequently collected aliquots were serially diluted until 10^{-4}. Drops of the last dilution were placed in the plates and incubated for 48 h at 30 °C. The percentage of cell viability was calculated, the average value of CFUs was divided by the average value at 0 h. Data are presented as the average of three independent experiments ± SD. The variance analysis was performed using t test for pair comparisons. The significance is compared to the control in each time point.

Annex 2

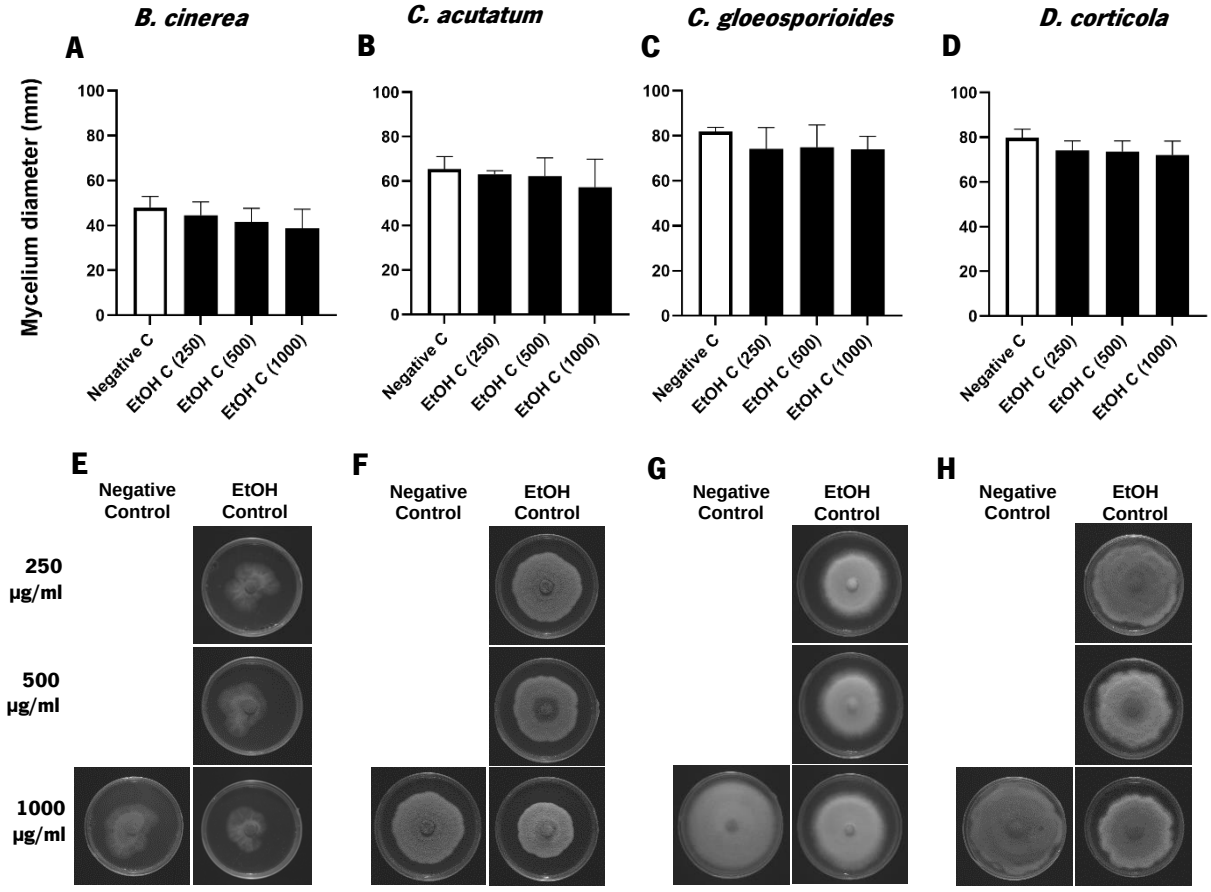

Figure 10 – Mycelium growth of *B. cinerea*, *C. acutatum*, *C. gloeosporioides* and *D. corticola* are not affected by the solvent extracts (EtOH). The treatments were incorporated in PDA medium: H_2O (negative control) and EtOH (solvent control). The volume of water is equal to the maximum volume of EtOH used in the viability assays with filamentous fungi. In each plate a mycelium disk of one fungus was placed in center and incubated for 9 days at 25 °C in the dark. The mycelium diameter (**A-D**) and the mycelium images (E-H) of fungi, *B. cinerea* (**A and E**), *C. acutatum* (**B and F**), *C. gloeosporioides* (**C and G**) and *D. corticola* (**D and H**), were determined and taken. The mycelium diameter was calculated, the average of two measured perpendicular diameters. Mycelium diameter data are presented as the average of three independent experiments ± SD. The variance analysis was performed using one-way ANOVA and Dunnett test for multiple comparisons. The significance was compared to the control. Mycelium images are representative of three experiments.

