Universidade do Minho
Escola de Ciências

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Analysis of antifungal extract production from plants and *in vivo* assessment of activity

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Professora Doutora Ana Cristina Gomes da Cunha

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Despacho RT - 31 /2019 - Anexo 3

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Despacho RT - 31 /2019 - Anexo 4

STATEMENT OF INTEGRITY

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

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

RESUMO

O uso constante e excessivo de fungicidas sintéticos contribui para o aumento da poluição dos solos, das águas, o surgimento de resistência dos fungos alvo bem como possíveis efeitos de toxicidade para a saúde humana. Estas adversidades têm estimulado cada vez mais a procura de novas alternativas mais sustentáveis para o ambiente. Os extratos vegetais normalmente ricos em metabolitos secundários, contêm frequentemente compostos com propriedades antifúngicas. Estes compostos poderão ser alternativas aos fungicidas sintéticos uma vez que não há o risco de acumulação no ambiente e atuam em vários locais do fungo alvo.

Assim, o principal objetivo deste trabalho foi avaliar a atividade antifúngica de várias espécies de plantas: *Digitalis purpurea*, *Eucalyptus globulus*, *Beta vulgaris* e *Hedera helix*. O extrato aquoso hera foi selecionado devido aos resultados promissores obtidos nos ensaios de viabilidade da levedura *Saccharomyces cerevisiae*, organismo modelo utilizado. Além disso, foram utilizadas estirpes mutantes de *S. cerevisiae* para estudar o possível mecanismo de ação do extrato aquoso de *Hedera helix*. Os ensaios de viabilidade sugerem que, pelo menos um dos alvos é a parede celular. Estes resultados foram reforçados através de ensaios de sensibilidade osmótica, onde foi observada uma menor viabilidade celular em células cultivadas na presença de 50 µg/ml de extrato em meio YPD contendo a maior concentração de NaCl utilizada (0,75 M).

O extrato de *Hedera helix* foi testado contra várias espécies de fungos fitopatogénicos tais como do género *Colletotrichum* (*C. acutatum*, *C. nymphaeae*, *C. gloeosporioides*, *C. godetiae*), *Diplodia corticola* e *Phytophthora cinnamomi*. Dos fungos utilizados, *Diplodia corticola* foi o mais suscetível à ação do extrato aquoso de *H. helix*, com uma inibição de 70 % após 3 dias de incubação. Adicionalmente, foram realizados dois ensaios *ex vivo* em plantas. Com o ensaio em folhas de morangueiro foi possível verificar um atraso da infeção por *C. acutatum* e ausência de efeitos fitotóxicos na concentração testada. O ensaio com estacas de sobreiro foi inconclusivo necessitando de otimização do protocolo.

Concluindo, o extrato de *Hedera helix* poderá ser uma fonte para novos fungicidas naturais, nomeadamente contra *Diplodia corticola*. Considerando que este fungo ameaça os sobreiros, fonte de um material economicamente importante e altamente valorizado, a cortiça, os resultados deste trabalho abrem a possibilidade de uma prevenção das infeções fúngicas de uma forma mais biossustentável e um aumento da produção de cortiça.

Palavras chave: atividade antifúngica, mecanismo de ação, *Diplodia corticola, Hedera helix*, *Saccharomyces cerevisiae*

ABSTRACT

The negative impact caused by the constant and excessive use of synthetic fungicides contributes to soil and water pollution, the emergence of resistance of target fungi as well as possible toxicity effects on human health. These adversities have increasingly stimulated the search for new and more sustainable alternatives for the environment. Plant extracts normally rich in secondary metabolites, often contain compounds with antifungal properties. These compounds could be alternatives to synthetic fungicides as they do not accumulate in the environment and act on several sites of the target fungus.

With this in background, the main aim of the work was to evaluate the antifungal activity of several plant species: *Digitalis purpurea*, *Eucalyptus globulus*, *Beta vulgaris* and *Hedera helix.* The ivy aqueous extract was selected because of the promising results obtained in the yeast *Saccharomyces cerevisiae* (model organism used) viability assays. In addition, mutant strains of *S. cerevisiae* were used to study the possible mechanism of action of the aqueous extract of *Hedera helix*. Viability assays suggested that, at least one of the targets is the cell wall. These results were reinforced through osmotic sensitivity assays, where lower cell viability was seen in cells grown in the presence of 50 µg/ ml extract and plated on YPD medium containing the highest NaCl concentration used, 0.75 M.

Hedera helix extract was tested against various species of phytopathogenic fungi such as of *Colletotrichum* genus *(C. acutatum, C. nymphaeae, C. gloeosporioides, C. godetiae)*, *Diplodia corticola* and *Phytophthora cinnamomi*. Among the fungi used, *Diplodia corticola* was the most susceptible to the action of the aqueous extract of *H. helix*, with an inhibition of 70 % after 3 days of inoculation. Two plant *ex vivo* assays were conducted. With the strawberry leaves assay it was possible to verify a delay of infection of *C. acutatum* and absence of phytotoxic effects at the concentration tested. The cork oak cutting assay was inconclusive and needs further protocol optimization.

In conclusion, it is believed that ivy extract could be a source for new natural fungicides, namely against *Diplodia corticola*. Considering that this fungus threatens cork oak stands, the source of an economically important and highly valued material, the cork, the results of this work open the possibility of an environmental-friendlier prevention of fungal infections and the improvement of cork production.

Keywords: antifungal activity, antifungal mechanism of action, *Diplodia corticola, Hedera helix*, *Saccharomyces cerevisiae*

LIST OF CONTENTS

LIST OF ABBREVIATIONS

dH_2O	Deionized water
CFU	Colony forming units
MIC	Minimum Inhibitory Concentration
YPDA	Yeast- Peptone- Dextrose- Agar
YPD	Yeast- Peptone- Dextrose
PDA	Potato- Dextrose-Agar
RPM	Revolutions per minute
OD	Optical Density
SD	Standard deviation
MS	Murashige and Skoog
WHO	World Health Organization
(FAOSTAT)	Food and Agriculture Organization of the United Nations
C^-	Negative control
CI	Infection control
CE	Extract control

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1 Agriculture sector: importance and challenges in crop management

Agriculture occupies nowadays 38 % of the Earth's land and is since ancient times recognized as crucial to provide food and assure food safety. It is also an important sector in human health as far as allows the improvement and diversification of human nutrition (Jones and Ejeta, 2016; Reganold and Wachter, 2016) and has an important role in the world economy (Jiménez-Reyes *et al.*, 2019). Between 2013 and 2017, according to the Food and Agriculture Organization of the United Nations (FAOSTAT), cereals, sugarcane, vegetables and fruits were some of the crops most produced in the world (Brauer *et al.*, 2019). Some of these crops, namely fruits, grains and vegetables, are an essential source of economic income nationally and internationally (Jiménez-Reyes *et al.*, 2019). The virtuous relationship between natural conditions, as soil and climate, and anthropogenic factors, such as advances in organization and in technical, chemical and biological sciences, is essential for the production of satisfactory agricultural quantities (Baer-Nawrocka and Sadowski, 2019). However, the expansion of food production has triggered several negative consequences for the environment, namely the disappearance of large parts of forestry areas culminating in the release of greenhouse gases, the rapid depletion of underground water sources and the decrease in biodiversity (FAO, 2017). Beyond this there are many threats to this sector such as climate change impacts, pests and diseases, urbanization and the rapid world population increase (Calicioglu *et al.*, 2019). The continuous decrease in agricultural productivity limits and hinders the access to the nutrients and vitamins. In India, Food Policy Research Institute (IFPRI) suggests that the low agricultural productivity is one of the reasons to the malnutrition, essentially in poor families (Blackie, 2014). Furthermore, it is known that the low consumption of fruits and vegetables is associated with the development of some pathologies, such as type 2 diabetes, hypertension and cardiovascular diseases (Hall *et al.*, 2009). According to Food and Agriculture Organization of the United Nations (FAOSTAT) in order to ensure food to the world population in continuous growth, which may reach 10 billion in 2050, the food production should increase by 70 % (Demestichas *et al.*, 2020).

Among the impacts on agriculture, plant diseases are associated with major production and economic losses (Sankaran *et al.*, 2010). So, to keep the sustainability of this sector, besides control measures, it is fundamental monitoring plant sanitary conditions and the detection of plant diseases (Sankaran *et al.*, 2010; Fang and Ramasamy, 2015). Since the beginning of agriculture that pests have impacts in agricultural production causing decreases in productivity. According to FAO, pests are defined as any species, breed or biotype of plant, animal or pathogen, harmful to

plants and plant products (FAO, 2009). Pests are generally grouped as animal pests (insects, mites, nematodes, rodents, slugs and snails, birds), plant pathogens (viruses, bacteria, fungi, chromista) and weeds (competitive plants), being fungi the most devastating plant pathogens (Oerk, 2006; Möller and Stukenbrock, 2017).

1.2 Phytopathogenic fungi

Fungi belong to Fungi kingdom and are organized in different phyla, mostly Ascomycota and Basidiomycota (Doehlemann *et al.*, 2017; Möller and Stukenbrock, 2017). These organisms have two major ways to obtain their food: saprobism and parasitism. The saprobic fungi, which cannot invade living tissues, obtain their nutrition from dead organic matter, while the parasitic fungi infect living organisms (Dube, 2013).

Sometimes plants can be invaded by infectious agents that cause diseases. Phytopathogenic fungi are the most common agents of plant infections (Knogge, 1996), causing significant losses in crops worldwide, in worth millions of dollars (Yoon *et al.*, 2013). According to Fang and Ramasamy (2015), pathogen infections are responsible for losses of 20 to 40 % in agricultural production. It is possible to divide the pathogens into two main categories: facultative pathogens that can live as saprobes and parasites, and obligate pathogens (Dube, 2013; Balloux and van Dorp, 2017). Normally, there is a distinction between facultative and accidental pathogens (occasionally infect weakened hosts; Balloux and van Dorp, 2017). These phytopathogenic fungi invade hosts in different ways, being classified into 3 levels of specialization. The first level includes opportunistic fungi that need plant wounds or weakened plants in order to invade and colonize the hosts. Although these fungi have a wide range of hosts, their virulence is low. The plant will show only mild symptoms of the disease. The second level includes fungi that need live plants to grow although in some cases they can survive outside their hosts. These are the true pathogens and most plant fungi belong to this level, causing disease in a small number of species. The last level includes obligate pathogens. For this type of fungi the live plant is a fundamental requirement for the completion of the life cycle (Knogge, 1996).

During the infection, these pathogens acquire different ways to acquiring nutrients being classified as biotrophic, necrotrophic and hemibiotrophic (Howlett, 2006). Biotrophic fungi do not cause the death of host cells (Idnurm and Howlett, 2001). These type of fungi can penetrate the host plasma membranes through appendages of fungal hyphae, named haustoria (Rodriguez-Moreno *et al.*, 2018). In this way, biotrophic fungi become able to use the hosts' metabolism for

their own benefit (Panstruga, 2003). In contrast to biotrophic, necrotrophic fungi feed on dead tissue. Generally, they release toxins, such as small peptides or secondary metabolites that cause the death of host cells (Panstruga, 2003; Howlett, 2006), initially only in a small part of the host. The continuous development of the fungus is ensured by the spread of necrosis from the initial infection (Doehlemann *et al.*, 2017). Hemibiotrophic fungi usually have a biotrophic phase, then become necrotrophic (González-Fernández *et al.*, 2010).

According to Dean *et al.*, (2012), the "Top 10" fungal plant pathogens are: *Magnaporthe oryzae*, *Botrytis cinerea*, *Puccinia* spp., *Fusarium graminearum*, *Fusarium oxysporum*, *Blumeria graminis*, *Mycosphaerella graminicola*, *Colletotrichum* spp., *Ustilago maydis*, *Melampsora lini,* and. These are some of the fungi species that will be explored in this work and are described in more detail in the next section.

1.2.1 *Diplodia corticola*

Diplodia corticola is an endophytic fungi of the Botryosphaeriaceae family that can affect plants of different ages (Paoletti *et al.*, 2007; Hodkinson *et al.*, 2019). Considering the top 10 of the most plant fungal pathogens, it is possible to see that *D. corticola* is not considered one of these fungi. However, this fungus is one of the most aggressive to *Quercus* species. In the Mediterranean region there is a high number of cork oaks (*Quercus suber* L.; Félix *et al.*, 2017) that have been attacked by *D. corticola*, namely in Italy, Morocco, Portugal, Spain and Tunisia (Moricca *et al.*, 2016).

The mechanism of pathogenesis to this fungus remains unknown. This leads to the increase interest in the characterization of phytopathogenic fungi secretome in order to better understand the mechanism of infection and consequently helping in the development of disease management strategies (Fernandes *et al.*, 2014). However, it is known that the opportunistic behavior is a characteristic of many cork oak pathogenic fungi. These fungi are endophytes, meaning that they can invade the host without causing any symptoms in the tree. Abiotic factors also play an important role in the decline of cork oaks since changes in environment, such as drought and frost, can weaken these trees. When the plant host becomes weaker, these endophytes, that are initially confined, may start to colonize adjacent tissues, causing a negative impact, namely the progressive decay of the tree and possibly its death.

Sunken cancers on collar, trunk and branches represent the most common symptoms observed in cork oaks affected by *D. corticola* (Moricca *et al.*, 2016). Cork bark cracks and the yellowing of leaves are also some visual alterations resulting from the infection (**Figure 1**) (Félix *et al.*, 2017;

Hodkinson *et al.*, 2019). Félix *et al.*, (2017) demonstrated that plant symptoms depend of the strain of *D. corticola*, suggesting that their metabolic and proteomic profiles may be different.

A large cork oak forestry area, about 715 thousand hectares, is found in Portugal (Dias, 2012b), playing important ecological and socio-economic roles. In addition to the abundant animal and plant species found in these forests, they also support human activities such as grazing, hunting, farming and tourism (Félix *et al.*, 2017). Portugal also became, in the last decades, the major cork producer and exporter in the world (Pestana and Tinoco, 2009).

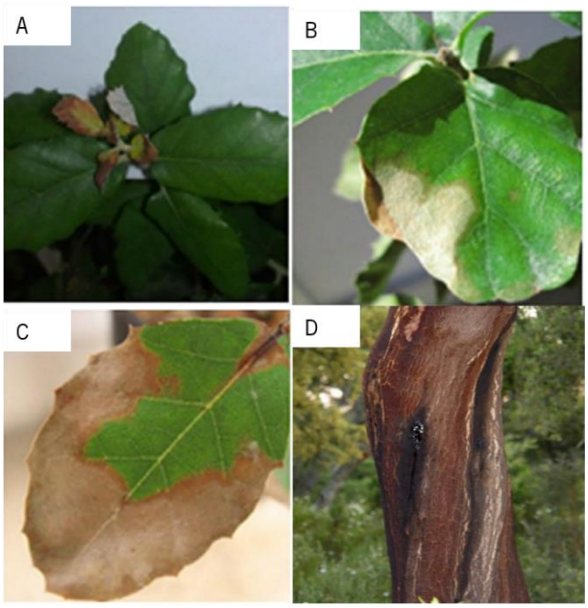

Figure 1. Representative images of the effects of infections by *Diplodia corticola*, dieback (A), leaf chlorosis (B and C), trunk with necrosis (D) with mucilaginous exudate (black area of the trunk). Adapted from Moricca *et al.*, (2016); Félix *et al.*, (2017).

1.2.2 *Colletotrichum* spp.

Colletotrichum genus, belonging to Glomerellaceae family, is described by fungal pathologists as one of the most important genus comprising phytopatogenic species (Dean *et al.*, 2012; Kim and Shim, 2019). This genus, with more than 200 species, has a negative influence on several plant crops, mainly in tropical, subtropical and temperate regions (Bragança *et al.*, 2016; Rogério *et al.*, 2020). The species belonging to this genus have a hemibiotrophic lifestyle, which is characterized by a biotrophic phase followed by a necrotrophic phase. At a certain moment the fungi switch to a necrotrophic phase where through the secretion of several toxins contribute to the degradation of plant tissues (da Silva *et al.*, 2020).

These fungi normally infect young fruits during preharvest stage through the pedicel and peel wounds but the infection remains latent until the fruit undergoes ripening (Boonruang *et al.*, 2017). The latent or quiescent infections of *Colletotrichum* are one of the causes of pre and postharvest

losses being these species associated with several diseases on fruits, legumes and ornamental plants (Farr *et al.*, 2006; Cavalcante *et al.*, 2019). Fruits production, such as strawberry, mango, citrus, avocado or even banana, are particularly affected by these species (**Figure 2**) (Cannon *et al.*, 2012).

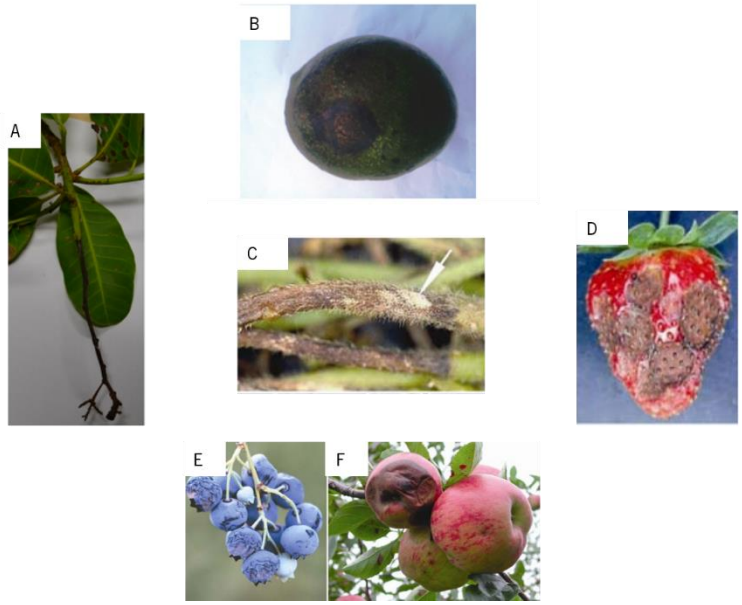

Figure 2. Anthracnose symptoms on floral peduncle and leaves of *Anacardium humile* (A) and on avocado (B). Lesions caused by the fungus *Colletotrichum acutatum* on strawberry petioles (C), on ripened strawberry (D), on blueberry (E) and on apple (F). Adapted from Peres *et al.*, (2005); Kimaru *et al.*, (2018); dos Santos *et al.*, (2019).

Colletotrichum spp. is responsible for one of the most problematic strawberry diseases, anthracnose (Anciro *et al.*, 2018). The symptomatology of this disease can be seen in different parts of the plant, such as on leaves, stems, flowers and fruits (Masi *et al.*, 2017). In the field, flower blight and fruit rot are common signs, while in plant nurseries is more likely to see lesions on stolons, petioles and leaves. Crown necrosis is characterized by reddish-brown necrotic patches, while root necrosis has been linked to stunting and chlorosis in some cases (Baroncelli *et al.*, 2015). Among the *Colletotrichum* spp., *C. acutatum*, *C. fragariae* and *C. gloeosporioides* are the three fungal species reported as active agents on strawberry disease, being *C. acutatum* the most responsible for anthracnose in Europe (Denoyes-Rothan *et al.*, 2003).

1.2.3 *Phytophthora cinnamomi*

Phytophthora species, which are essentially plant pathogens, belong to the oomycetes class of a phylum pseudofungi (Hardham and Blackman, 2018; de Andrade Lourenço *et al.*, 2020). They have a hemibiotrophic lifestyle, where the biotrophic phase plays an important role in the

initiation of the infection. In this initial phase, specialized infection structures, haustoria, are formed. Through this structure, the rupture of the plant cell wall occurs and, consequently, the establishment of contact between the host membrane and the fungus (Jupe *et al.*, 2013). The development of these species can occur mainly below ground (soilborne pathogens), where the fungi can cause root losses, root and collar rots and bleeding bark cancers, or above ground (airborne pathogens) resulting not only in bleeding bark cancers but also in necrosis of leaves, shoot blights and fruit rots (Jung *et al.*, 2018). Santos *et al.*, (2015) reported the typical symptoms caused by *Phytophthora cinnamomi* infection in plantlets of different genotypes, resulting from the artificial crossing between *Castanea sativa* vs *Castanea crenata* and *Castanea sativa* vs *Castanea mollissima*. The most sensitive plants showed root rot, characterized by large necrotic lesions, and sometimes an extension of these lesions to the collar and shoots (**Figure 3**).

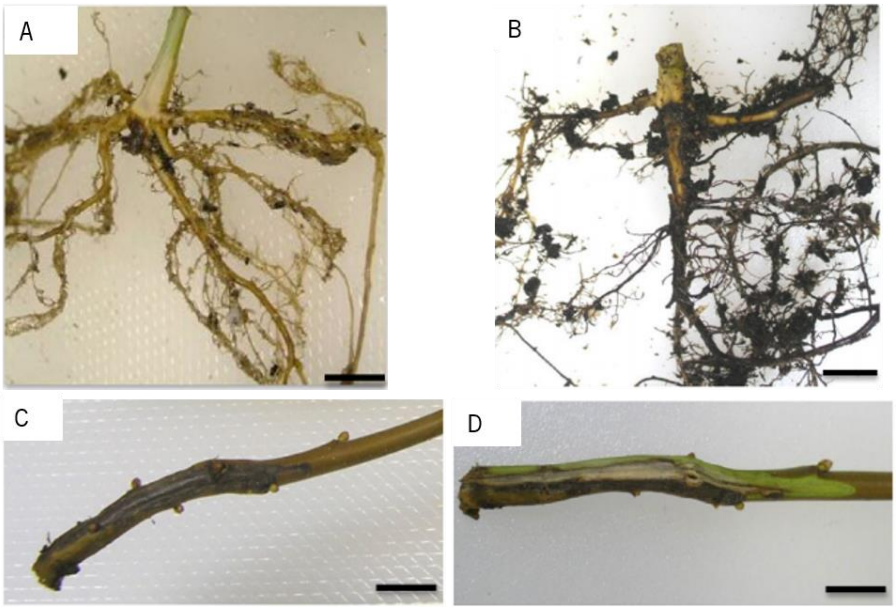

Figure 3. Representative images of normal root (A) and root infected with *Phytophthora cinnamomi* with high level of rot root (B). External (C) and internal (D) lesion of the longest shoot. Adapted from Santos *et al.*, (2015).

Although the colonization can occur through natural wounds or breaks in the peridermal layer, normally these species infect the roots (Hardham and Blackman, 2018). The hampering in nutrient and water transport by the xylem vessels due to root infection can result in visible consequences, such as sparse ramification, influences on a normal growth of lateral shoots that can cause the clustering of leaves at the end of branches, chlorosis, defoliation and dieback of branches, and probably mortality (Jung *et al.*, 2018; Hamberg *et al.*, 2021). This genus is associated with several economic losses, namely in potato, tobacco, soybean and chestnut (de Andrade Lourenço *et al.*, 2020). Amongst all *Phytophthora* species, *P. cinnamomi* has the largest host range, affecting

approximately 5000 species, mainly in Mediterranean climate (Cardillo *et al.*, 2018; Hardham and Blackman, 2018; Costa *et al.*, 2020). This fungus, with asexual and sexual phases during its life cycle, can develop saprophytically or as a parasite on hosts. The capacity of this pathogen to grow saprophytically contribute to the long-term survival (Hardham and Blackman, 2018). In southwestern Australia, the impact of *Phytophthora* has been seen and reported in jarrah (*Eucalyptus mardinata*), while in southwestern Europe this fungus is one of the major responsible to the cork and oak decline. It is also responsible to declines in chestnuts and avocados (Cardillo *et al.*, 2018).

In Portugal, the fruits and wood obtained from *Castanea sativa* Mill, also called European chestnut tree, display an important role to the national economy. The infection of these trees by *Phytophthora* culminates in the reduction of productivity and consequently in economic losses (de Andrade Lourenço *et al.*, 2020), being imperative to control the spread of this oomycete. Despite that, the procedures that are used nowadays are expensive and limited (Sena *et al.*, 2018).

1.3 Pesticides

Approximately 85 % of the world production of pesticides is intensively used in agriculture as a way to combat, prevent, or both, the development of harmful animals, mainly insects, weeds, invasive plants, fungi and bacteria (Kumar and Singh, 2015; Kim *et al.*, 2017; Pietrzak *et al.*, 2019). The utilisation of pesticides has been increasing over the years due to the perturbations and losses caused by these agricultural pests (Sherf and MacNab, 1986). It is estimated that approximately 5 billion kg of pesticides are applied around the world annually (Ali *et al.*, 2020). The efficacy of these chemicals is due to the presence of active agents. These agents can be a chemical, plant extract, pheromone or microorganism with activity against pests that, in case of Europe, has to be approved by the European Commission before a pesticide can come into the market (Commission European, no date).

Taking into account the target organism, it is possible to distinguish different types of pesticides: insecticides, fungicides, herbicides, rodenticides, molluscicides and nematicides (Aktar *et al.*, 2009). In the European Union, 40 % of total pesticides sales correspond to fungicides, representing the synthetic organic fungicides 60 % of fungicide sales (Zubrod *et al.*, 2019). After selecting the adequate pesticide to the target organism, there are some factors that affect the efficiency of the pest control, such as: the quality of pesticide, the timing of application and the quality of application and coverage (Rakhimol *et al.*, 2019). Although the use of these chemicals is

seen as an economic and efficient way of managing pests, this way increasing crop production, there are many disadvantages associated with this increased use (Ali *et al.*, 2020). Usually, the long use of pesticides to control pest diseases has effects in human health and on the environment and contributes to the appearance of resistant strains (Brauer *et al.*, 2019; Varah *et al.*, 2020). Regarding human health, not only workers but also consumers may be exposed to the pesticides by different ways: contact, inhalation or even by ingestion of contaminated water or food (Brauer *et al.*, 2019). According to the World Health Organization (WHO) 300,000 deaths occur worldwide, per year, due to pesticide poisoning (Sharma *et al.*, 2020). There are some studies that demonstrate high levels of organophosphate pesticide in the urine of children that ate food from conventional agriculture (Reganold and Wachter, 2016). The exposure of the farmers of developing countries is more dangerous due to the practice of incorrect techniques (Damalas and Eleftherohorinos, 2011). Normally, this exposure results in several problems, such as endocrine, immunological, neurological, carcinogenic and premature births (Brauer *et al.*, 2019). In addition, pesticides are also a threat to the environment by contamination of soils, water or air (Teysseire *et al.*, 2020). It is inevitable that the application of pesticides in the target areas ends up contaminating and affecting undesirable targets (Rice *et al.*, 2007). Moreover, pesticides reach less than 1 % of target pests, while the remaining contaminate soil, vegetables, fruits, food grain and water (Ali *et al.*, 2019). After the application of pesticides, factors such as wind erosion can help the spreading of these substances across long distances, culminating in the formation of deposits on the soil or surface waters (Teysseire *et al.*, 2020). The main source of pesticide contamination is water, as rain and irrigation lead to a runoff of these pesticide particles which can result in groundwater contamination (Richards *et al.*, 2016). This leads to the exposure of non-target organisms, such as beneficial insects and humans (Teysseire *et al.*, 2020).

Some rules have been established by the European parliament to allow a sustainable use of pesticides. For example, awareness campaigns should be promoted in order to better inform the general public about the impact of these compounds. Also, the aquatic environment is one of the most susceptible to pesticides, so it is advisable to use safeguard zones or to plant hedges in order to reduce exposure to contaminated water (Jornal Oficial da União Europeia - *EUR-Lex*, 2009). Despite the implemented rules for the good use of pesticides, the presence of pesticides residues has been reported in some agricultural products. The European Food Safety Authority, analysing 80,000 samples of different agricultural products from several countries (third world countries and countries of the European Union), regarding the presence of pesticides residues, showed that both

set of countries presented residues above the recommended levels, with the third world countries showing a higher rate (5.7 %) than European Union countries (1.7 %).

1.3.1 Fungicides

As an attempt to control fungal infections that are the major threat to crop production, the use of fungicides plays an important role in maintenance of food quantity and quality. These kind of compounds are mostly used in vegetables and fruits, representing more than 35 % of total market of pesticides worldwide (Zubrod *et al.*, 2019). Some fungicides can prevent disease, protecting plants from the infection, and other fungicides can eliminate an infection already established (Dias, 2012a). So, there are two categories of fungicides: contact and systemic fungicides, the latter being the more modern ones (Dias, 2012a). Introduced in 1960, systemic fungicides have been replacing the old ones for giving better levels of disease control (Gullino *et al.*, 2000).

Contact fungicides, with a preventive action, are used as protectants of plants from fungal infections diseases, killing the fungi before the development of mycelia within the plant tissues (Dias, 2012a). Contrarily, systemic fungicides can move within the plant system after its application, protecting against possible fungi attacks or even eliminate an existing infection (Cremlyn, 1961). Relatively to the site of action, the older fungicides are multisite in action, while the modern fungicides have a specific site of action, typically acting in a specific receptor or process (Smart, 2003; Dias, 2012a). There are many compounds that can be used to combat agricultural relevant fungal pathogens such as benzimidazoles, phenylamides, dicarboximides, anilinopyrimidines, quinone outside inhibitors (Qois) and carboxylic acid amides (CAAs), however the azoles antifungals are the preferred treatment (Price *et al.*, 2015). In addition to their low cost and long-lasting stability, these compounds can be active against several fungal infections, such as mildews and rusts of grains, fruits, vegetables, and ornamentals; powdery mildew in cereals, berry fruits, vines and tomatoes (Hof, 2001).

The mechanism of action differs between the different fungicides. In the case of azoles, the inhibition of ergosterol synthesis occurs through the ligation that can establish with 14α-demethylase (CYP51). The complex formed by both molecules, does not allow the demethylation of lanosterol and eburicol and therefore the biosynthesis of ergosterol, which is a fundamental sterol in fungal cell membranes (Price *et al.*, 2015; Sousa, 2019). The combination of ergosterol failure in the cell with the increased amount of intermediate compounds hampers membrane integrity and the morphology of the cell culminating in the inhibition of fungal growth (Ribas e Ribas

et al., 2016). This fungistatic action that facilitate the adaptation of fungi in conjunction with the high and repeated usage, or even the use of the drug in a concentration below the effective concentration, contributes to the appearance and development of resistant fungal strains to the class of azoles (Rosam *et al.*, 2020). On the other hand, excessive use of azoles causes contamination of soil, air and plants owing to their lipophilic nature, which allows them to adsorb into soil and organic matter (Brauer *et al.*, 2019).

Despite the benefits of fungicides in combating fungal diseases, the use of fungicides on plants will also affect other fungi especially biologically similar aquatic fungal species. Aquatic fungi play an important role in decomposition of organic matter and some of them can interact symbiotically with macrophytes forming mycorrhiza with benefits to the plant host and fungus. Fungicides in water can also affect the normal role of fungi in the ecosystem since the loss of fungal biodiversity will impact on the next trophic levels (Sousa, 2019). On the other hand, because the ability of ecosystems to offer particular services is dependent on both the number and type of species present, practices that modify species composition or reduce biodiversity in non-agricultural systems may also affect goods and ecosystem services. Therefore, the successive increase of toxins and nutrients from agricultural practices present in groundwater and surface water can trigger increased costs for water purification and a decrease in fishing. Despite the effectiveness of pesticides for increasing agricultural production and thus increasing access to food, there has been a growing demand for alternatives due to the negative impact that they have on the ecosystem (Tilman *et al.*, 2002). Comparative studies have already been done between conventional agriculture and organic agriculture (where virtually no synthetic pesticides are used) also called biological agriculture (Reganold and Wachter, 2016). As can be seen in the **Figure 4**, some of these studies demonstrate that the yields in biological agriculture are lower but this type of agriculture is more profitable and eco-friendlier. In addition, foods are similar, in terms of nutrients, and in biological agriculture they have less pesticides residues.

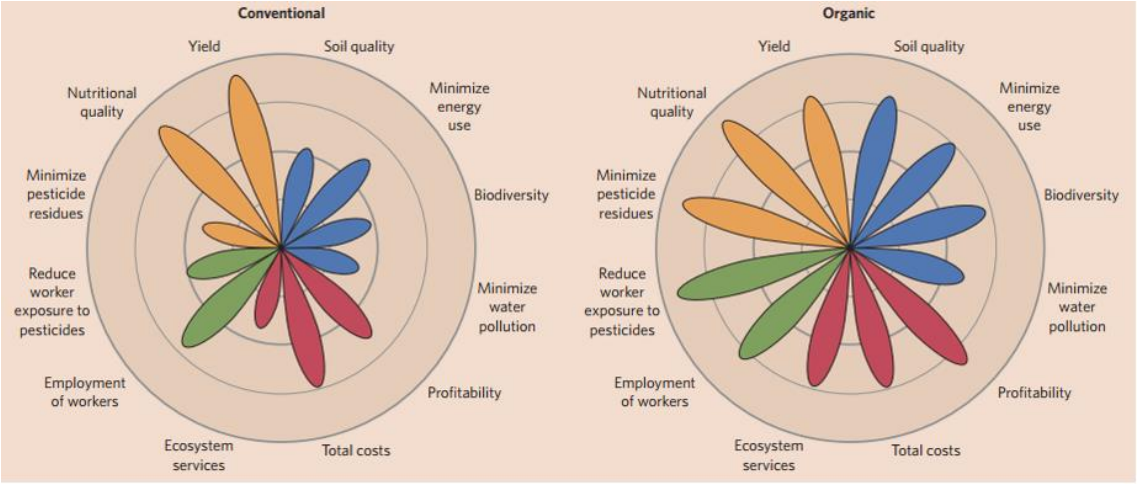

Figure 4. Evaluation and comparison of four areas of sustainability between two types of agriculture: Conventional and organic. Areas of production are represented by orange petals, environmental sustainability by blue petals, economic sustainability by red petals, and wellbeing by green petals. Based on the length of the petals, in the organic agriculture are higher balance between the four areas. Adapted from Reganold and Wachter (2016).

Another disadvantage due to the increased use of the same fungicide or other with chemical/biochemical similarities, is the development of resistance by fungi through various mechanisms (Brent and Hollomon, 2007; Zubrod *et al.*, 2019).

1.3.2 Resistance to fungicides

The resistance that some plant pathogens have to fungicides is one of the reasons to the constant search for new antifungal agents (Leadbeater, 2014). The terms qualitative and quantitative resistance are used to describe the mechanisms associated to the fungicide resistance. The first one, is based on genetic mutations that will be culminating in the failure of the fungicide treatment. This failure can be explained by the fact that the occurrence of a mutation in the gene that encodes the target protein can lead to the absence of an amino acid necessary for the binding and action of the fungicide (Deising *et al.*, 2008). This can help understanding why the fungicides with one single site target are more likely to induce fungicide-resistance problems comparatively to the fungicides that act in several target sites, since one single mutation is sufficient to make the target much less vulnerable to the fungicide (Brent and Hollomon, 2007). With the continuous treatment with the same drug the resistant strain will be selected and, at this stage, biological factors such as life short cycle, abundant sporulation, and long-distance spore dispersal speed up the selection process (Deising *et al.*, 2008; Brauer *et al.*, 2019).

In the second one, the quantitative, the pathogens are able to maintain the intracellular concentration of the fungicide below the lethal concentration by several mechanisms (Deising *et al.*, 2008). Some of these mechanisms are the synthesis of efflux pumps, which are responsible to the secretion of the drug molecule to the extracellular space; the reduction of membrane permeability to the fungicide due to alterations in the plasma membrane; or by the enzymatic degradation of the fungicide (Deising *et al.*, 2008; Revie *et al.*, 2018; Brauer *et al.*, 2019). In addition, upregulation of the target protein can also occur, increasing the function, or the fungi can use other metabolic pathways as an alternative (Revie *et al.*, 2018).

1.4 Biopesticides: as an alternative to synthetic pesticides

The current use of synthetic pesticides has a major impact in the environment, affecting biotic and abiotic components (Kumar, 2012). This fosters the search for biopesticides that are natural substances or obtained from living organisms (Costa *et al.*, 2019). Comparatively to the synthetic pesticides these natural compounds have a short life cycle, reducing their field persistence, as well as a more restricted spectrum due to its high specificity, requiring the use of various biopesticides to combat different phytopathogenic agents and a slower killing rate (Marutescu *et al.*, 2017; Kumar *et al.*, 2021). Despite that, the use of biopesticides is more beneficial than the use of conventional pesticides since they are less toxic to the farmers and consumers, and normally affect only the target pest and closely organisms (Abbey *et al.*, 2019; Kumar *et al.*, 2021). They are also biodegradable with a quick decomposition time, reducing potential negative impacts in the environment, as well as in flora and fauna (Abbey *et al.*, 2019). In addition, and in opposition to synthetic pesticides, biopesticides are less likely to induce pest resistance (Fenibo *et al.*, 2021). These compounds can be classified into three categories: microbial pesticides, plant-incorporated protectants (PIPs) and biochemical pesticides (Sporleder and Lacey, 2013). Entomopathogenic microorganisms such as bacteria and fungi, and also viruses, the majority of which are target-specific, are used to make microbial pesticides (Idris *et al.*, 2020). The second class, PIPs, include substances that are produced by genetically modified organisms (GMOs). This genetic material, incorporated into the plant confers protection against pests or chemical stressors (Sudakin, 2003). Lastly, the biochemical pesticides are natural substances provided by plants and also by animals, like insects, capable to fight the pest through non-toxic mechanisms to the target pest, humans and the environment (Costa *et al.*, 2019; Fenibo *et al.*, 2021). This class includes pheromones and other semiochemicals, plant extracts and natural insect growth regulators (Sporleder and

Lacey, 2013). A semiochemical is a chemical signal produced by an organism that can influence the behaviour of another organism from the same or from different species. An example are the sex pheromones that are currently used due to its capacity to induce mating disruption. Normally, the pheromones are also the vehicle in the mass trapping and lure-and-kill systems techniques used by farmers to help pest monitoring (Chandler *et al.*, 2011). The use of compounds that mimic semiochemicals, also naturally produced by an organism, is a promising tool in pest control (Seiber *et al.*, 2014). Although the biopesticide market has been increasing over the years, approximately 10 % annually, it remains only a small percentage of the total market used for crop protection (Damalas and Koutroubas, 2018). As reported by Chandler *et al.*, (2011), 68 active substances were already registered in European Union, representing only 2.5 % of the total pesticide market. Among these substances, 34 are microbials, 11 biochemical and 23 semiochemical products. However, due to their advantages, biopesticides are estimated to equal the synthetic pesticide market, in terms of size, by 2040-2050 (Damalas and Koutroubas, 2018).

Regarding the plant extracts, it is known that plants produce several secondary metabolites that although not directly associated with fundamental main processes such as growth, development and reproduction, play an important role in the interaction and adaptation to the environment, and also have various applications in medicine, cosmetic and nutrition (Akula and Ravishankar, 2011; Rafińska *et al.*, 2017). There are several studies that suggest that these compounds could be an important source to the formulations of novel biopesticides (Marutescu *et al.*, 2017). Tannins, terpenoids, saponins, alkaloids, flavonoids are some of the secondary metabolites produced by plants with several biological activities, including antifungal properties (Arif *et al.*, 2009). It is thought that the majority of the secondary compounds are involved in plant defence against herbivores and pathogens, however, there are several factors, such as extraction methods, stabilization of the active compound, purification steps, that affect the possible use of plant extracts as botanical pesticides (Marutescu *et al.*, 2017; Rafińska *et al.*, 2017). In this work four plant species were selected for their reported content in secondary compounds with important biological activities. These plants are further described in the following section.

1.4.1 *Digitalis* L.

Digitalis L. genus, including biennial or perennial herbs, belongs to the Plantaginaciaeae family and is native to the Balkans, Hungary, Italy, Spain, Lebanon, Romania, Transcaucasia, Turkey, Japan and India (Patil *et al.*, 2013; Verma *et al.*, 2016). In 1785, the potential use of an active

compound was recognized for the first time. Present on digitalis extract, it was later identified as a cardiotonic steroid and has applications in the treatment of heart ailments (Verma *et al.*, 2016). This genus is also used in Turkish traditional medicine due to its diuretic, stimulant and tonic characteristics (Benli *et al.*, 2009). *Digitalis purpurea* and *Digitalis lanata* are two of the species with important pharmacological properties due to the presence of cardiac glycosides such as digitoxin, gitoxin and digoxin (Katanić *et al.*, 2017). In addition to the cardiac glycosides there are other compounds detected in the chemical composition of *Digitalis* species, namely steroidal saponins, pregnane glycosides and phenlyethanoid glycosides (Kirmizibekmez *et al.*, 2014). *D. purpurea*, also called purple foxglove or lady's glove, is recognized as an important medicinal plant (Pérez-Alonso *et al.*, 2016; Verma *et al.*, 2016). Due to the presence of cardenolides that could be useful in the regulation of heart rhythms in humans, the leaves of this plant have been widely used in treatment of heart diseases (Pérez-Alonso *et al.*, 2016).

To the best of our knowledge there are only few studies regarding the antimicrobial activity of *Digitalis* spp. Benli *et al.*, (2009), reported the study of antimicrobial activity of *D. lamarckii* against bacteria (*Enterococcus gallinarum* CDC-NJ-4, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* RSHI, *Escherichia coli* RSHI, *Shigella* RSHI, *Escherichia coli* ATCC 25922, *Streptococcus pyogenes* ATCC 19615, *Staphylococcus aureaus* ATCC 29213, *Listeria monocytogenes* ATCC 7644, *Pseudomonas aeruginosa* ATCC 27853) and yeast (*Saccharomyces cerevisiae*, *Candida albicans* 845981, *C. albicans* 900628 and *C. crusei* ATCC 6258) strains and concluded that *D. lamarckii* extracts has antibacterial activity. Regarding antifungal activity we only found studies of activity of two other species of *Digitalis*: *D. lamarckii* and *D. ferruginea*. Thus, due to the possible similarity between species and the presence of several compounds normally associated with antifungal activity, such as saponins, we decided to study *D. purpurea* against several phytopathogenic fungi.

1.4.2 *Eucalyptus globulus* Labill.