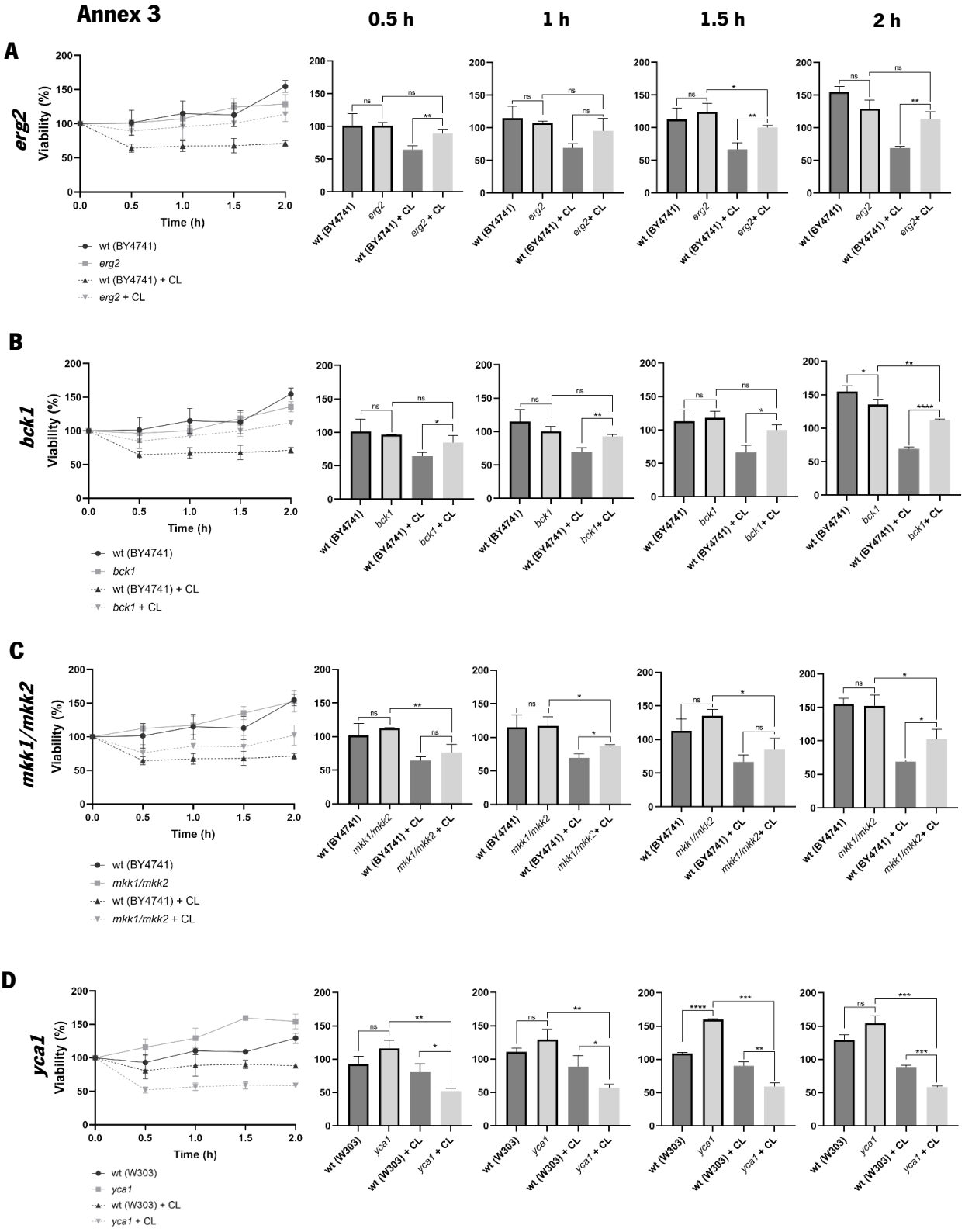

Figure 11 – *Erg2, bck1 and mkk1/mkk2* mutant strains appears to be more resistant and *yca1* mutant more sensitive to *C. ladanifer* (CL) extract than wild type (wt) strains. The cell suspension was incubated at 30 °C and 200 rpm to reach the OD_{600} between 0.4-0.6. The treatments were applied in (**A**) *erg2*, (**B**) *bck1*, (**C**) *mkk1/mkk2* and (**D**) *yca1* mutant strains, as well as the respective wild types (wt), BY4741 and W303. The treatments were: 5 % EtOH, the solvent extract (solvent controls, wt), and CL extract at 400 µg/ml. The cell suspensions treated were incubated at different times: 0, 0.5, 1.0, 1.5 and 2 h and subsequently collected aliquots were diluted until 10^{-4}. Drops of the last dilution were placed in the plates and incubated for 48 h at 30 °C. The percentage of cell viability was calculated, the average value of CFUs was divided by the average value at 0 h. Data are presented as the average of three independent experiments ± SD. The variance analysis was performed using t tests for each pair of comparisons: ns – not significant, *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. The significance is compared between pairs: wt untreated with mutant untreated, mutant untreated with mutant treated and wt treated with mutant treated.