The Myrtaceae family comprises 140 genera and approximately 3800 species, mainly in tropical and subtropical regions. Trees of the genus *Eucalyptus* are one of the most cultivated plants with an important role in traditional medicine (Elansary *et al.*, 2017) besides the paper and wood industries. Plants from this genus are also considered a rich source of essential oils (EOs) and bioactive compounds, mainly alkaloids, terpenoids, steroids, phenols, glycosides and tannins (Javed *et al.*, 2012; Bakht *et al.*, 2018). The intense odor of eucalyptus leaves is due to the presence of EOs, abundant in monoterpenes but sometimes also rich in sesquiterpenes (Barbosa *et al.*, 2016). The presence of these compounds contribute to the pesticide potential demonstrated

for several eucalyptus species (Puig *et al.*, 2018b). On the other hand, EOs have other applications, namely in the food sector, as flavoring, and also in perfumes, soaps and detergents due to its fragrance. Additionally, the EOs of eucalyptus ingested orally have been used against catarrh and topically are used as a rubefacient (Ghaffar *et al.*, 2015). Traditionally, eucalyptus leaves extracts have been used with different proposes. Leaves extracts have an antiseptic application being used in wounds, cuts and skin infections (Vuong *et al.*, 2015), and are also used in the treatment of respiratory diseases such as asthma and bronchitis (González-Burgos *et al.*, 2018).

Regarding the antifungal activity of *Eucalyptus globulus,* studies in the literature concern mostly to EOs characterization. The EO of *E. globulus* possesses activity against the fungi *Trichophyton mentagrophytes,* a dermatophyte such as *Candida* spp., and *Pityrosporum orbiculare* (Batish *et al.*, 2008). Ameziane *et al.*, (2007) demonstrated that the EO, as well as the methanolic extract of *E. globulus,* showed antifungal activity against *Penicillium digitatum* and *Geotrichum candidum*. In addition, Puig *et al.*, (2018b), indicated the potential use of aqueous extract of *E. globulus* leaves as a pre-emergency bioherbicide since phytotoxic effects on germination and early growth in *Lactuca sativa* and *Agrostis stolonifera* were obtained similar to a bioherbicide, metolachlor. Chemical analysis of this extract showed the presence of phenolic acids such as chlorogenic and *p*-coumaric derivatives, flavonoids such as hyperoside, rutin, quercitrin, ellagic acid and kaempferol 3-*O*-glucoside and organic acids with low molecular weight, namely citric, malic, shikimic, succinic and fumaric acids (Puig *et al.*, 2018a).

1.4.3 *Beta vulgaris* L.

Beta vulgaris L., also known as chukandar or beet root, belongs to the Chenopodiaceae family (Sulakhiya *et al.*, 2016). This herbaceous biennial crop, native from the Mediterranean region, grows in various climate conditions being broadly cultivated in Europe, America and India (Mikołajczyk-Bator *et al.*, 2016; Sulakhiya *et al.*, 2016; Rehman *et al.*, 2021). The presence of betalains, water-soluble compounds, impart the characteristic reddish color of this root (Mroczek *et al.*, 2012). Several biological activities are attributed to betalains, such as antioxidant, anti-inflammatory, hepatoprotective and antitumor (Georgiev *et al.*, 2010). Beet root also contains several other phytochemicals such as ascorbic acid, carotenoids, phenolic acids and flavonoids (Clifford *et al.*, 2015). Since 1000 B.C. that this plant is largely used in the Mediterranean region due to its high concentration of sugars and antioxidants; also, the Romans use *B. vulgaris* roots for medicinal applications while the leaves were used mainly in food (Ninfali and Angelino, 2013; Silva

et al., 2020). *B. vulgaris* is used as a folk medicine for liver and kidney problems and is reported its stimulation capacity of the immune and hematopoietic systems (Kapadia, 2003). Only recently, this plant received scientific attention due to the possibility of helping in cardiovascular health (Clifford *et al.*, 2015).

To the best of our knowledge, the antifungal potential of beet root remains poorly known. We only found in the literature a study that assessed the activity of aqueous extract of *B. vulgaris* against three phytopathogenic fungi: *Fusarium oxysporum*, *Rhizoctonia solani* and *Macrophomina phaseolina* presenting percentages of inhibition of 10.3 % and 40.3 %, against *F. oxysporum* and *R. solani* respectively (Cherkupally *et al.*, 2017). So, we decided to explore this extract against other phytopathogenic fungi.

1.4.4 *Hedera helix* L.

Hedera helix L., also known as ivy or English ivy, is a climbing plant that belongs to Araliaceae family (Prescott *et al.*, 2014; Yu *et al.*, 2015). It is native of Europe and Western Asia and is widely cultivated around the world (Roşca-Casian *et al.*, 2017; Sun *et al.*, 2017). Leaf morphology and color depends on the age and when they come with flowers, produced from summer to autumn, they are small and exhibit greenish-yellow color. In winter, *H. helix* produces fruits in the form of small black berries (Roşca-Casian *et al.*, 2017).

Several activities have been reported for the saponins present in leaves of *H. helix*, namely antifungal, anthelmintic, molluscicidal, antileishmanial and anti-mutagenic, having this plant species an important role in traditional medicine (Bedir *et al.*, 2000; Yu *et al.*, 2015). Since the nineteenth century, leaf extracts have been used due to their expectorant and bronchospasmolytic effects to treat respiratory diseases (Yu *et al.*, 2015). Leaf extracts of *H. helix* are also used as an attempt to reduce fever and cause diaphoresis (Hooshyar *et al.*, 2014). Furthermore it has been reported that the extracts of this plant possess several properties, such as anti-inflammatory, antimicrobial, antioxidant, anti-tumor, antispasmodic and hepatoprotective (Sun *et al.*, 2017). Nowadays, there are many products based on *H. helix* extracts, namely drops, syrups and suppositories that could be used to treat cough, common cold and in inflammatory bronchial disorders (Gul *et al.*, 2018).

Phytochemical analysis of this plant confirmed the presence of various secondary metabolites such as alkaloids, terpenoids, saponins and tannins, which depends on the part(s) of plant used and also on the solvent used for the extraction (Roşca-Casian *et al.*, 2017). Among these bioactive

compounds, saponins, such as hederacoside C and α-hederin, are the main active compounds in *H. helix* (Sun *et al.*, 2017). The cytotoxic effect of α-hederin has already been demonstrated against a wide range of cancer cell lines (Lorent *et al.*, 2013). This compound was also described as being active against *C. albicans* (Prescott *et al.*, 2014). However, studies of these compounds and of ivy extracts against plant pathogenic fungi are less frequent. To our knowledge, the first study of the antifungal activity was reported in 2017 for English ivy ethanolic extract against a range of plant pathogens such as *Aspergillus niger*, *B. cinerea*, *B. tulipae*, *Penicillium gladioli* and *Sclerotinia sclerotiorum*. The results were promising since the minimum inhibitory concentration (MIC) obtained was lower than MICs obtained with the synthetic drug fluconazole (Roşca-Casian *et al.*, 2017). The abundant presence of secondary compounds, mentioned above, in *H. helix* as well as the antifungal activity already demonstrated against some phytopathogenic fungi led to the interest in studying extracts of this plant species.

1.5 Scientific problem and objectives

As already mentioned, pesticides are chemicals widely used in agriculture. The negative effects of these substances are significant both environmentally and on human health (Damalas and Koutroubas, 2018). An alternative to pesticides are biopesticides. Usually their lower toxicity, rapid biodegradation and targeted action on a particular species are advantageous features of this type of substances/products compared to conventional pesticides (Marutescu *et al.*, 2017). Because of this, the use of biopesticides has been encouraged to reduce the use of synthetic pesticides and thus contribute to a decrease in the adverse effects caused by these chemicals (Costa *et al.*, 2019).

Plant extracts are currently indicated as possible sources for the formulation of biopesticides (Marutescu *et al.*, 2017). Taking this into account, the main objective was to study the antifungal activity of plant extracts, with the possible formulation of a new biopesticide for application in agriculture to combat phytopathogenic fungi in a more bio-sustainable way.

To find plants with antifungal properties, four plants species were chosen based on the information available in the literature, because they have several compounds with important biological activities in their constitution. The plant species selected to be studied in this work were *H. helix*, *E. globulus*, *D. purpurea* and *B. vulgaris*, which, according to our knowledge, are still not completely characterized in terms of antifungal activity against phytopathogenic fungi. Subsequently to achieve the main goal, tasks were established as follows.

Firstly, a screening was carried out through cell viability assays using *Saccharomyces cerevisiae* as a model organism. Afterwards, the remaining tasks was carried out with *H. helix* extract, that were: a) the use of mutants to study the mechanism of action also through cell viability assays, b) the assessment of *in vitro* activity against several phytopathogenic fungi: *C. acutatum*, *C. gloeosporioides*, *C. godetiae*, *C. nymphaeae*, *D. corticola* and *P. cinnamomi,* and c) the assessment of antifungal activity by *ex vivo* assays using infected leaves of strawberry plants and in *Quercus suber* apical cuttings.

CHAPTER 2
MATERIALS AND METHODS

2.1 Plant species selection and preparation of plant extracts

Beta vulgaris was bought in a local supermarket, chopped into pieces, frozen with liquid nitrogen, grounded in a mill and left to dry at room temperature. Leaves of *H. helix* and *Eucalyptus globulus* were collected in October 2020, in Braga, and leaves of mature plant of *D. purpurea* were collected in March 2020 in a private field, away from roads, in Ponte de Lima. All the leaves were air dried at room temperature, in the dark, during approximately four weeks. Subsequently, the leaves were frozen in liquid nitrogen and grounded into a fine powder with a pestle and mortar. Then, in order to obtain extracts rich in secondary metabolites two extractions were performed: ethanolic and aqueous. The exception was for *B. vulgaris* with which only the aqueous extract was done. To prepare the aqueous extract of *E. globulus*, the powdered plant material was extracted with deionized water at 100 °C, in a proportion of 1:10 (w/v) for 10 min, filtered through Whatman filter paper No.1 and stored at -20 °C till use. In the case of *H. helix* and *D. purpurea*, the same procedure was followed but the extraction was for 30 min. For the aqueous extract of *B. vulgaris*, the powered material was extracted with deionized water at 100 °C, in a proportion of 1:20 (w/v), for 30 min, and then filtered and stored as described above. To obtain the ethanolic extracts, the plant materials were extracted with 96 % (v/v) ethanol (98 % v/v for *H. helix;* Gumushan-Aktas and Altun, (2016)) in the dark and at room temperature. After 2 days, the ethanolic solutions were filtered through Whatman filter paper No. 1 and the solvent was evaporated using a rotavapor at approximately 40 °C and 60 rpm.

After evaporation of the solvent, the extract was resuspended in deionized water and stored at -20 °C. All extracts, aqueous and ethanolic, were lyophilized and stored in the dark at room temperature until use. The freeze-dried ethanolic extracts were dissolved with ethanol, which had the same concentration that was used for the extraction, and the aqueous extracts were dissolved with deionized water to a final concentration of 50 mg/ml and 5 mg/ml, respectively. To avoid ethanol toxicity, the extract was prepared in higher final concentration compared to the aqueous extract to allow the use of smaller volumes in treatments with *Saccharomyces cerevisiae* cultures. The freeze-dried extract of *H. helix* was dissolved in deionized sterile water to a final concentration of 2.5 mg/ml.

2.2 Microorganisms and culture conditions

The *Saccharomyces cerevisiae* strain used in this work was **BY4741** (genotype MAT*a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0)* as well as some mutants such as ***erg2*** (genotype MAT*a; his3Δ1;*

leu2Δ0; met15Δ0; ura3Δ0; YMR202w::kanMX4, *bck1* (MAT*a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YJL095w::kanMX4*), ***mkk1/mkk2*** (BY4741; Mat*a*; *his3Δ1* ; *leu2Δ0* ; *met15Δ0* ; *ura3Δ0*; *YPL140c::kanMX4*; *mkk1::LEU2* and ***yca1*** (W303 MAT*a ade2–1 ura3-1 leu2-3,112 trp1-1 his3-11,15 can1-100 yca1::KANMX4).* These strains were cultivated in YPDA medium (2 % w/v peptone (Bacto™), 2 % w/v dextrose (Difco™), 2 % w/v agar (Labchem), 1 % w/v yeast extract (FisherBioreagents), at 30 °C for 2 days. After that, the plates were stored at 4 °C. **Table 1** includes all the fungal species that were used in this work. These microorganisms were cultivated on Potato Dextrose Agar (PDA; Biolife) in the dark at 25 °C.

Table 1 Fungi used in this work and corresponding suppliers.

Fungus	Provided by
Phytophthora cinnamomi PH107	Helena Machado (National Institute of Agrarian and Veterinary Research)
Colletotrichum acutatum 15-015	Pedro Talhinhas (Instituto Superior de Agronomia, School of Agriculture, University of Lisbon)
Colletotrichum nymphaeae CA397	Pedro Talhinhas (Instituto Superior de Agronomia, School of Agriculture, University of Lisbon)
Colletotrichum gloeosporioides 15-025	Pedro Talhinhas (Instituto Superior de Agronomia, School of Agriculture, University of Lisbon)
Colletotrichum godetiae 15-019	Pedro Talhinhas (Instituto Superior de Agronomia, School of Agriculture, University of Lisbon)
Diplodia corticola CAA500	Ana Cristina Esteves (Centre for Environmental and Marine Studies, University of Aveiro)

2.3 Viability test: colony forming units (CFU)

To prepare the initial inoculum, 1-2 colonies of a selected yeast strain were collected from a solid YPDA medium, inoculated in Yeast Peptone Dextrose (YPD; YPDA without agar) liquid medium and the culture was incubated overnight at 30 °C, in an orbital shaker (200 revolutions per minute; rpm). The optical density was measured at 600 nm (OD_{600}) to monitor cell proliferation. After this incubation period, the suspension was diluted to OD_{600} = 0.1 using fresh YPD medium and incubated again for 4 h, under the same conditions. When OD_{600} reached 0.4, the suspension was divided into aliquots. With all the plant extracts, except for aqueous extract of *H. helix*, three aliquots were used for the different extract concentration tested: 500, 1000 or 2000 µg/ml. The fourth aliquot was the negative control, which was prepared by replacing the extract by the solvent (96 % (v/v) ethanol or sterile deionized water) at the highest volume of extract used. In the case of aqueous extract of *H. helix,* five aliquots were used for the treatments with extract: 10, 50, 75, 100

or 250 µg/ml. The sixth aliquot was the negative control, which was prepared by replacing the extract volume by the solvent (sterile dH_2O) at the highest volume of extract used. All suspensions were incubated under the same conditions and after 0, 30, 60 and 90 min an aliquot of 100 µl was collected and serially diluted in sterilized dH_20 from 10^{-1} to 10^{-4}. Three 40 µl-drops of the highest dilution were placed on Petri dishes with YPDA (3 replicates per condition) and incubated at 30 °C for 48 h. The number of colonies was counted in each plate and the percentage of viability (percentage of colony-forming units; % CFUs) was calculated considering 100 % viability for time 0 min in each condition.

2.3.1 Evaluation of osmotic stress susceptibility

An assay was performed to evaluate the osmotic sensitivity of *Saccharomyces cerevisiae* to NaCl solutions and if it varied with the presence of the extract and extract concentrations. Knowing that *Saccharomyces cerevisiae* BY4741 cells are resistant to salt concentrations up to 0.5 M (Dhar, Sägesser, Weikert, Yuan, & Wagner, 2011), four concentrations of the salt were tested - 0, 0.25, 0.5 and 0.75 M -, and the influence of the extract, NaCl and combination of both on CFU was assessed. To prepare the cultures, the procedure described in the previous section (section 2.3) was followed, in this case using Petri dishes with the salt incorporated in the PDA medium. Briefly, in the day before of the assay, the inoculum was prepared and, in the subsequent day, a dilution was made to OD_{600} = 0.1 and the culture was incubated as above, till reaching the OD_{600} = 0.4. Two aliquots were used for the different extract concentrations tested: 10 or 50 µg/ml, and a third aliquot was used as a negative control. In the latter, the volume of extract was replaced by the solvent (sterile dH_2O) at the highest volume of extract used. All suspensions were incubated under the same conditions and after 0, 30 and 60 minutes an aliquot was collected and serially diluted in sterilized dH_20 to form dilutions from 10^{-1} to 10^{-4}. After that, three 40 µl drops of the highest dilution were placed in the Petri dishes with different concentrations of NaCl (3 replicates per condition). All the Petri dishes were incubated at 30 °C for 48 h. After this, colonies were counted and the percentage of cell viability was calculated, considering 100 % of cell viability at 0 min for each condition and for each concentration of salt.

2.4 Evaluation of antifungal activity *in vitro*

The antifungal activity of *H. helix* aqueous extract was assessed *in vitro* against *C. acutatum*, *C. gloesporioides*, *C. nymphaeae, C. godetiae*, *Diplodia corticola*, *Phytophthora cinnamomi*

measuring mycelium growth. Briefly, the extract was added at different concentrations – 50, 100, 500, 1000 or 1500 µg/ml - to PDA medium before its solidification, and the negative control, was prepared with sterilized deionized water in equal volume of the extract used to prepare the highest concentration. Using a sterile 1000 µl tip, a small portion (diameter = 5 mm) of fungal mycelium was excised from the margins of 12-days old cultures and placed in the middle of the Petri dishes. The plates were maintained at 25 °C, protected from light, and the mycelial colony diameter was measured after 3, 6 and 9 days. Two perpendicular centered lines marked on the bottom of each plate were used to guide the measurement of two diameters and the data was expressed as the mean value. The percentage of growth inhibition was determined using the formula $\frac{dc-dt}{dc}*100$, where d_c corresponds to the average mycelial diameter in the controls and d_t is the mycelial diameter in each triplicate, being the treatment mean value obtained by the average of the percentage of growth inhibition of the replicates.

2.5 Evaluation of antifungal activity in *ex situ* bioassays

2.5.1 Preparation of *C. acutatum* spore suspension

A culture of *C. acutatum* with 9 days was used to prepare the spores solution. Briefly, a 5-mm mycelium disc of *C. acutatum* was removed from the margins with a sterile 1000 µl tip, suspended in 5 ml of 0.05 % tween 20, and after the spores were observed and counted under a microscope with a haemocytometer. The suspension was adjusted to a final concentration of 9×10^5 spores/ml and filtered through sterile gaze.

2.5.2 *Ex situ* assay using strawberry leaves

Cultivated garden strawberry *Fragaria* x *ananassa* variety Camarosa was used to evaluate the antifungal effects of the *H. helix* extract. Strawberry trifoliate leaves were collected and surface sterilized according to an adaptation of the protocol described by Šernaitė, Rasiukevičiūtė & Valiuškaitė (2020). Briefly, the leaves were immersed in 70 % ethanol during 30 s and then rinsed in sterile distilled water. Leaves (3 replicates per condition) were subjected to different conditions: treatment with two concentrations of extract -1000 and 2000 µg/ml (with the extract and the spore suspension), the negative control (just with water), the extract control (just with the extract at 2000 µg/ml) and the infection control (just with spore suspension). For each leaf, 0.5 ml of extract or/and sterile distilled water and spore suspension were used. For the treatments with extracts and *C. acutatum* spore suspension, the leaves were firstly brushed in both sides with *H. helix*

extract and after air-dried the adaxial side was brushed with the spore suspension. The negative control was prepared by brushing both sides of the leaves with sterile distilled water, twice (after the first one dried out). In the extract control, the extract was used to brush the leaves on both sides, also twice. Finally, to prepare the infection control, the leaves were brushed with sterile distilled water on both sides and, after dried out, the adaxial side was brushed with the spore suspension. Each trifoliate leaf was transferred to Petri dishes containing moistened filter paper and two moistened pieces of cotton. All the Petri dishes were placed near the window during 12 days replenishing sterile water to maintain high humidity. The leaves were observed every day and photographed in a laminar flow chamber.

2.5.3 *Ex situ* assay using *Quercus suber* apical cuttings

Apical cuttings (20 cm) were obtained from mature trees of *Quercus suber* growing at *Campus* of Gualtar (University of Minho, Braga), the cut end was immersed in water and immediately taken to the laboratory. The cuttings were disinfected according to a modified Newhouse protocol (Newhouse *et al.*, 2014). After washing with abundant tap running water, the cuttings were immersed on 0.01 % Tween 20 for 30 s and then rinsed in sterile dH_2O and left to dry. Similarly to the leaf assay, five cuttings (replicates) were used per condition: negative control, control of extract, infection control and the treatments with two concentrations of extract: 1000 or 2000 µg/ml. To apply the fungal inoculum a small longitudinal incision (0,5-1 cm) was made with a sterile scalpel on each cutting at 5 cm from the base, and half of a 5-mm plug of *D. corticola* mycelium or of PDA medium was attached to the wound and wrapped with parafilm. To apply the extract, the 5-mm apical region of each cutting was cut and a small cotton ball impregnated with a fixed volume (28 µL) of the extract or sterile deionized water was attached and wrapped with parafilm. This volume was estimated to test the same extract concentrations as in the leaf bioassay (1000 and 2000 µg/ml), considering that the vascular tissues account for roughly 50 % of the total cutting volume. As for the leaf assay, for the extract control the highest concentration (2000 µg/ml) was used.

The cuttings were adjusted to small holes made in Styrofoam platforms to maintain the upright position and placed on a recipient with the basal ends immersed in MS medium (Murashige and Skoog, 1962). The setting was placed near a window and the cuttings were monitored during one month.

2.6 Statistical analysis

Results were statistically analyzed by one-way ANOVA followed by post-hoc Tukey test for multiple comparisons using the GraphPad Prism version 8.0 for Windows, GraphPad Software (San Diego, California USA, www.graphpad.com). The data was presented as mean ± standard deviation (S.D.) obtained from at least three independent experiments, in the case of viability tests with *Saccharomyces cerevisiae* and *in vitro* assays. For the *ex vivo* assays on strawberry leaves and in cork oak cuttings, the data was presented as mean ± standard deviation (S.D.) obtained from three and five replicates, respectively.

CHAPTER 3
RESULTS AND DISCUSSION

3.1 Evaluation of the effect of plant extracts using *Saccharomyces cerevisiae* as a model organism

The budding yeast *Saccharomyces cerevisiae* is a model organism that has been used to understand the mode of action of various antifungal and therapeutic compounds (Simons *et al.*, 2006). So, firstly *S. cerevisiae* strain BY4741 was used to perform viability tests in order to study the effects and activity of the plant extracts with possible activity against phytopathogenic fungi.

3.1.1 Effect of aqueous and ethanolic extracts of *Digitalis purpurea* leaves on *Saccharomyces cerevisiae* BY4741

To evaluate the antifungal activity of *D. purpurea* extracts, cells from an exponentially growing culture were incubated with different concentrations of extract and viability was assessed by CFU after 0, 30, 60 and 90 min. In the negative controls of both ethanolic and aqueous extracts of *D. purpurea* an increase in viability was observed along time (**Figure 5**).

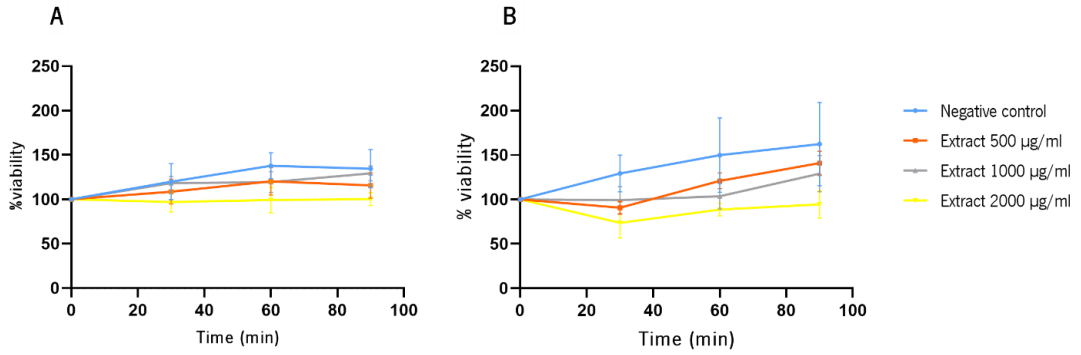

Figure 5. Viability of *Saccharomyces cerevisiae* BY4741 in the presence of ethanolic (A) or aqueous (B) extract of *Digitalis purpurea* leaves. Cells from an exponentially growing culture were exposed to 500, 1000 or 2000 µg/ml extract at 30 °C, 200 rpm, and viability was assessed by CFU after 0, 30, 60 and 90 min of incubation. The negative control was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. The data represent the mean ± S.D. of three independent experiments. The results from the statistical analysis can be found in the supplementary material (**Figure A1**, **Figure A2**).

With the ethanolic extract, the viability reached 135 %, while with the aqueous extract reached 162 % after 90 min of incubation. Probably, this difference is due to the presence of ethanol that can delay culture growth. With the ethanolic extract, in all concentrations, viability reached statistically similar values as compared to the negative control in all timepoints (**Figure A1A**). On the other hand, with the aqueous extract of *D. purpurea*, all treatments displayed lower viability than the negative control. After 30 min incubation, the percentage of viability in the presence of 500 µg/ml and 2000 µg/ml significantly decreased to 91 % (p<0.05; **Figure A1B**) and 73 % (p< 0.001; **Figure A1B**) of the control, respectively. At lower concentrations of extract, 500 µg/ml and 1000 µg/ml, a

slower culture growth was observed, but at the highest concentration, 2000 μg/ml, there seems to be a stagnation of proliferation after 1 h of incubation and cell viability was statistically lower compared to the negative control ($p < 0.05$; **Figure A2**). A possible explanation for this difference could be the adaptation of cells to the extract when present at lower concentrations (**Figure 5**).

Comparing both extracts of *D. purpurea,* the aqueous extract seems to exhibit activity against *Saccharomyces cerevisiae* BY4741. To the best of our knowledge there is no reference to antifungal activity of extracts from this plant species. However, Katanić *et al.*, (2017) studied the antifungal activity of methanolic extracts of two other species of *Digitalis*: *Digitalis ferruginea* subsp. *ferruginea* and *Digitalis lamarckii.* These extracts possess antifungal activity against two *Penicillium* species (MIC = 5 mg/ml). Also, these authors verified that the *Fusarium oxysporum* and *Phialophora fastigata* were the fungi more susceptible to the action of *D. ferruginea* subsp. *ferruginea* extract (MIC = 2.5 mg/ml). *C. albicans* and *Aspergillus niger* were the most resistant fungi to both extracts.

3.1.2 Effect of aqueous and ethanolic extract of *Eucalyptus globulus* on *Saccharomyces cerevisiae* BY4741

The effect of ethanolic and aqueous extracts of *E. globulus* leaves were also evaluated against *S. cerevisiae* BY4741 using viability experiments as above for *D. purpurea*. In a similar way to what was observed for *D. purpurea* extract, both negative controls showed increasing viability over time (**Figure 6**). The presence of ethanol in the negative control of the ethanolic extracts treatments might account to the differences in viability between both negative controls. After 90 min of incubation, the viability of the negative control in the ethanolic extract was around 120 % and in the aqueous extract was around 164 %. The general behavior of both ethanolic and aqueous extracts were similar. As we can see in **Figure 6**, all tested concentrations did not appear to interfere with cell viability when compared with the negative controls. The percentage of viability always remains quite similar to the respective negative control. Statistical analysis showed that none of the extract concentrations was able to induce a significant reduction in cell viability compared to the negative control, after 30 min of incubation with ethanolic and aqueous extract of *E. globulus* (**Figure A3A** and **Figure A3B**). This suggests an absence of either fungistatic and fungicidal activity against *S. cerevisiae*. Bakht *et al.*, (2018) demonstrated the activity of an aqueous fraction of *E. globulus* against *C. albicans*. Thus, it was thought that the eucalyptus extract could decrease the cell viability of *S. cerevisiae* BY4741 used in this work. Perhaps the differences in the extraction method as well as the timing of leaf harvest may influence the composition in secondary metabolites of the extract.

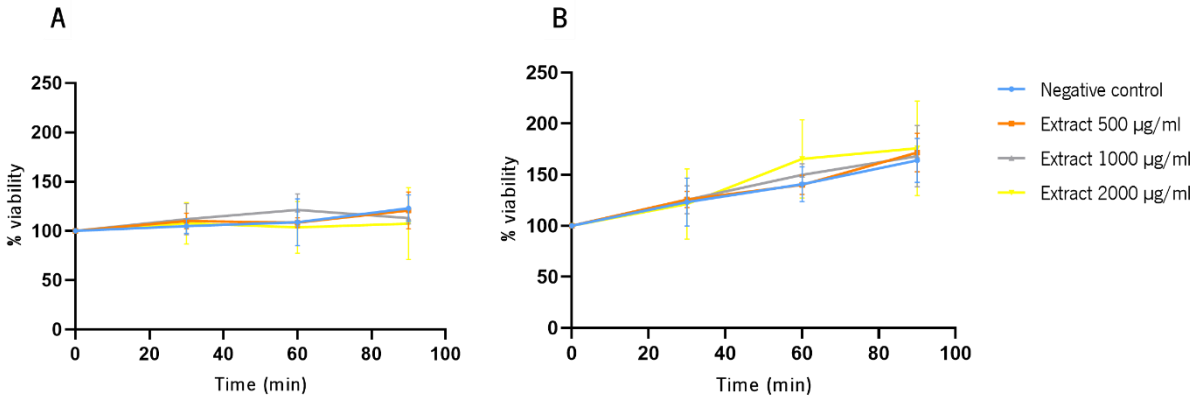

Figure 6. Viability of *Saccharomyces cerevisiae* BY4741 in the presence of ethanolic (A) or aqueous (B) extract of *Eucalyptus globulus* leaves. Cells from an exponentially growing culture were exposed to 500, 1000 or 2000 µg/ml extract at 30 °C, 200 rpm, and viability was assessed by CFU after 0, 30, 60 and 90 min of incubation. The negative control was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. The data represent the mean ± S.D. of three independent experiments. The results from the statistical analysis can be found in the supplementary material (**Figure A3**).

3.1.3 Effect of aqueous extract of *Beta vulgaris* on *Saccharomyces cerevisiae* BY4741

To assess antifungal activity of *B. vulgaris* aqueous extract, also similar viability experiments were performed. In the negative control, as expected, the viability increased over time, reaching approximately 174 % of cell viability after 90 min (**Figure 7**).

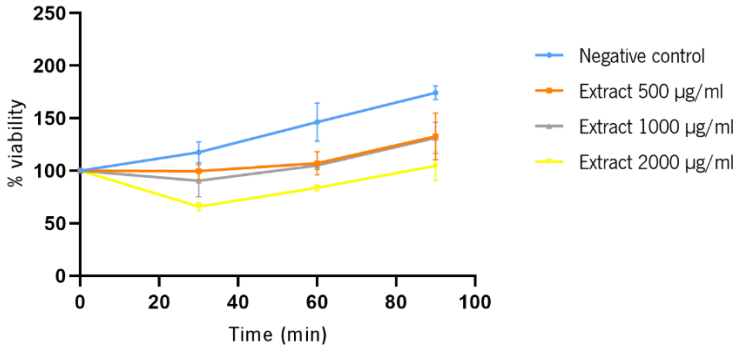

Figure 7. Viability of *Saccharomyces cerevisiae* BY4741 in the presence of aqueous extract of *Beta vulgaris*. Cells from an exponentially growing culture were exposed to 500, 1000 or 2000 µg/ml extract at 30 °C, 200 rpm, and viability was assessed by CFU after 0, 30, 60 and 90 min of incubation. The negative control was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. The data represent the mean ± S.D. of three independent experiments. The results from the statistical analysis can be found in the supplementary material (**Figure A4**).

Unlike to what was obtained with *D. purpurea* and *E. globulus* extracts, viability decreased in the presence of *B. vulgaris* extract when compared with the negative control. As we can see in the **Fig. 7**, after 30 min of incubation, the negative control and the lowest concentration (500 µg/ml) reached very similar values of cell viability, but at higher concentrations (1000 and 2000 µg/ml),

a significant decrease in cell viability was observed, respectively to 90 % (p<0.05; **Figure A4A**) and 66 % (p< 0.001; **Figure A4A**) of the control. Although there was a gradual increase in cell viability over time, after 30 min, in the presence of the different concentrations of extract, viability values were consistently lower than those of the control at all timepoints. After 90 min, cell viability in all treatments showed significant differences compared to the control, with these differences being more evident for the highest concentration tested (p<0.01; **Figure A4B**), suggesting a dose-dependent response to the extract. This behavior suggests antifungal activity of the extract.

These results suggest that *B. vulgaris* extract is a candidate for a possible natural antifungal agent. These are in line with the results obtained by Cherkupally *et al.*, (2017) who found antifungal activity of aqueous extract of *B. vulgaris* against *Fusarium oxysporum* and *Rhizoctonia solani* with percentage of inhibition of 10.3 % and 40.3 %, respectively.

3.1.4 Effect of aqueous and ethanolic extract of *Hedera helix* leaves on *Saccharomyces cerevisiae* BY4741

The last plant species used in the screening for antifungal activity was *H. helix*, for which aqueous and ethanolic extracts were prepared from its leaves. In the experiment with the ethanolic extract, viability in the negative control increased along time, as expected, reaching approximately 150 % after 90 min (**Figure 8A**).

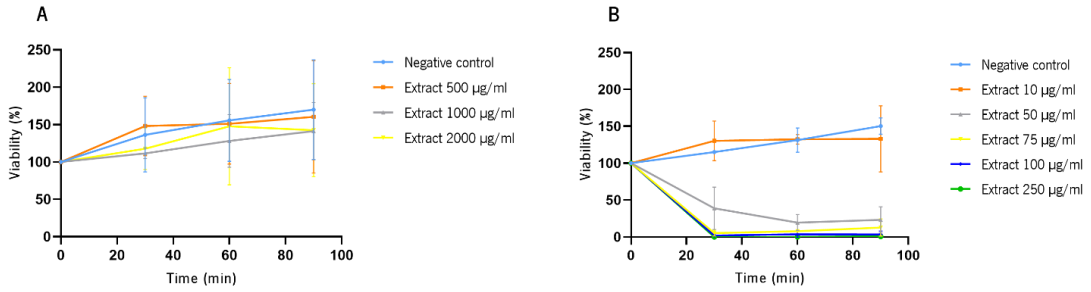

Figure 8. Viability of *Saccharomyces cerevisiae* BY4741 in the presence of ethanolic (A) or aqueous (B) extract of *Hedera helix*. Cells from an exponentially growing culture were exposed to 500, 1000 or 2000 µg/ml extract, in the case of ethanolic extract, or 10, 50, 75, 100 or 250 µg/ml, in the case of aqueous extract, and incubated at 30 °C, 200 rpm. Viability was assessed by CFU after 0, 30, 60 and 90 min of incubation. The negative control was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. The data represent the mean ± S.D. of three independent experiments. The results from the statistical analysis can be found in the supplementary material (**Figure A5**).

For the treatments no decrease in viability was detected even with the highest concentration tested. With all the concentrations tested, viability was similar to the control after 30 min of incubation (**Figure A5A**). This suggests that the ethanolic extract of *H. helix* leaves does not have toxic effects in yeast. Contrarily, with the aqueous extract the viability was remarkably lower when using the

same set of concentrations (not shown), so we adjusted to 0, 10, 50, 75, 100 and 250 µg/ml. The results obtained for this extract are depicted in **Figure 8B**. The control and the lowest concentration of *H. helix* extract tested (10 µg/ml) showed the highest viability. After 30 min of incubation, the percentage of viable cells incubated with 50 µg/ml decreased to values below 50 % (p<0.001; **Figure A5B**) and kept decreasing till 60 min. For the concentrations 75, 100 and 250 µg/ml, the viability was already around 0 % after 30 min of incubation (p<0.0001; **Figure A5B**).

Among the various compounds present in *H. helix* leaves, there is reference in the literature to a saponin, α-hederin, with antifungal activity. This compound, as demonstrated by Favel *et al.*, (1994), possesses antifungal activity against *Candida glabrata* and *C. albicans*, with MICs of 25 and 5 µg/ml, respectively. However, Roşca-Casian *et al.*, (2017) demonstrated antifungal activity of 50 % ethanol extract of *H. helix* against phytopathogenic fungi namely: *A. niger*, *Botrytis cinerea*, *B. tulipae*, *F. oxysporum*, *Penicillium gladioli* and *Sclerotinia sclerotiorum.* This does not totally concord with the results of this work, chemical analysis may be useful to explain the differences since methods of extraction and time of harvest, among other factors, may contribute to different chemical compositions.

3.2 Mechanism of action of *Hedera helix* aqueous extract

In order to better understand the mechanism of action of this extract, viability tests were also performed with mutant strains such as *erg2*, *bck1*, *mkk1/mkk2* and *yca1*. This strategy was based on the assumption that if a mutant strain is more resistant to the extract than the wild type, then the bioactive component(s) of the extract would have as receptor the protein encoded by the mutated gene or another protein with which it interacts.

The ergosterol biosynthesis, an essential component of fungal plasma membranes, involves the participation of 30 enzymes known as Erg proteins (Liu *et al.*, 2019; Jordá and Puig, 2020). Mevalonate biosynthesis, farnesyl pyrophosphate biosynthesis, and ergosterol biosynthesis are the three modules that make up the ergosterol biosynthesis pathway (Liu *et al.*, 2019). Squalene synthase, Erg9, catalyzes the conversion of two molecules of farnesyl pyrophosphate to squalene, which is the precursor of all steroids of the final module. Squalene epoxidase, Erg1, converts squalene to squalene epoxide, which is then transformed into lanosterol by lanosterol synthase, Erg7. After this, the enzymes lanosterol 14-demethylase, Erg 11 (also known as Cyp51), the C-14 reductase, Erg24, and the C-4 demethylation complex, Erg25-Erg26-Erg27, through several processes such as demethylation, reduction and desaturation reactions, convert the lanosterol into

zymosterol. Finally, the Erg6 coverts the zymosterol into fecosterol, which is converted into episterol, by sterol C-8 isomerase encoded by Erg2. In the final step, episterol is desaturated and reduced to ergosterol through the activity of Erg3, Erg5 and Erg4 (Liu *et al.*, 2019; Jordá and Puig, 2020; **Figure 9**). Usually, the proteins involved in the last module are the targets of azoles, antifungal drugs widely used in agriculture (Liu *et al.*, 2019).

Figure 9. Ergosterol biosynthetic pathway in *Saccharomyces cerevisiae*. The synthesis of ergosterol comprises 3 modules (distinguished by colors). The first module culminates in the synthesis of mevalonate (blue color), the second in the synthesis of farnesyl pyrophosphate (green) and finally, the third module, in the synthesis of ergosterol (yellow). The red circle corresponds to the gene encoding Erg2, the enzyme that is absent in the *erg2* mutant strain used in this work. Adapted from Hu *et al.*, (2017).

To study if aqueous extract of *H. helix* leaves could target ergosterol biosynthesis we used the mutant strain *erg2*, unable to synthesize the enzyme Erg2. As depicted in **Figure 10A**, viability of this mutant strain is similar to the wild type under the same treatment conditions (**Figure 8B**). Regardless of the concentration, except for 10 µg/ml, viability at 30 min was significantly lower when compared with the control (p<0.0001; **Figure A6A**). These results suggest that the extract does not target ergosterol biosynthesis.

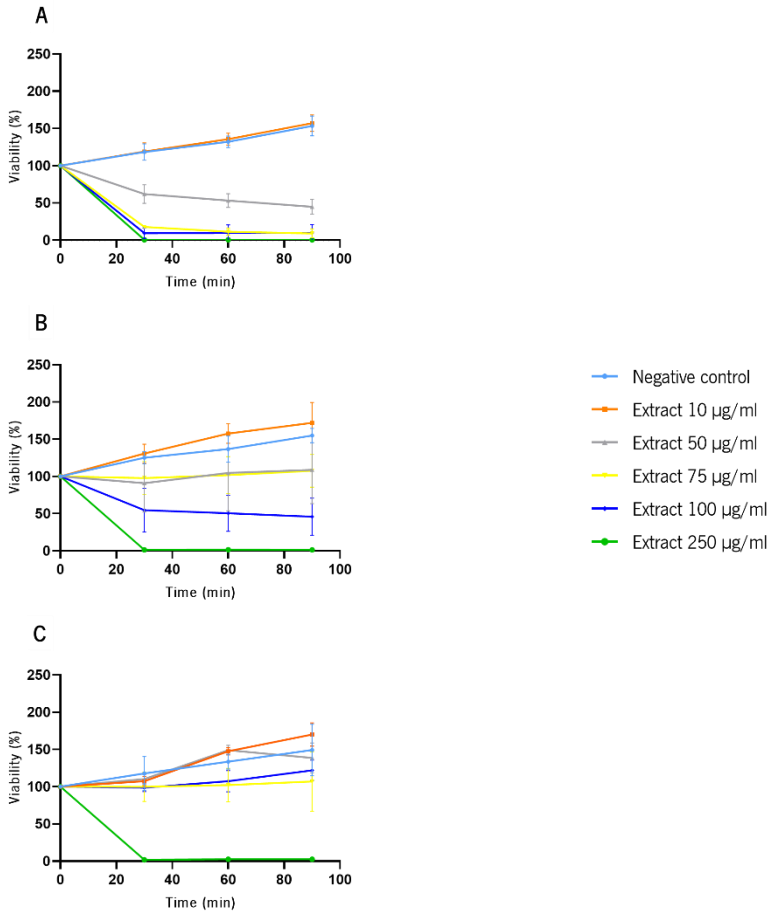

Figure 10. Viability of mutant strains *erg2* (A), *bck1* (B) and *mkk1/mkk2* (C) in the presence of aqueous extract of *Hedera helix*. Cells from exponentially growing cultures were exposed to 10, 50, 75, 100 or 250 µg/ml extract at 30 °C, 200 rpm, and viability was assessed by CFU after 0, 30, 60 and 90 min of incubation. The negative control was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. The data represent the mean ± S.D. of three independent experiments. The results from the statistical analysis can be found in the supplementary material (**Figure A6**).

As ergosterol biosynthesis is unlikely to be targeted by the extract, we evaluated if the target could be the cell wall, using strains with the genes *BCK1* or *MKK1/MKK2* blocked. These genes are involved in the same protein kinase signaling pathway, which through successive phosphorylations, contribute to the cell wall integrity (Traven *et al.*, 2009). In yeast, the protein kinase C (Pkc1) is involved in the regulation of polarized growth and stress responses, specifically in the modulation of the cell wall integrity (CWI) signaling pathway, which involves cell wall remodeling and stress response gene expression (Nomura *et al.*, 2017). This protein activates the MAPK kinase kinase (MAPKKK) Bck1. Bck1, when active, phosphorylates MAPK kinases (MAPKKs) Mkk1 and Mkk2 (Udom *et al.*, 2019). In turn, Mkk1/Mkk2 activate the MAPK Slt2 that activates two transcription factors: Rlm1 and the SBF complex (**Figure 11**). Rlm1 and the SBF complex are involved in the expression of genes responsible for cell wall synthesis as well as in the cell cycle

(Jendretzki *et al.*, 2011; Udom *et al.*, 2019). Normally, this pathway is activated due to osmotic shocks or also due to the action of cell wall stressing agents, such as zymolyase, calcofluor white, or Congo red (Nomura *et al.*, 2017).