Annex 4

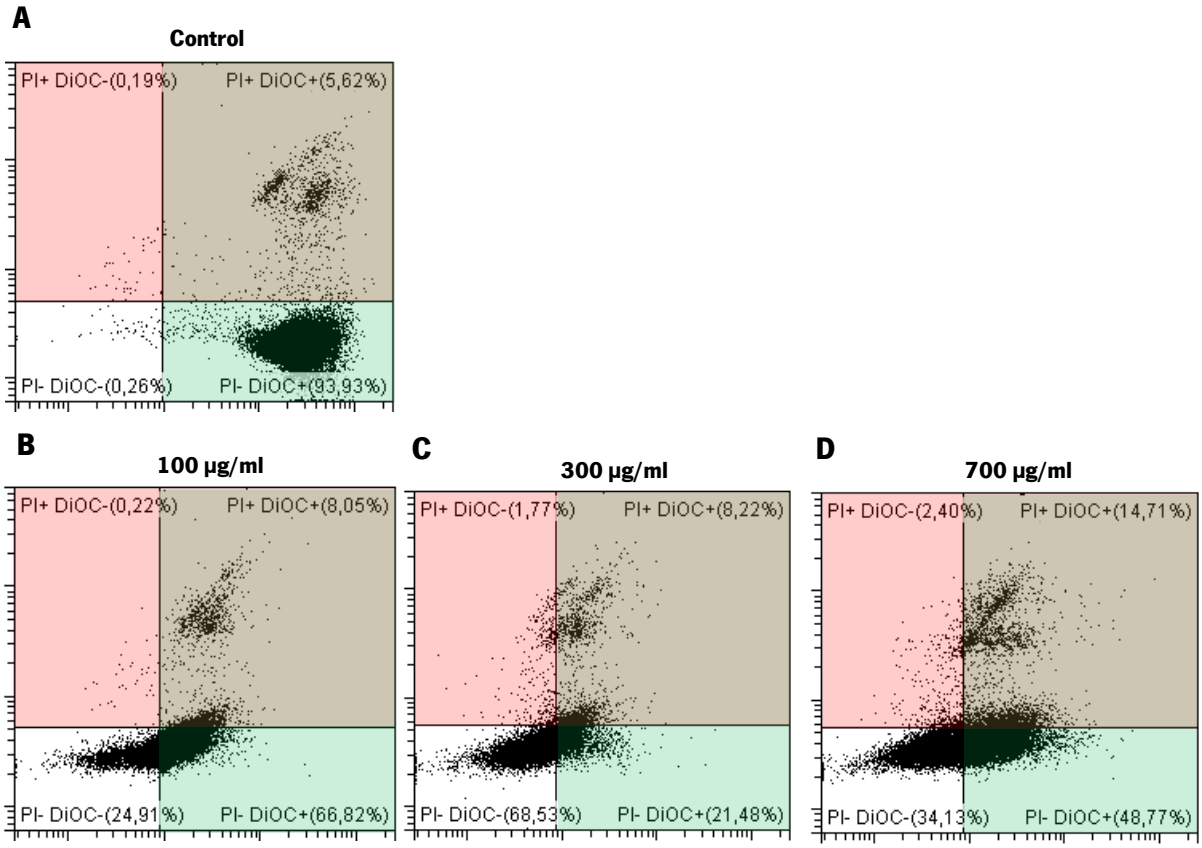

Figure 12 – CL extract triggers the depolarization of inner mitochondrial membrane in *S. cerevisiae.* The cell suspension was incubated in YPEtOH medium at 30 °C and 200 rpm to reach the OD_{600} between 0.4-0.6. The treatments were applied: (**A**) 5 % EtOH, the solvent extract, (solvent control) and CL extract at different concentrations, (**B**) 100, (**C**) 300 and (**D**) 700 µg/ml. The cell suspensions treated were incubated for 1 h at 30 °C and 200 rpm. The fluorochromes were applied: DiOC6(3), for 30 min, and PI, for 10 min, at 30 °C and 200 rpm. The flow cytometry analysis was performed, whereby of the total events only PI negative events were selected to analyse the intensity of DiOC6(3) fluorescence. Dot-polt - events without (-) and with (+) marking of PI and/or DiOC6(3): **white** (PI- DiOC-), **red** (PI+ DiOC-), **green** (PI- DiOC+) and **brown** (PI+ DiOC+) areas. Data representative of two independent experiments.

Annex 5

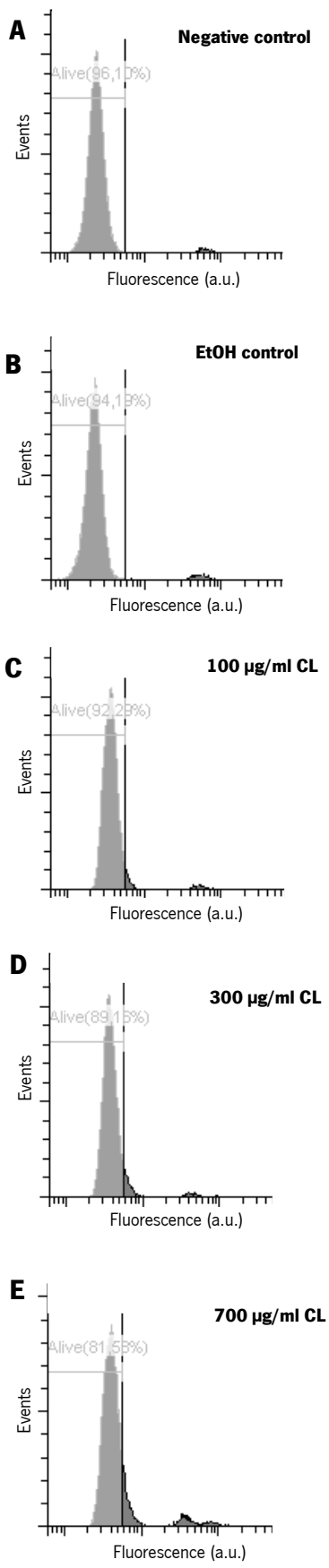