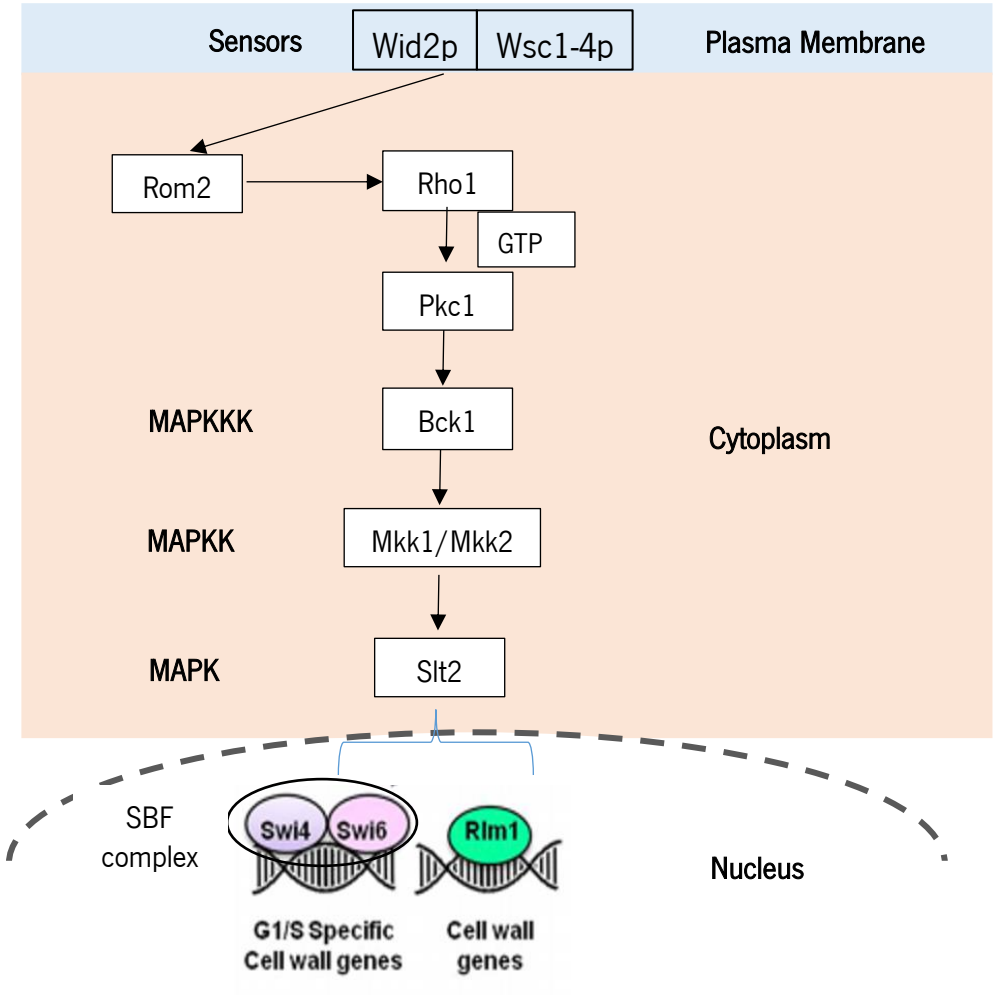

Figure 11. Representative image of the Cell wall integrity (CWI) pathway. Sensors in the plasma membrane, due to various cell wall stresses, activate the PKC signaling pathway via MAP kinase. The cytoplasmic tail of Wsc1 sensor interacts with GDP/GTP exchange factor Rom2, which catalyzes the nucleotide exchange and thereby activates the small GTPase Rho1. The interaction of Roh1-GTP with protein kinase C (Pkc1) triggers a Mitogen-Activated Protein Kinase (MAPK) cascade composed by the MAPKKK Bck1, a dual pair of MAPKKs (Mkk1/Mkk2) and the MAPK Slt2. Target transcriptions factors are the SBF complex (Swi4 and SWi6) and Rlm1. The first one regulates cell cycle progression and Rlm1 activates genes involved in cell wall synthesis, composition and remodeling. Adapted from Reinoso-Martín *et al.*, (2003); Kock *et al.*, (2015); Udom *et al.*, (2019).

When the *bck1* mutant was used (**Figure 10B**), the viability of the negative control increased along time, similarly to *erg2* (**Figure 10A**), and the wild type (**Figure 8B**). This behavior was similar when cells were exposed to 10 µg/ml however, unlike the wild type strain, with 50 or 75 µg/ml viability was constant throughout the experiment. Only with the higher concentrations tested, a significant

decrease to 50 % (100 µg/ml; $p<0.05$; **Figure A6B**) and 0 % (250 µg/ml; $p<0.001$; **Figure A6B**), respectively, was observed after 30 min. These results suggest that the mutant was more resistant to the extracts when compared to the wild type BY4741. Significant differences between both strains were observed when statistical analysis was performed for the time-point 30 min (75 µg/ml; $p<0.001$; **Figure A8B**). With respect to the double mutant strain *mkk1/mkk2* (**Figure 10C**), viability with the negative control and with 10 µg/ml extract has also increased over time, however, when cells were exposed to 50, 75 or 100 µg/ml, viability did not change significantly till the end of the experiment (90 min of incubation). This could indicate that the extract at these concentrations possesses a fungistatic action. Only at the concentration of 250 µg/ml it was possible to see a clear reduction of viability and highly significantly different from the control, reaching approximately 0 % after 30 min ($p<0.0001$; **Figure A6C**).

The data obtained with both mutants affected in the cell wall integrity signaling pathway, indicate that *bck1* was more sensitive than *mkk1/mkk2* to the toxic action of aqueous extract of *H. helix* leaves. Compared to the wild type strain and *erg2,* the *bck1* mutant and the double mutant strain *mkk1/mkk2* are more resistant to the *H. helix* extract. In the case of the *bck1* mutant, the statistical analysis indicates significant differences between viability with the wild type strain treated with 75 µg/ml after 30 min ($p< 0.001$; **Figure A8B**). For the *mkk1/mkk2* mutant, 50 µg/ml was sufficient to see significant differences in cell viability compared to the wild type after 30 min ($p< 0.01$; **Figure A8C**). An explanation for these results could be that the extract has the cell wall as target of its activity.

3.3 Evaluation of osmotic stress susceptibility

Previous results with yeast mutants pointed to the cell wall as, at least, one of the targets of the extract. Therefore, the extract would be more active under osmotic stress since the cell wall provides osmotic protection to cells. Accordingly, mutants affected in the PKC signaling pathway display an osmotic sensitive phenotype (Gerik *et al.*, 2008). In an attempt to corroborate the hypothesis of the cell wall being a target of the extract, cells from cultures grown in the absence of extract (negative control) and in the presence of two concentrations of extract (10 or 50 µg/ml) were harvested, diluted and plated on plates containing different concentrations of NaCl as osmotic stressor. The cell viability in the negative control (without extract) was not influenced by the NaCl concentrations used up to the highest concetration tested (**Figure 12**) although smaller colonies were observed as the NaCl concentration increased (**Figure A9**). In accordance with **Figure 8B**, the

activity of the extract at 50 µg/ml is visible in the absence of NaCl with similar percentage of viability. The same was observed when viability was measured in the presence of NaCl but with a clear decrease along increasing concentrations of the osmotic stressor. This effect was more pronounced in 0.75 M NaCl in which a significant decrease of viability was observed when 50 µg/ml extract was used to incubate the cells (**Figure 12**). As cells are more sensitive to 50 µg/ml extract when challenged with 0.75 M NaCl than in its absence of NaCl, the toxicity of the extract might be related with the cell wall.

According to the results obtained in yeast mutants the cell wall may well be one of the targets of the tested extract. It is well known that the cell wall is the barrier between the cell and the extracellular environment and it is fundamental to keep the cell viable (Okada *et al.*, 2016; Wang *et al.*, 2018). The cell wall is able to detect changes in the extracellular environment and triggers various biological processes contributing to the adaptation of the cell to that environment (Wang *et al.*, 2018). It is therefore crucial that the cell wall is being constantly remodelled so that cell integrity is maintained under unfavourable environmental conditions (Novačić *et al.*, 2020). So, it was expected that the PKC signaling pathway mutants, *bck1* and *mkk1/mkk2*, would be more sensitive to the extract. This unexpected higher resistance (**Figure 10B** and **Figure 10C**, respectively) might be due to the complex downstream effects of this signaling pathway. It has been reported that the PKC pathway controls not only cell wall remodeling but also the actin cytoskeleton organisation, autophagy, apoptosis, nutrient sensing, ribosome biogenesis, cell cycle, cytokinesis and genome stability (recently reviewed by Heinisch and Rodicio, 2018). So, further studies would be required to determine the cellular effects of the activity of the extract on the PKC pathway.

Taken together, the results with the *bck1* and *mkk1/mkk2* mutants and from the osmotic susceptibility assays, clearly point to perturbance of the PKC signaling pathway and cell wall integrity. The chemical complexity of the extract might account to multiple toxic effects such as direct action on the cell wall and interference with the PKC signaling to downstream effects other than the cell wall. The fact that the wild type strain is more sensitive than the mutant strains can be explained by a hyperactivation of the pathway with downstream toxic effects, which are not possible when the cell has a deficient pathway by mutations in *BCK1* and *MKK1* and *MKK2*.

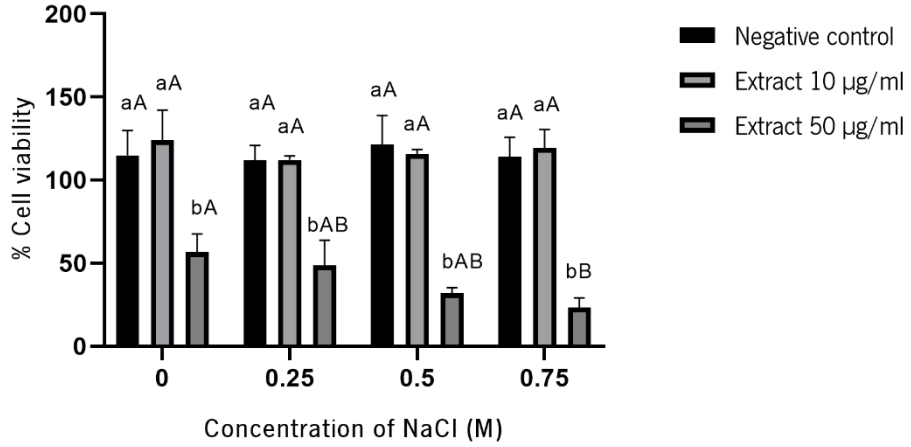

Figure 12. Viability of *Saccharomyces cerevisiae* BY4741 in the presence of aqueous extract of *Hedera helix* and osmotic stress. Cells from an exponentially growing culture were exposed to 10 or 50 µg/ml extract at 30 °C, 200 rpm, and viability was assessed by CFU on plates containing different concentrations of NaCl (0, 0.25, 0.5 or 0.75 M) after 0, 30 and 60 min of incubation (only results for 30 min are represent here). The negative control was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. The data represent the mean ± S.D. of three independent experiments. For statistical analysis the letter code was used (lowercase letters for comparisons of extract concentrations effects within each salt concentration and capital letters for comparisons between salt concentrations for each extract concentration). Mean values followed by the same letters are not statistically different.

In addition to ergosterol biosynthesis and cell wall integrity, the extract was evaluated for the capacity to trigger apoptosis with the *yca1* mutant of *S. cerevisiae*. Despite the fact that yeast is a single-cell eukaryote, it is widely assumed that it undergoes programed cell death (PCD), in response to harmful environmental factors (Wong *et al.*, 2012). It is thought that when *S. cerevisiae* is under stress conditions or when defects are detected in DNA replication or normal mitochondrial function, the metacaspase Yca1 is activated and triggers apoptosis (Mazzoni and Falcone, 2008; Shrestha *et al.*, 2019). Mutations in *YCA1* gene are normally associated with an increase of cell survival after different stresses due to the absence of apoptotic markers (Severin *et al.*, 2008). Because the *yca1* mutant was constructed in W303-1A genetic background (Vendrell *et al.*, 2011), this strain was used as the wild type phenotype reference in the viability assays.

Saccharomyces cerevisiae W303 cells treated with 10 µg/ml of extract show a similar behavior when compared to the control cells. For all other concentrations, viability values were lower than the control at all the time points (**Figure 13A**) and the differences were statistically significant (**Figure A7**). In a similar way to the wild type, the control and mutant cells treated with 10 µg/ml displayed the same behavior of increase of viability along time (**Figure 13B**). With *H. helix* extract at 50 µg/ml, a substantial decrease of viability was observed after 30 min, reaching approximately 20 %. For 75 µg/ml, 30 min was sufficient to decrease the cell viability to 10 % and

for higher concentrations total inhibition was observed at the same timepoint. When compared with the control, the percentage of viability was significantly different in all concentrations of extract tested, except with 10 µg/ml (p<0.001; **Figure A7B**). Therefore, results obtained with the *yca1* show that this mutant strain is more sensitive than the wild type, suggesting that the *H. helix* extract does not trigger apoptosis. Statistical analysis show that this difference is significant when compared with the wild type in the presence of 50 µg/ml over 30 min of incubation (**Figure A8D**).

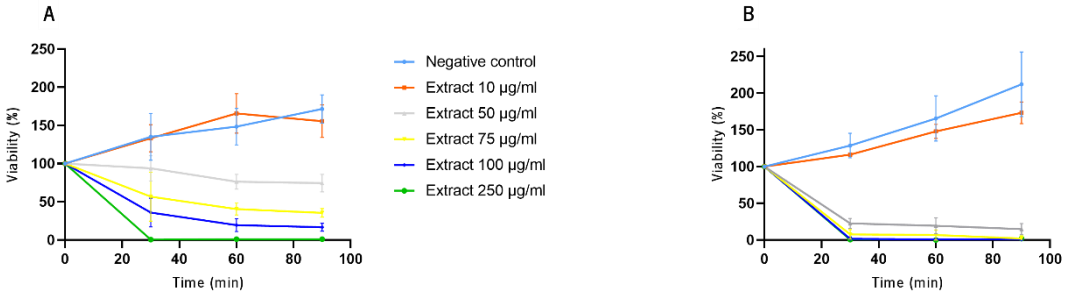

Figure 13. Viability of *Saccharomyces cerevisiae* W303 (A) and mutant strain *yca1* (B) in the presence of aqueous extract of *Hedera helix*. Cells from an exponentially growing culture were exposed to 10, 50, 75, 100 or 250 µg/ml extract at 30 ° C, 200 rpm, and viability was assessed by CFU after 0, 30, 60 and 90 min of incubation. The negative control was prepared by replacing the extract by the solvent at the highest volume of extract used in the assays. The data represent the mean ± S.D. of three independent experiments. The results from the statistical analysis can be found in the supplementary material (**Figure A7** and **Figure A8**).

When comparing all mutants to the corresponding wild type strains it was possible to observe that *erg2* shows similar behavior as the wild type strain unlike *bck1* and *mkk1/mkk2* mutants that show increased resistance to the extract. The normal functioning of the plasma membrane, such as functions related to transport and the activity of membrane-bound enzymes, is related to membrane organization. This organization is influenced by the constitution, namely of sterols, present in the membrane. Normally this organization is expressed in terms of the order parameter, S, whose high value reflects membranes with a high organization and conversely, low values of S reflect membranes with high fluidity/lower order (Lees *et al.*, 1979). Abe and Hiraki (2009), demonstrated that a decrease of the order parameter of the plasma membrane and the existence of spaces in the membrane affect its rigidity as well as the sensitivity to a drug. The authors tested the sensitivity of *erg* mutants to cycloheximide and among the *erg* mutants tested, they found that the *erg2* mutant showed more sensitivity to the compound compared to the wild type. Passive diffusion across the membrane due to altered sterol composition was the reason given by the authors. However, according to our results, the mutant is not more sensitive than the wild type, which could mean that the lack in Erg2 for ergosterol synthesis does not impair cell viability at least

under normal conditions (without the extract). Also, under the influence the activity of the extract differences between wild type and *erg2* were not noticeable. Taken together, we deduced that ergosterol biosynthesis would not be the target of our extract.

With the *bck1* and *mkk1/mkk2* mutants we found that they are more resistant to the extract compared to the wild type. This suggests that the target of the aqueous extract of *H. helix* might be the cell wall. According to Prescott *et al.*, (2014), a cytotoxic saponin present in *H. helix* extract, α-hederin, acts similarly to the antifungal drug caspofungin by inhibiting β-glucan synthesis. However, Reinoso-Martín *et al.*, (2003) demonstrated that the integrity of the PKC signaling pathway is related to tolerance to caspofungin action. These authors found that in the absence of Slt2, the MAPK of the PKC MAP kinase signaling cascade, *S. cerevisiae* exhibited delayed growth in the presence of low amounts of caspofungin and an inability to grow at higher concentrations of the same compound. However, when we used two mutant strains affected in different steps of the PKC pathway, we saw an increase in resistance to the ivy extract. A possible explanation is that the *H. helix* extract is a complex mixture, which may culminate in different mechanisms of activity. It was also thought that since the remodeling of the cell wall is disrupted due to mutations in the *BCK1* and *MKK1/MKK2* genes, the active agent of our extract may be missing the target, thus preventing or hindering its action. Furthermore, the existence of a crosstalk between signaling pathways may contribute to the increased resistance of mutants to the extract. In yeast, there is crosstalk between this PKC signaling pathway and the calcineurin pathway. Calcineurin-responsive zinc finger transcripton factor, Crz1 also appears to contribute to cell wall integrity (Wang *et al.*, 2020). Thus, another possible explanation could be the hyperactivation of this pathway rather than the PKC pathway.

Regarding the mechanism of loss of viability, our results do not point to apoptosis. The *yca1* mutant, which is affected in the apoptosis mechanism, is more sensitive to the extract than the wild type strain, indicating that loss of viability is mediated by other process than apoptosis. However, since metacaspase Yca1 is not the only one involved in programmed cell death (PCD), we cannot exclude the hypothesis that the extract might trigger another metacaspase and cause apoptosis. As reported by Chin *et al.*, (2014), the action of the antifungal agent caspofungin requires the pro-apoptotic gene *AIF1* but does not require the metacaspase Yca1. On the other hand, a different death mechanism may be involved upon the action of *H. helix* extract. For instance, according to Mazzoni and Falcone (2008), Yca1 is known to be involved in triggering PCD in response to two physiological conditions: chronological age and killer toxins. In the case of toxins

that kill more sensitive cells, three toxins have been identified in *S. cerevisiae* namely K1, K2 and K28. However, the presence of the same toxins but in different concentrations triggers different responses. Low concentrations of all the toxins lead to the triggering of Yca1-mediated apoptotic cell death while in the presence of high concentrations of the same toxins cell death occurs by non-apoptotic mechanisms, not depending on Yca1 caspase activity.

3.4 Antifungal activity of *Hedera helix* aqueous extract on phytopathogenic fungi

Assays of *H. helix* toxicity on *S. cerevisiae* as fungal model clearly show a remarkable antifungal activity. In accordance with the objectives of this work, this activity was then assessed against phytopathogenic fungi, so that the potential application in farming can be evaluated. Different concentrations of the extract were incorporated in solid medium and the antifungal activity was estimated by measuring the mycelium growth after 3, 6 and 9 days of incubation. By comparing the mycelium diameter in the control with the diameter of fungi growing on the different concentrations of *H. helix* extract it was possible to calculate the percentage of inhibition.

3.4.1 Effect of *Hedera helix* extract on mycelium growth of *Colletotrichum* species

Disks of *Colletotrichum* mycelia were placed in the center of Petri dishes with PDA medium supplemented with different concentrations of *H. helix* aqueous extract and then allowed to incubate at 25 °C in the dark. Regarding the results obtained with *C. gloeosporioides* after three days of incubation (**Figure 14A**), there is only a slight difference in diameter of mycelium between all concentrations and the negative control. Despite this apparent slower growth in the presence of the different extract concentrations, a significant inhibition (7 %) was only detected at 1500 µg/ml ($p<0.05$; **Figure A10**) on the sixth day, being lost after 9 days of incubation. This suggests that possibly the fungus *C. gloeosporioides* have adapted to the extract. These results also suggest that the aqueous extract of *H. helix* has no influence on the normal growth of the fungus; and we also found that the morphology of the colony as well as the mycelium density were not affected by increasing extract concentration (**Figure 14E**). The results obtained with the other fungi of the genus *Colletotrichum* used in this work - *C. godetiae*, *C. nymphaeae* and *C. acutatum* - followed the same behavior. For all *Colletotrichum* species none of the concentrations tested were able to significantly inhibit the mycelium growth, for any day of incubation (**Figure A11**).

To the best of our knowledge, the aqueous extract of *H. helix* has not yet been tested against these phytopathogenic fungi. However, Kim *et al.*, (2018) studied the antifungal activity against

Colletotrichum coccodes of some compounds isolated from *Trevesia palmata*, a plant species of the same family as *H. helix*. *C. coccodes* was shown to be resistant to the activity of these compounds, which is in concordance with the results obtained in this work for all the species of *Colletotrichum* used.

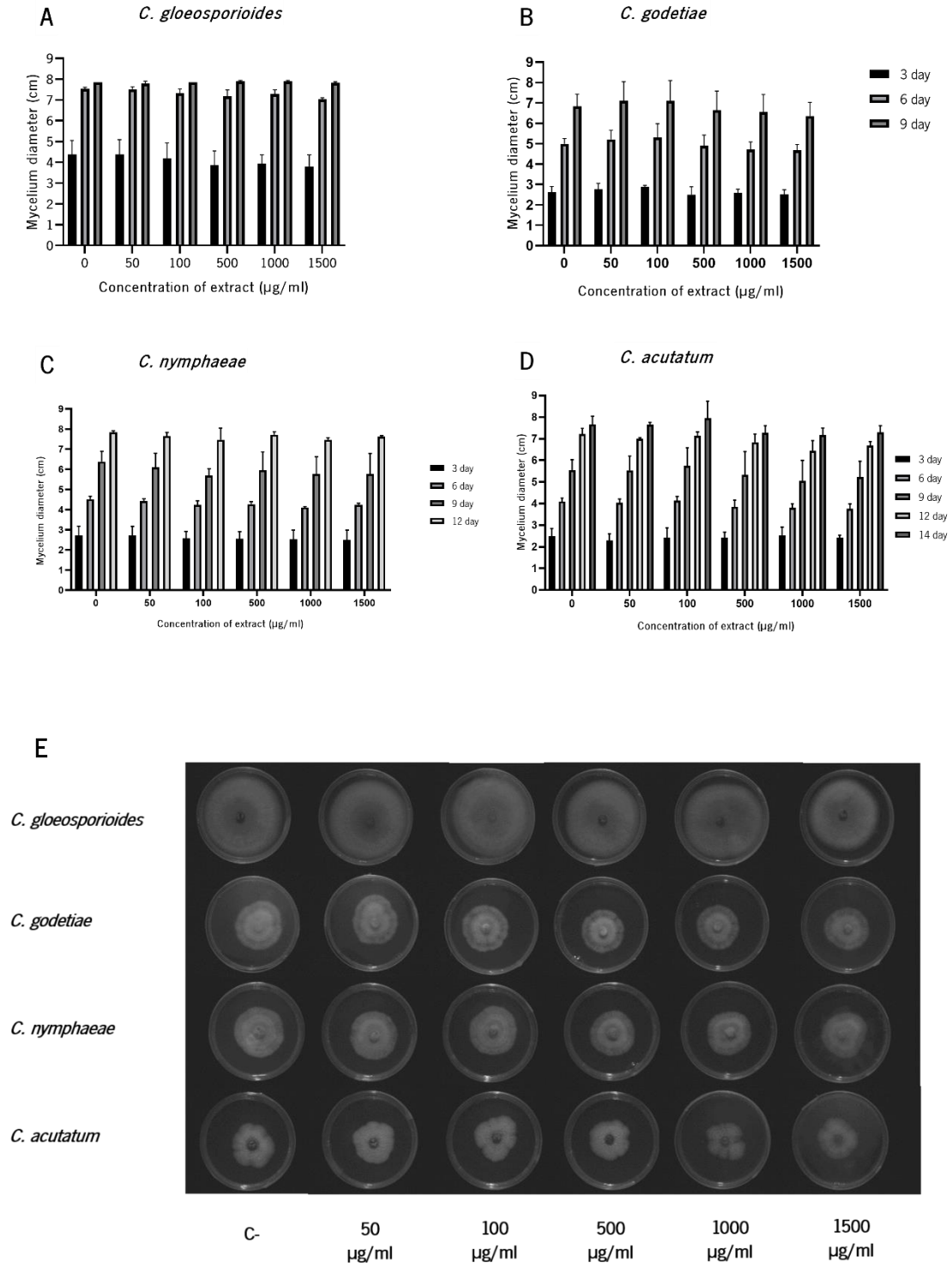

Figure 14. Evaluation of antifungal effect of *Hedera helix* aqueous extract on *Colletotrichum* species. A small portion of fungal mycelium was excised from the margins of 12 days old cultures and placed in the middle of Petri dishes with PDA medium with different concentrations of extract: 50, 100, 500, 1000 or 2000

µg/ml. In the negative control (C-), the highest volume of extract used in the assays was replaced by the solvent. The mycelia diameter of *Colletotrichum gloeosporioides* (A), *Colletotrichum godetiae* (B), *Colletotrichum nymphaeae* (C) and *Colletotrichum acutatum* (D) were measured along time. Each graph corresponds to the average mycelium diameter of each *Colletotrichum* species, measured along time for each extract concentration. Each bar represents the mean ± S.D. of three independent experiments. Representative images of the antifungal activity of *Hedera helix* extract at different concentrations compared to the negative control (C-) against *Colletotrichum* species after 6 days of incubation (E). The results from the statistical analysis can be found in the supplementary material (**Figure A10** and **Figure A11**).

3.4.2 Effect of *Hedera helix* aqueous extract on mycelium growth of *Phytophthora cinnamomi*

The results obtained with the evaluation of the antifungal activity of *H. helix* aqueous extract against *P. cinnamomi* were very similar to those obtained with *Colletotrichum* species. It was possible to observe that mycelial growth was similar to the control for all the tested concentrations (**Figure 15A**). With the statistical analysis it was verified that no concentration of extract was able to significantly reduce the size of the mycelium during the experiment (**Figure A12**). Our results indicate that the extract was not able to affect the mycelial growth of this oomycete nor the density or morphology of the colony (**Figure 15B**).

To the best of our knowledge no antifungal activity of *H. helix* extracts has yet been reported against this oomycete. However, Kim *et al.*, (2018) studied five compounds isolated from the *Trevesia palmata,* belonging to the same family as ivy, and found relatively low half maximal inhibitory concentration (IC_{50}) values for four of the compounds tested, demonstrating satisfactory inhibition of *Phytophthora infestans*. One of the triterpene glycosides studied, α-hederin, exhibited one of the lowest IC_{50} values thus reinforcing its possible antifungal potential. A possible explanation for this difference to our results could be that it is not the pure compound tested which could influence the ability to inhibit fungal growth. On the other hand, the presence of several compounds could influence the mechanism of action, which seems to agree with the results obtained in yeast.

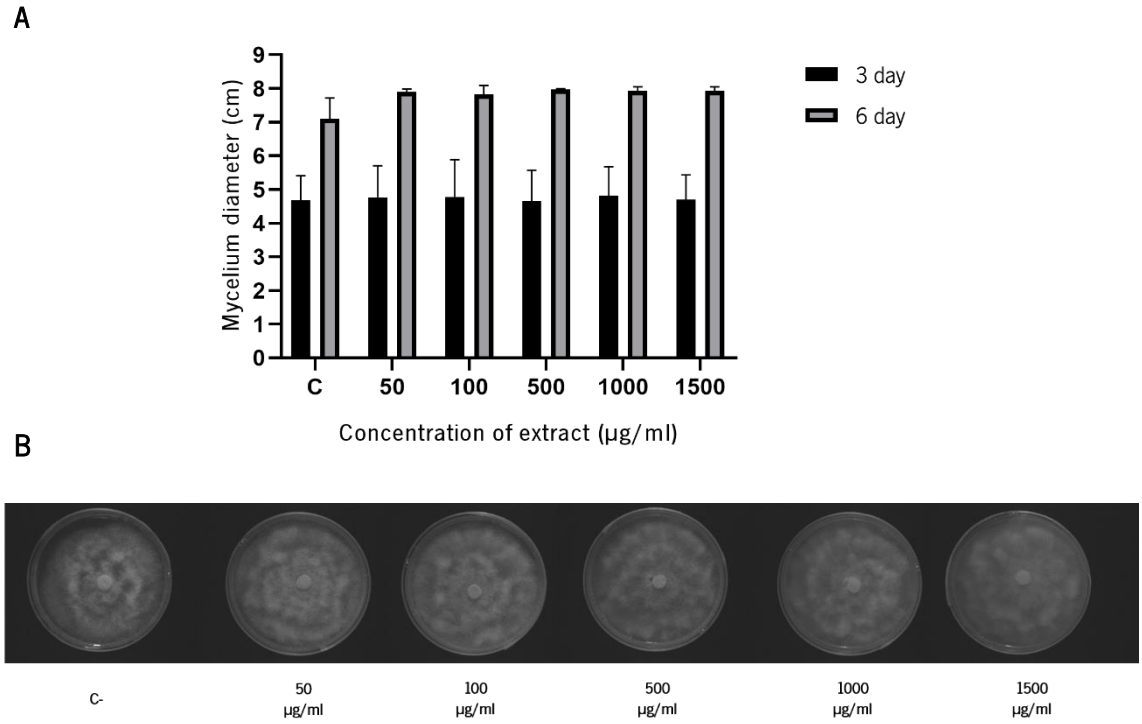

Figure 15. Evaluation of antifungal effect of *Hedera helix* aqueous extract on *Phytophthora cinnamomi*. A small portion of fungal mycelium of *Phytophthora cinnamomi* was excised from the margins of 12 days old cultures and placed in the middle of Petri dishes with PDA medium with different concentrations: 50, 100, 500, 1000 or 1500 µg/ml. In the negative control (C-) the highest volume of extract used on the assays was replaced by the solvent. The mycelium diameter of *Phytophthora cinnamomi* was measured along time for each extract concentration (A). Each bar represents the mean ± S.D. of three independent experiments. Representative images of *Hedera helix* antifungal activity at different concentrations compared to the negative control (C-) against *Phytophthora cinnamomi* after 6 days of incubation (B). The results from the statistical analysis can be found in the supplementary material (**Figure A12**).

3.4.3 Effect of *Hedera helix* aqueous extract on mycelium growth of *Diplodia corticola*

The activity of *H. helix* extract was also evaluated against *D. corticola*. In **Figure 16A**, which represents the diameter of mycelium under different concentrations of extract over 9 days, it is possible to see that in the presence of 50 µg/ml of extract the mycelium size reaches values similar to the control with no significant differences, except on the third day (p<0.05; **Figure A13**). For all other concentrations tested, growth was slower than the control (**Figure 16C**), with significant differences on the third and sixth days of incubation (p<0.0001; **Figure A13**). The concentration of 1000 µg/ml of *H. helix* extract caused the highest percentage of inhibition at the third day, reaching approximately 70 %. Regarding the other concentrations, 100, 500 and 1500 µg/ml, we also verified remarkably high values, approximately 50 %, 65 % and 64 % of inhibition, respectively (**Figure 16B**). After 9 days, the fungus shows approximately the same diameter when compared to

the control. A possible explanation for this behavior is the adaptation of the fungus to the action of the extract, ending in a decrease of its susceptibility.

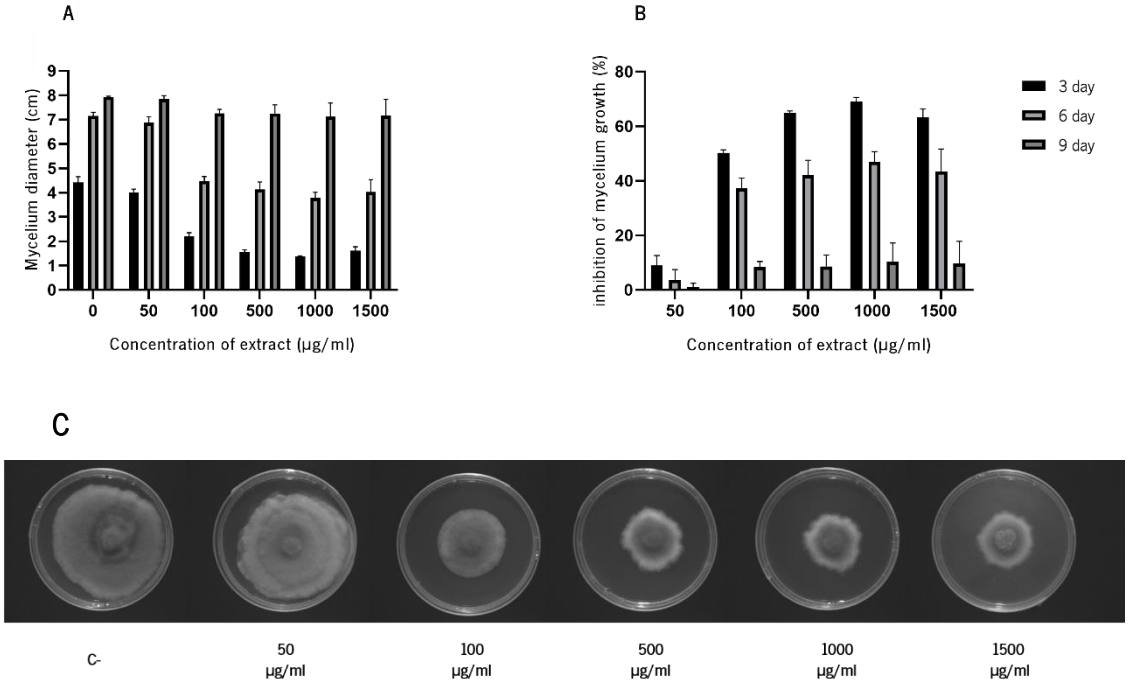

Figure 16. Evaluation of antifungal effect of *Hedera helix* aqueous extract on *Diplodia corticola*. A small portion of fungal mycelium of *Diplodia corticola* was excised from the margins of 12 days old cultures and placed in the middle of Petri dishes with PDA medium with different concentrations of extract: 50, 100, 500, 1000 or 1500 µg/ml. In the negative control (C-) the highest volume of extract used on the assays was replaced by the solvent. The mycelium diameter was measured over 9 days (A) and the percentage of mycelium growth was calculated (B). Each bar represents the mean ± S.D. of three independent experiments. Representative images of *Hedera helix* antifungal activity at different concentrations compared to the negative control (C-) against *Diplodia corticola* after 6 days of incubation (C). The results from the statistical analysis can be found in the supplementary material (**Figure A13**).

The results obtained with the fungus *D. corticola* were totally different when compared with the other fungi tested. In this work, we present evidence indicating that the target of the extract is the cell wall. As we know, the cell wall plays an important role in cell viability, morphogenesis and pathogenicity, and its composition is strongly regulated in response to environmental or stress conditions. The proteins that exist in the cell wall of fungi that can contribute to cell wall modification, adherence to surfaces and also help protect the fungus from toxic environments are usually species-specific. The inner cell wall of most fungal species is made up of a core of covalently connected branched β-1,3, -1,6 glucan linked through β-1,4 linkage to chitin (Latgé, 2007). The proteins and other polysaccharides that bind to branched β-(1,3):β-(1,6) glucan may vary with the fungal species (Gow *et al.*, 2017). It is possible that even between true fungi, small differences in the cell wall may contribute to increased sensitivity to the extract, in this case in *D. corticola* and increased resistance in other cases, as observed in *Colletotrichum* species. Furthermore, there is

a notable and relevant difference between fungi and oomycetes, which are not true fungi. Normally, oomycetes have cellulose instead of chitin in their cell wall (Badreddine *et al.*, 2008). So, the cell wall can then vary between fungal species and be one of the reasons for the observed differences.

To our knowledge, extracts of this plant have not yet been tested against *D. corticola*, so these results seem to be promising for future application in the control of infections caused by this phytopathogenic fungus that, as already mentioned, infests the cork oak. The raw material obtained from this tree, cork, is a major economic source, especially for Portugal, which is the world leader in the production, export and marketing of this product. Portugal has more than 730,000 hectares of cork oaks, producing around 100,000 tons of cork (Associação Portuguesa da cortiça, 2018). This evidence highlights the importance of the results obtained since it may be possible to use the aqueous extract of *H. helix* and thus ensure high productivity and high quality of a product of high economic importance in an environmentally sustainable manner. Furthermore, it should be noted that the extract, being aqueous, does not raise additional concerns for hypothetical application in real natural conditions since it does not contain solvents potentially dangerous to the ecosystem.

3.5 *Ex vivo* assays using strawberry leaves

In order to evaluate the viability of a future application of the extract as an antifungal in agricuture, an *ex vivo* assay was performed with strawberry mature leaves to assess the effectiveness and eventual phytotoxicity of the ivy extract. Three controls were made, the negative control where the leaves were brushed twice on both surfaces with sterile deionized water only; extract control, where the leaves were brushed twice and both surfaces with the highest concentration of extract used (2000 µg/ml) and the infection control, where the leaves after being brushed on both surfaces with sterile deionized water were brushed (adaxial part) with a previously prepared suspension of *C. acutatum* spores. For the treatments, the extract (1000 or 2000 µg/ml) was brushed on both surfaces of leaves and, after drying, the adaxial side was brushed with *C. acutatum* spore suspension. The leaves were placed in Petri dishes, maintained with a humid atmosphere, near to the window, and were monitored for symptoms of fungal infection during 12 days. After 3 days, brown spots were visible only on the leaves inoculated with the fungus (CI), proving an effective infection with *C. acutatum* (**Figure 17**), while leaves from the other groups displayed an appearance without evidence of extract toxicity or fungal infection, being similar to the negative control (C-). According to Arroyo *et al.*,(2005), the infection of leaves results in macroscopic symptoms such as the appearance of these brownish spots. After 6 days, no

significant differences were observed in the leaves, as compared to 3 days incubation. At the end of the ninth day, the intensification of the brown spots and leaf yellowing began to be observed in control leaves infected with the fungus (CI). Interestingly, in leaves treated with 1000 µg/ml extract and infected (C1000), the yellowish symptom was also emerging, although much less extensive than in the CI, but in leaves treated with 2000 µg/ml (C2000) no symptoms were detected. On the twelfth day, the yellowish area on C1000 leaves increased and appeared for the first time on C2000 leaves, however, in both cases in a much less extent than in CI. As the yellowing symptom did not appeared on CE leaves, together these results suggest that the aqueous extract of *H. helix* leaves is retarding the infection of *C. acutatum* on strawberry leaves and in a dose-dependent way. The fact that symptoms are visible after 9 or 12 days, for 1000 µg/ml and 2000 µg/ml, respectively, suggests that the tested concentrations were not lethal allowing the fungus to adapted and resume colonization and/or infections process later on, or that the active components of the extract are degraded over time. This outcome is of utmost importance and needs further investigation for designing treatment protocols before an antifungal product is released to the market. Remarkably, the control used to investigate potential effects of the extract (CE) remained free of symptoms until the last day of the experiment, suggesting that the aqueous extract of *H. helix* leaves has no phytotoxic effects. Also, the normal and healthy aspect of strawberry leaves of the negative control (C-) is an indication that the conditions of humidity and surface sterilization of leaves were appropriate, as was the time lapse of the experiment, validating this way the results obtained in the treatments.

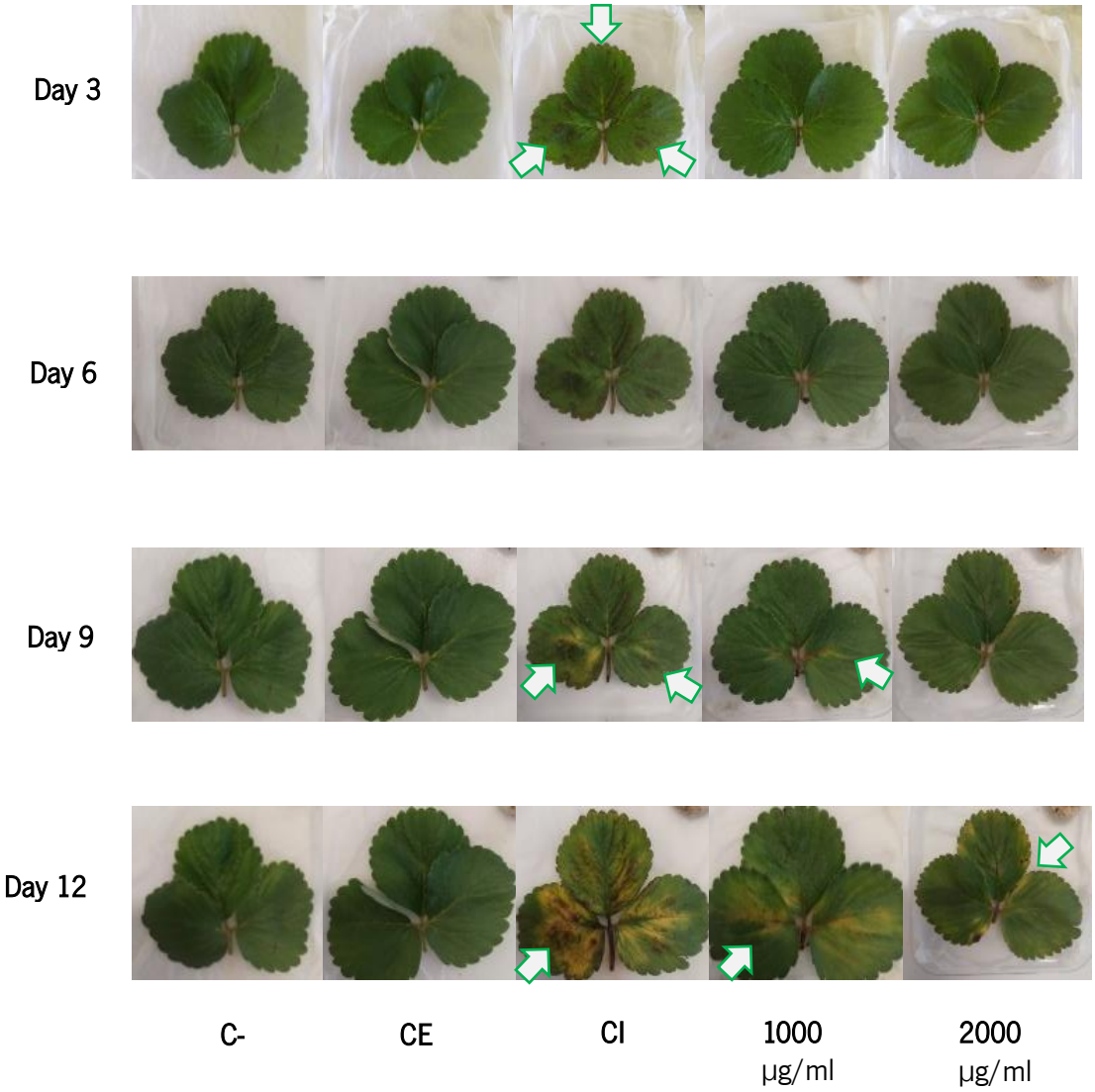

Figure 17. *Ex vivo* assay for antifungal activity of *Hedera helix* leaves extract against *Colletotrichum acutatum* infection in strawberry leaves. Trifoliate leaves of strawberry plant, variety Camarosa, were collected from healthy mature plants and treated with the extract and/or infected with *Colletotrichum acutatum* spores. Leaves were incubated for 12 days and symptoms of infection were recorded along time. The C- correspond to the negative control, without fungal inoculation and brushed twice with deionized sterile water. CE corresponds to control of extract; leaves were brushed twice with the highest concentration tested, 2000 µg/ml. CI is the control of infection; leaves were brushed with sterile deionized water and afterwards brushed with *Colletotrichum acutatum* spore suspension. 1000 µg/ml and 2000 µg/ml correspond to leaves brushed with 1000 or 2000 µg/ml *Hedera helix* extract and afterwards brushed with *Colletotrichum acutatum* spore suspension. These images are representative of 3 independent experiments.