Figure 13 – Selection of metabolically active cells. The selected cells (negative for PI fluorescence, represented by the region named alive) in each tested situation for CL extract described in section 2.5 and represented in Fig. 8. (**A**) H_2O (negative control), (**B**) EtOH (solvent control), (**C**) 100 µg/ml CL extract; (**D**) 300 µg/ml CL extract (**E**) 700 µg/ml CL extract.

Annex 6

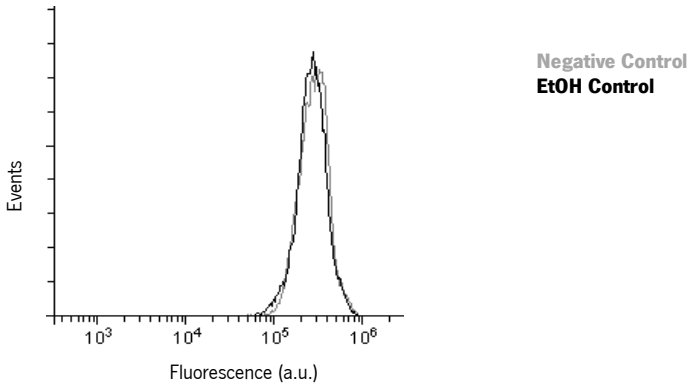

Figure 14 – The CL extract solvent did not affect the polarization of inner mitochondrial membrane in *S. cerevisiae*. The cell suspension was incubated in YPEtOH medium at 30 °C and 200 rpm to reach the OD_{600} between 0.4-0.6. The treatments were applied: H_2O (negative control, grey line) and EtOH, (solvent control, black line). The cell suspensions treated were incubated for 1 h at 30 °C and 200 rpm. The fluorochromes were applied: DiOC6(3), for 30 min, and PI, for 10 min, at 30 °C and 200 rpm. The flow cytometry analysis was performed, whereby of the total events only PI negative events were selected to analyse the intensity of DiOC6(3) fluorescence. Data representative of two independent experiments.