3.6 *Ex vivo* assay using *Quercus suber* apical cuttings

Promising results were obtained with the aqueous extract of *H. helix* leaves against *D. corticola in vitro* (**Figure 16**). This fungus is one of the main responsible for the decline of *Quercus* species (Félix *et al.*, 2017), so it is important to investigate if the ivy extract could be used directly in the field to control *D. corticola* infection and related diseases. For a more field realistic approach, small

cork oak apical 20-cm branches were collected from mature trees in field stands and submitted to different treatments in the lab in a set-up detailed in Material and Methods (section 2.5.3).

Briefly, five apical cuttings (replicates) were used per condition: negative control, extract control, infection control and treatment with two extract concentrations (1000 or 2000 µg/ml). A small longitudinal incision (0.5- 1 cm) was made with a sterile scalpel on each cutting at 5 cm from the base. Here was placed a half of 5-mm plug of *D. corticola* mycelium or of PDA medium and wrapped with parafilm (**Figure 18B**). To apply the extract, the 5-mm apical portion of each cutting was cut and a small cotton ball impregnated of the extract or water was attached and wrapped with parafilm (**Figure 18A**). The experiment was monitored every day for one month.

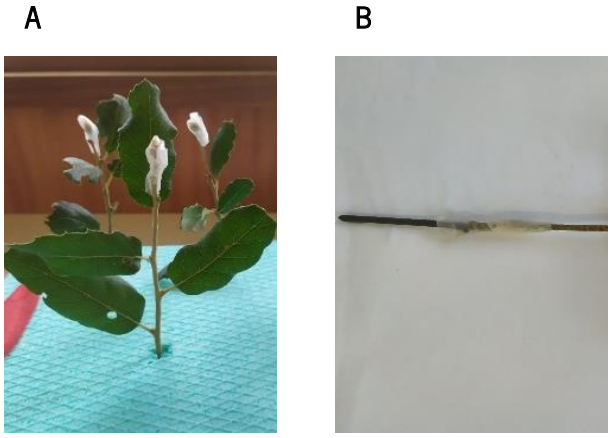

Figure 18. Representative images of the cuttings used to study the activity of the *Hedera helix* extract against artificial infection of the fungus *Diplodia corticola.* Each cutting was approximately 20 cm with 4-5 leaves (A). The lesion for fungal inoculation was done approximately 5 cm from the base and wrapped with parafilm (B).

With this *ex vivo* assay it was possible to observe that after 3 days the leaves started to show signs of dehydration in all the conditions, including the negative control (**Figure 19**). This was somewhat unexpected because a similar protocol was already reported for inoculation in 3-year-old holm oak seedlings (Linaldeddu *et al.*, 2014).

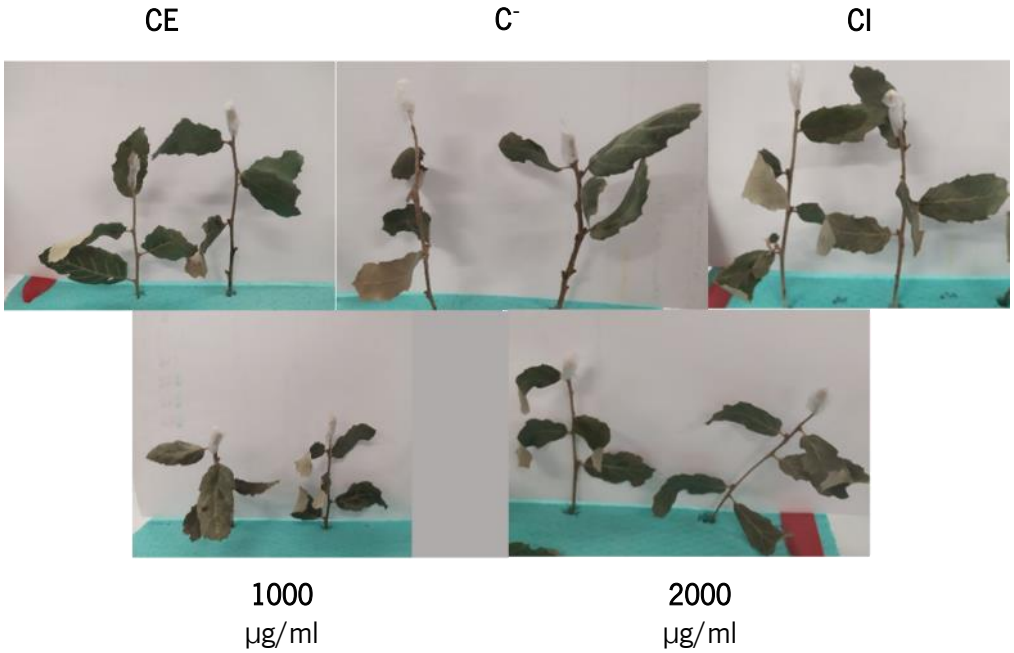

Figure 19. *Ex vivo* assay for antifungal activity of *Hedera helix* leaves against artificial *Diplodia corticola* infection in cork oak cuttings. Small wounds were made with a sterile scalpel on the cork oak cuttings. CE corresponds to control of extract; cotton with extract at highest concentration tested (2000 µg/ml) placed on the apical cut and an agar disc placed on the wound. The C- correspond to the negative control; cotton with sterile deionized water and agar disc on wound. CI is the control of infection; cotton with sterile deionized water and a mycelium disc of fungus on the wound. 1000 µg/ml and 2000 µg/ml corresponds to cotton with extract 1000 or 2000 µg/ml *Hedera helix* with mycelium disc of fungus on the wound. These images are representative images of two replicates.

Over time, leaves got drier and starting to curve and visually it was not possible to observe relevant differences in the appearance of the leaves comparing the conditions. Also, no signs of infection were noticed. To evaluate this dryness symptomatology, the number of curved leaves in each cutting was counted in all replicates and the percentage calculated (**Figure 20**). As can be verified no statistical differences were observed between conditions. However, it is also evident a high intragroup variability (represented by the large S.D.), what means that more replicates and eventual better procedures are needed to optimize this assay. Altogether these results suggested an imbalance between the transpiration demand (transpiration rate) and the water supply (rate of water absorption by the cut basal end).

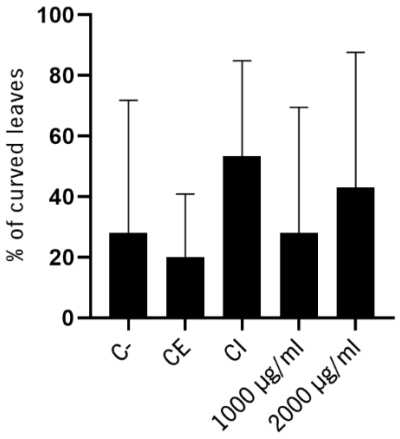

Figure 20. Percentage of curved leaves per cutting submitted to different treatments. Cuttings only with agar disc on the wound and water on the top (C) or with the maximum concentration of extract on the top (CE). Cuttings with discs with *Diplodia corticola* mycelium and water on the top (CI) or with 1000 µg/ml of extract (1000 µg/ml) or with 2000 µg/ml of extract (2000 µg/ml) on the top. Data represent the mean ± S.D. of five independent experiments. Statistical analysis was performed running one-way ANOVA and no significant differences was observed.

Essential physiological processes in the leaf, such as photosynthesis and transpiration, are required for plant growth and development. The leaf must have a balanced water status in order to operate effectively, mainly during evapotranspiration (Heinen *et al.*, 2009). Normally, when an imbalance between water absorption and leaf transpiration occurs tissue dehydration begins (Aroca *et al.*, 2012). We thought that this could help explaining the results obtained, because, although the experiment was performed in lab conditions (low light intensities, a constant temperature of around 21 °C, no wind), which promote low transpiration rates, the supply function (a cut surface in MS medium) could be very compromised. A new experiment was set to investigate if the number of leaves in each cutting could influence the drying symptoms in order to improve this procedure. Here, 20-cm apical cuttings were again collected in the field, brought to the lab where the number of leaves was adjusted to 1, 2, 3 4 and 5 leaves per cutting (5 replicates), and maintained under to the same conditions as above. It was observed that the cuttings not only maintained healthy leaves for a longer period, but also that those with 1 to 4 leaves did not show leaf curling after 20 days (**Figure 21**). At the end of 20 days, the 5-leafed cuttings appeared dried and with curved leaves, and the 1-leaf cutting remained greener than the rest.

These results seemed to agree with the hypothesis that the transpiration rate is higher than the water absorption, culminating in a rapid dehydration of the leaves in the *ex vivo* assay. So, one way to optimize this experimental procedure would be to reduce the leaf area. Although results are encouraging, we cannot exclude a season effect because these second experiment was performed

on November and the first in October. In November, the days are shorter and there was less solar incidence on the leaves, leading to potential lower transpiration rates. We also think that only after having a set-up with healthy leaves for a longer period it would be possible to have success in the infection procedure as with the *ex vivo* strawberry leaf assay.

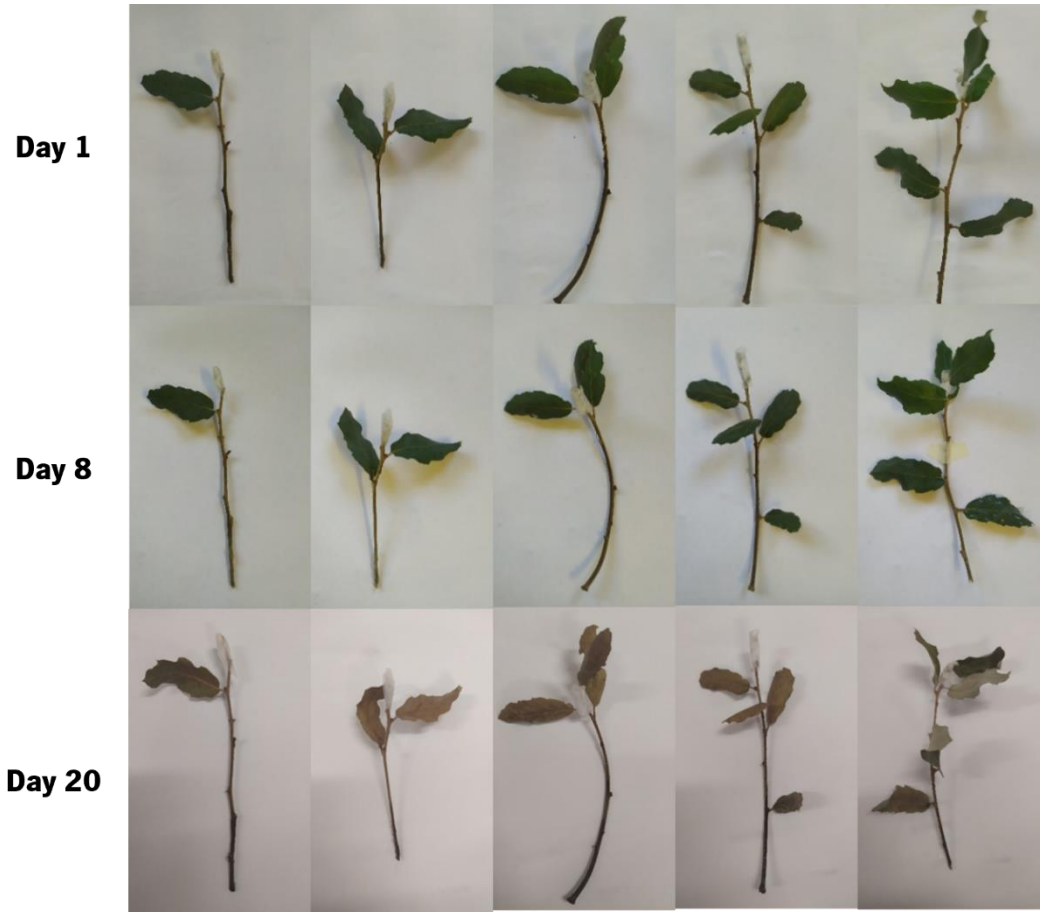

Figure 21. Representative images of cork oak branches with different number of leaves in each branch (1-5) submitted to the same conditions as those reported in **Figure 19** for the negative controls.

CHAPTER 4

FINAL REMARKS AND FUTURE PERSPECTIVES

4.1 Final remarks

The constant use of pesticides over time has led to severe environmental consequences, triggering, more and more, the need and the search for more environmentally friendly alternatives. Plants produce many secondary metabolites, usually exhibiting important biological activities, which could be used as an important source for the formulation of biopesticides.

Among the plant species used and extracts tested, ivy aqueous extract was the most active. An attempt to unravel the mode of action of this extract was made through the use of mutated yeast strains. Results of these assays suggest that the extract targets at least the cell wall.

This extract was further characterized assessing its antifungal activity against six phytopathogenic fungi *in vitro*. To our surprise, the ivy extract was not as active against the filamentous fungi as it was against yeasts, with the exception for the fungus *D. corticola*, where the extract promoted a high percentage of inhibition after three days of incubation (70 %). Considering the economic importance of cork, extracted from cork oaks that are heavily attacked by *D. corticola*, these results seem very promising. Trying to get closer to what would be a possible future application at the field level, *ex vivo* assays were carried out with cork oak cuttings collected form mature trees. The *ex vivo* results were only preliminary and the experimental protocol still needs to be optimized in order to effectively cause fungal infection and accurately evaluate antifungal activity.

The results of this thesis highlight aqueous ivy extract as a possible source of natural antifungal agent against *D. corticola*. To the best of our knowledge the mechanism of action of aqueous extract of *H. helix* as well as the high *in vitro* antifungal activity of this extract against *D. corticola* have never been studied. Thus, this work reveals a high importance and possibly a contribution to combat infectious diseases in agricultural productions with high economic interest. The fact that it is an aqueous extract also presents numerous advantages as it does not raise concerns about the solvents used for the extraction of the active compounds, as well as the negative impact they could have when applied in the field, to plants and soil. The exploitation of these results and further research could be useful for the design of a plant extract-based formulation.

4.2 Future perspectives

The presence of secondary metabolites, essentially saponins, are usually associated with the defensive capacity of plants against pathogens. In this work, we presumed the possible antifungal activity of ivy extract due to the compound α-hederin. This is a bioactive compound highly reported in studies of *H. helix*. Therefore, chemical analysis, which is already in progress, would be the next fundamental step to confirm if this compound is present in the aqueous extract and also to find other candidates for the antifungal activity. It would also be important and relevant to deepen the studies on the mechanism of action of the aqueous extract of ivy. For this, and once cellular activity at the cell wall level is pointed out, a Western Blot could be performed to verify the activation by phosphorylation of proteins Pkc1, Mkk1/Mkk2 and Bck1. Other effects on the cell influenced by the Pkc1 pathway could also be assessed, namely cell cycle by flow cytometry or autophagy and polarization of the actin cytoskeleton by fluorescence microscopy. Furthermore, since there is the possibility of the extract triggering the apoptosis mechanism through the pro-apoptotic gene *AIF1* it would be important to carry out viability assays with strains with this gene mutated, similarly to the assay performed with *yca1* mutant strain.

On the other hand, it will also be interesting to test pure compounds in order to determine which compound(s) are responsible for the antifungal activity. In addition, since *H. helix* extract has been shown to be more active on yeast than on filamentous fungi the reverse may also be true. Thus, extracts whose activity on yeast was not so highlighted, as the case of *D. purpurea* and *B. vulgaris*, could be more active on filamentous fungi. So, it would be interesting to carry out *in vitro* tests with these extracts against phytopathogenic fungi.

Among the phytopathogenic fungi studied, *D. corticola* was to most susceptible to ivy extract. Considering the percentage of inhibition obtained against *D. corticola* and since the *ex vivo* test carried out in this thesis did not allow to draw conclusions, it would be important to optimize the experimental protocol using infected cuttings, in order to assess the application of the extract in conditions closer to the field level.

In addition, the spectrum of fungi can also be extended for both *in vitro* and *ex vivo* assays. Since in this work the ivy extract did not seem to inhibit the growth of the fungus *C. acutatum in vitro* but in the *ex vivo* leaf bioassay there was a delay in the progression of the infection it would be interesting to investigate this extract in other fungi that cause economic and production losses.

Finally, and considering that an ultimate objective is to formulate a biological product based on ivy extract for commercialization, it would be important to understand if this extract is not toxic

to humans, starting with animal cells models. Also, it would be important to study optimal culture conditions of *H. helix* and to calculate extract yield per cultivated area in order to assess viability of this plant source. Finally, product design, market research, advertising of the product will be crucial for the commercialization of the natural fungicide based on aqueous extract of *H. helix*.

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SUPPLEMENTARY MATERIAL

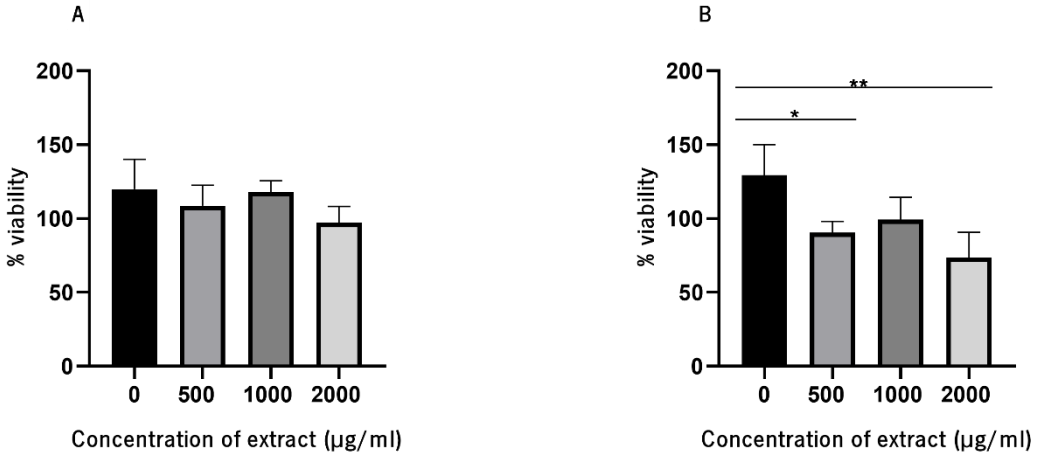

Figure A1. Statistical analysis of the data form of **Figure 5** after 30 min of incubation with ethanolic *Digitalis purpurea* (A) and aqueous extract of *Digitalis purpurea* (B) on *Saccharomyces cerevisiae* BY4741. One-way ANOVA was used for the analysis and the differences were considered statistically different if * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

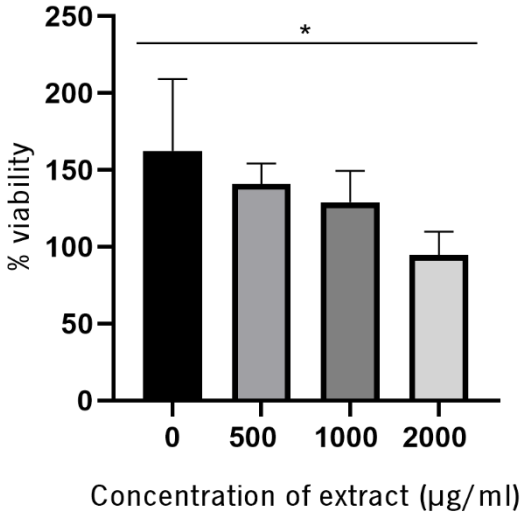

Figure A2. Statistical analysis of the data form of **Figure 5B** after 90 min of incubation with aqueous extract of *Digitalis purpurea* on *Saccharomyces cerevisiae* BY4741. One-way ANOVA was used for the analysis and the differences were considered statistically different if * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

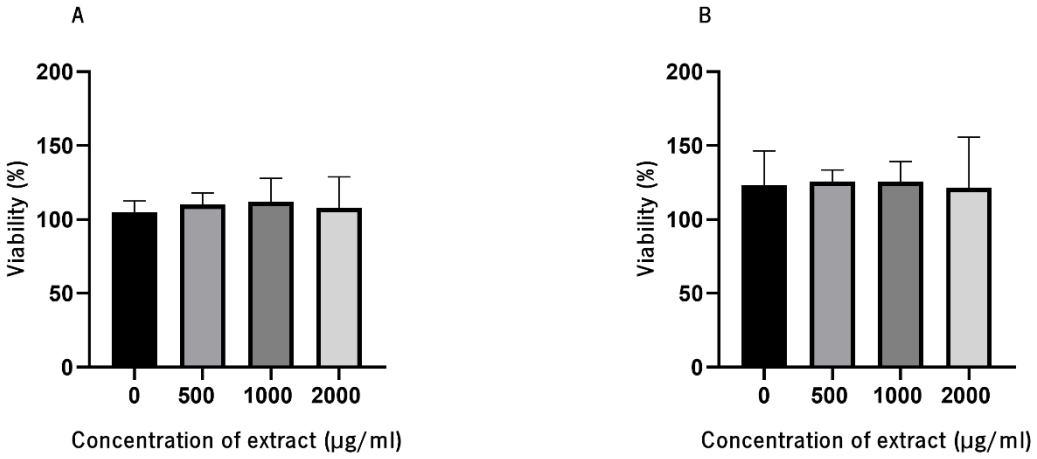

Figure A3. Statistical analysis of the data form of **Figure 6** after 30 min of incubation with ethanolic *Eucalyptus globulus* (A) and aqueous extract of *Eucalyptus globulus* (B) on *Saccharomyces cerevisiae* BY4741. One-way ANOVA was used for the analysis and the differences were considered statistically different if **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

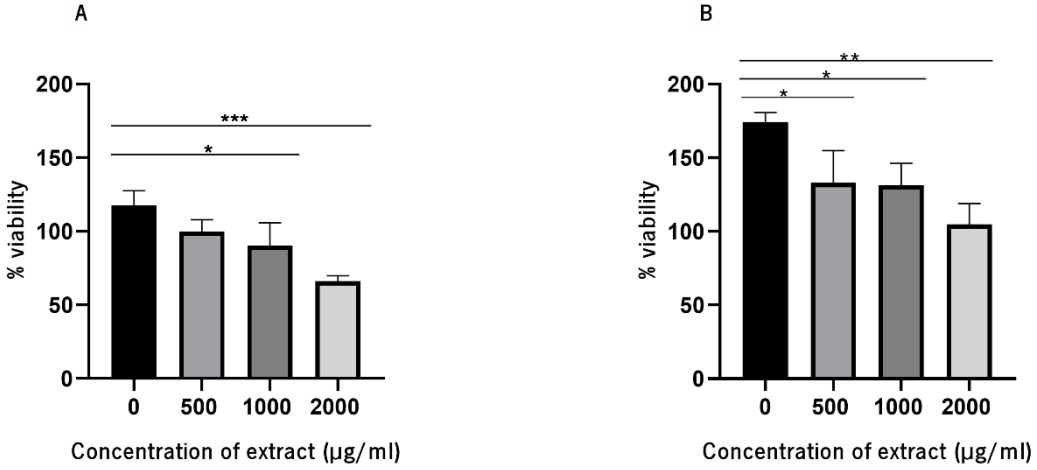

Figure A4. Statistical analysis of the data form of **Figure 7** after 30 (A) and 90 (B) min of incubation with aqueous extract of *Beta vulgaris* root on *Saccharomyces cerevisiae* BY4741. One-way ANOVA was used for the analysis and the differences were considered statistically different if **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

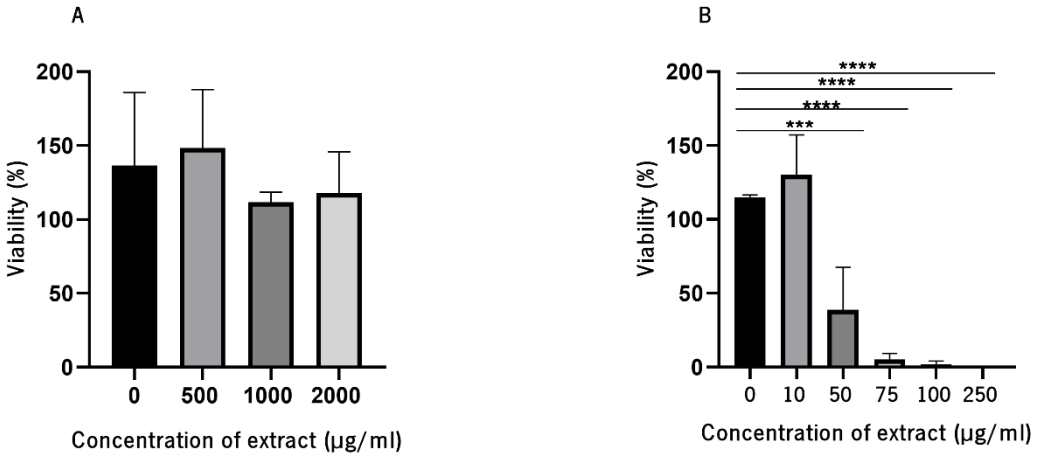

Figure A5. Statistical analysis of the data form of **Figure 8** after 30 min of incubation with ethanolic *Hedera helix* (A) and aqueous extract of *Hedera helix* (B) on *Saccharomyces cerevisiae* BY4741. One-way ANOVA was used for the analysis and the differences were considered statistically different if **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

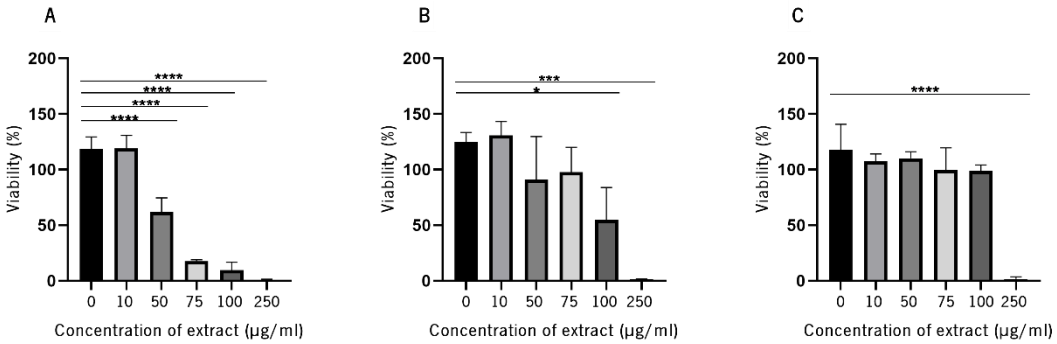

Figure A6. Statistical analysis of data form, **Figure 10A**, **Figure 10B**, **Figure 10C**, respectively at 30 min incubation of *Hedera helix* aqueous extract with the yeast mutant *erg2* (A), the yeast mutant *bck1* (B) and the yeast mutant *mkk1/mkk2* (C). One-way ANOVA was used for the analysis and the differences were considered statistically different if **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

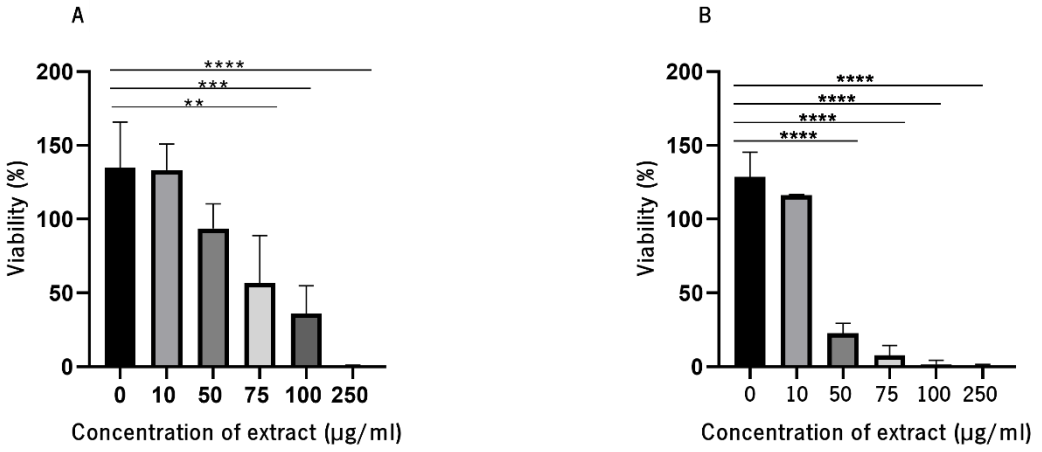

Figure A7. Statistical analysis of data form **Figure 13A and Figure 13B** after 30 min of incubation of *Hedera helix* aqueous extract with *Saccharomyces cerevisiae* W303 cells (A) and from the yeast mutant *yca1* (B). One-way ANOVA was used for the analysis and the differences were considered statistically different if $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$.

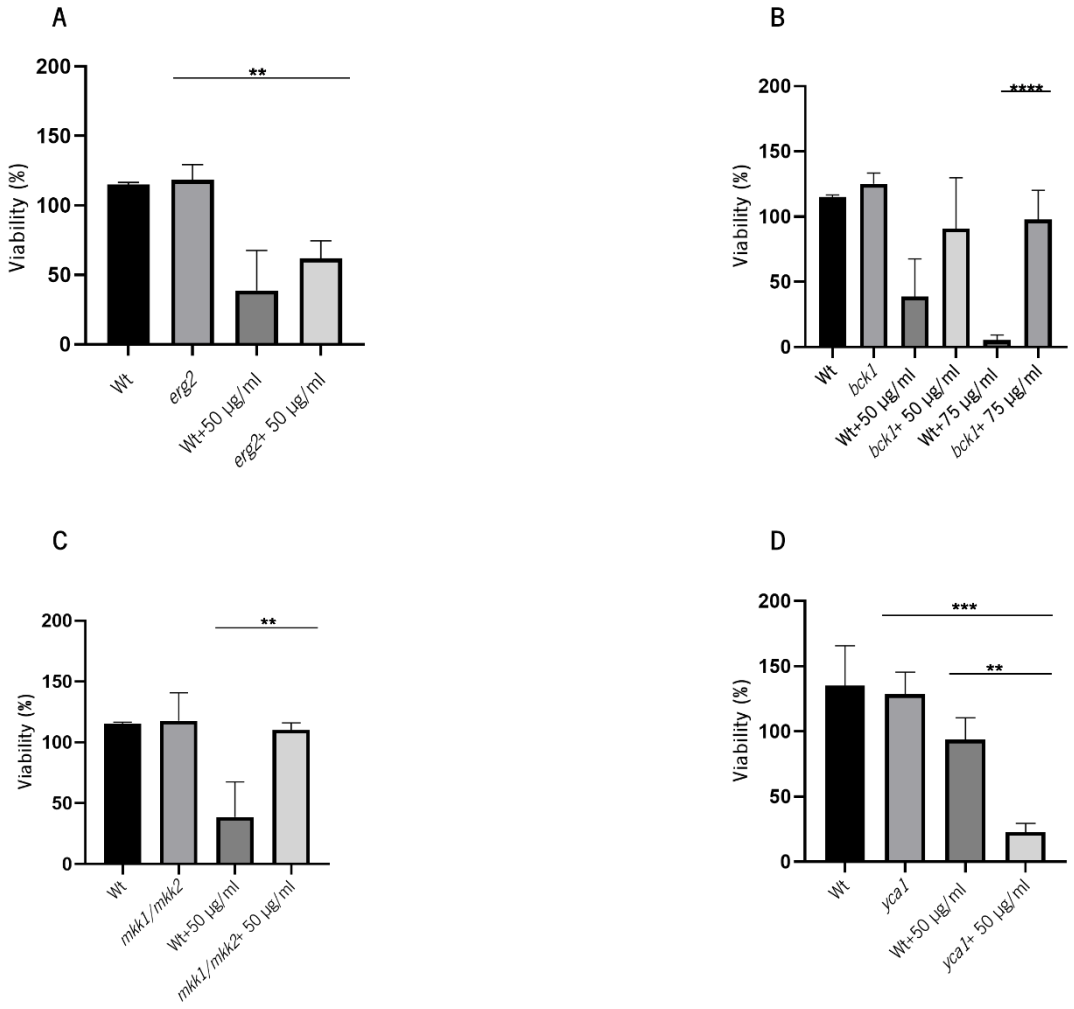

Figure A8. Statistical analysis of data between the wild type BY4741 and each mutant: *erg 2* (A), *bck1* (B), *mkk1/mkk2* (C) at 30 min of incubation of 50 µg/ml of *Hedera helix* aqueous extract. In the case of mutant

bck1 two concentrations of extract, 50 µg/ml and 75 µg/ml were represented. Statistical analysis of data between the wild type W303 and their mutant *yca1* (D). One- way ANOVA was used for the analysis and the differences were considered statistically difference if *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

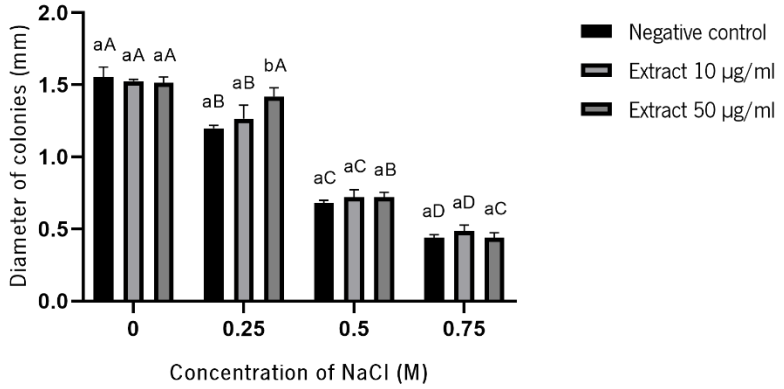

Figure A9. Results obtained by measuring the diameter of colonies of *Saccharomyces cerevisiae* BY4741 incubated during 60 min in YPD medium in the presence of different concentrations of *Hedera helix* aqueous extract - 0, 10 or 50 µg/ml and plated during two days on different concentrations of NaCl (0, 0.25, 0.5 or 0.75 M). The data represent the mean ± S.D. For statistical analysis the letter code was used (Lowercase letters for comparisons of extract concentrations effects within each salt concentration and capital letters for comparisons between salt concentrations for each extract concentration. Mean values followed by the same letters are not statistically different.

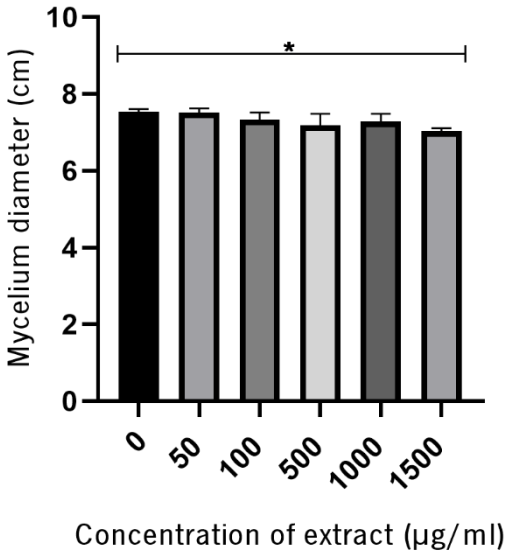

Figure A10. Statistical analysis to the data form **Figure 14A** at 6 days of incubation of *Colletotrichum gloeosporioides* with *Hedera helix* extract at different concentrations: 0, 50, 100, 500, 1000 or 1500 µg/ml. One-way ANOVA was used for the analysis and the differences were considered statistically different if *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

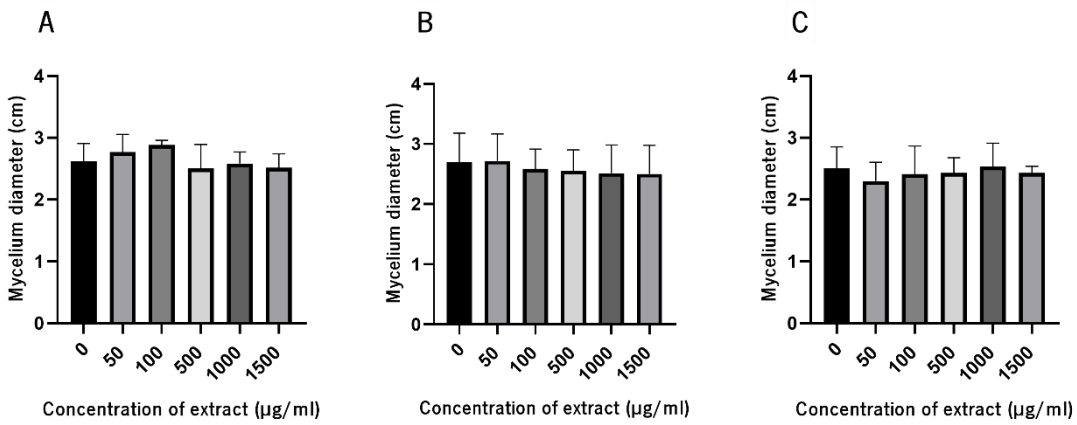

Figure A11. Statistical analysis to the data form **Figure 14B**, **Figure 14C**, and **Figure 14D** at 3 days of incubation of *Colletotrichum godetiae* (A), *Colletotrichum nymphaeae* (B) and *Colletotrichum acutatum* (C) with *Hedera helix* at different concentrations: 0, 50, 100, 500, 1000 and 1500 µg/ ml . One-way ANOVA was used for the analysis and the differences were considered statistically different if $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$. Only day 3 is represented but there were no significant differences in all the days during the experiment.

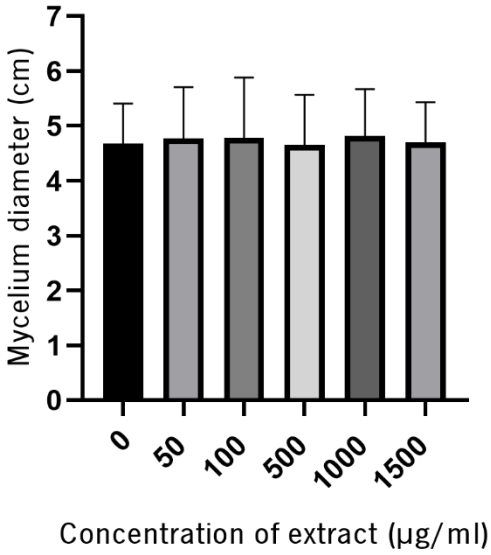

Figure A12. Statistical analysis to the data form of **Figure 15A** at 3 days of incubation of *Phytophthora cinnamomi* with *Hedera helix* at different concentrations: 0, 50, 100, 500, 1000 or 1500 µg/ ml. One-way ANOVA was used for the analysis and the differences were considered statistically different if $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$. Only day 3 is represented but there were no significant differences in all the days during the experiment.

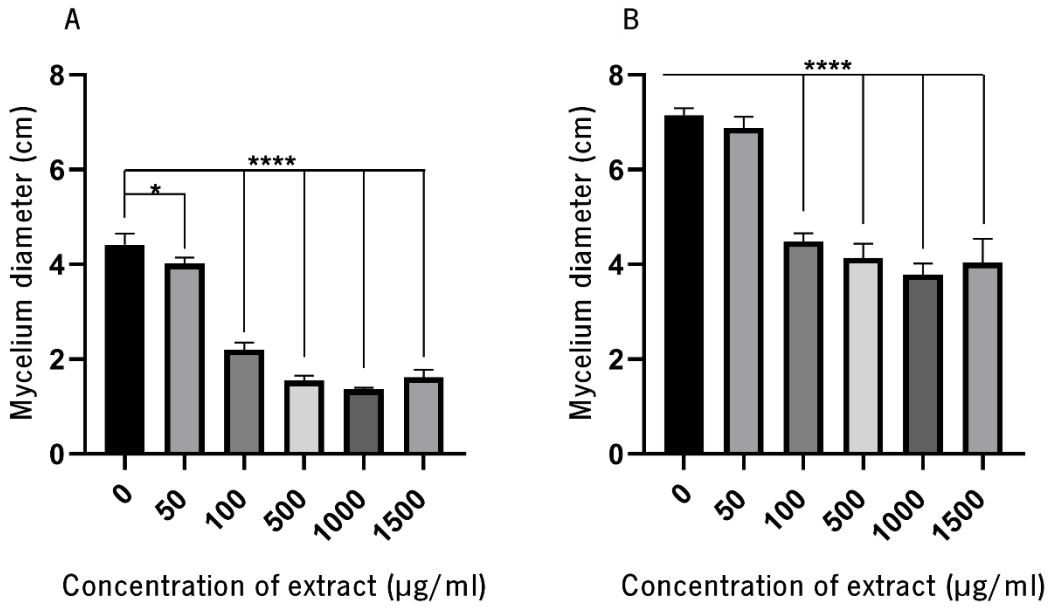

Figure A13. Statistical analysis to the date from **Figure 16A** at 3 (A) and 6 (B) days of incubation of *Diplodia corticola* with *Hedera helix* extract at different concentrations: 0, 50, 100, 500, 1000 or1500 µg/ ml. One-way ANOVA was used for the analysis and the differences were considered statistically different if * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.