Universidade do Minho
Escola de Ciências

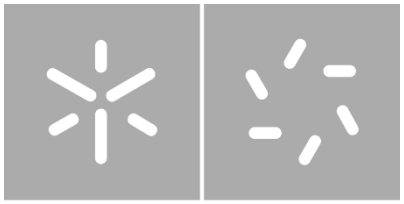
João Manuel Almeida Noversa

Natural antifungals from plants and the potential application in sustainable farming

January 2023

Natural antifungals from plants and the potential application in sustainable farming

UMinho| 2023 João Noversa



Universidade do Minho
Escola de Ciências

João Manuel Almeida Noversa

Natural antifungals from plants and the potential application in sustainable farming

Master Thesis

Master's Degree in Molecular Biology, Biotechnology and Bioentrepreneurship in Plants

Work developed under supervision of

Professor Doctor Rui Pedro Soares Oliveira

Professor Doctor Ana Cristina Gomes Cunha

January 2023

Despacho RT - 31 /2019 - Anexo 3

Direitos de autor e condições de utilização do trabalho por terceiros

Este é um trabalho académico que pode ser utilizado por terceiros desde que respeitadas as regras e boas práticas internacionalmente aceites, no que concerne aos direitos de autor e direitos conexos.

Assim, o presente trabalho pode ser utilizado nos termos previstos na licença abaixo indicada.

Caso o utilizador necessite de permissão para poder fazer um uso do trabalho em condições não previstas no licenciamento indicado, deverá contactar o autor, através do RepositóriUM da Universidade do Minho.

Agradecimentos

Depois desta jornada, preciso de apresentar os meus agradecimentos a todos aqueles que de forma direta ou indireta contribuíram para que isto fosse possível.

Começo por agradecer aos meus orientadores, Professor Rui Oliveira e à Professora Ana Cunha, em primeiro lugar por terem tornado possível este desafio, por me terem apoiado incondicionalmente, por partilharem conhecimento e por me permitem fazer aquilo que tanto gosto. Obrigado pela vossa paciência e por toda a ajuda.

Aos investigadores, Ana Cristina Esteves (Universidade de Aveiro), Helena Machado (Instituto Nacional de Investigação Agrária e Veterinária) e Pedro Talhinhas (Universidade de Lisboa) pelo fornecimento dos organismos fitopatogénicos sem os quais este trabalho não seria possível.

Ao Sr. Machado pelo fornecimento do material vegetal usado e por todo o conhecimento que me transmite diariamente. Ao Dr. Luís, por todo o apoio dado durante este ano, por nos facilitar a vida.

Aos meus colegas de laboratório, um obrigado pela entreajuda, pela partilha de conhecimento e pelo bom ambiente. Aos meus colegas de laboratório que se tornaram amigos, Ana Teixeira, Luara Simões e João o meu obrigado de coração por tudo o que fizeram por mim, pela amizade bonita que criamos e por me terem apresentado a equipa de laboratório de sonho, os calaceiros da bolsa.

A todos os meus amigos que aturaram as minhas más horas, me ajudaram e me apoiam incondicionalmente. Vocês são um dos meus pilares, como sabem.

Á minha namorada por estar presente 365 dias por ano, por me ajudar em tudo, por perceber o amor que tenho pela ciência e por ser a melhor companheira de vida que podia pedir.

Á minha família, que me suporta todos os dias, que me completa e que me permitiu sempre sonhar com tudo que quisesse. Obrigado por acreditarem sempre em mim e por serem um exemplo a seguir.

A ti, minha mãe, tudo isto é teu também. Todos os esforços que fizeste estão descritos nestas páginas, obrigado pelo amor e por acreditares no meu sonho tanto como eu.

Obrigado a todos.

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

Antifúngicos naturais à base de plantas e a sua potencial aplicação na agricultura sustentável

Resumo

O crescimento da população mundial e da consciência da importância da qualidade dos produtos na saúde do consumidor e dos ecossistemas, tornou necessário aumentar a produção agrícola de um modo sustentável. Os pesticidas sintéticos têm sido eficazes no controlo de doenças das plantas cultivadas, reduzindo perdas alimentares e económicas, mas têm impactos negativos no ambiente e na saúde. Os fungos fitopatogénicos são responsáveis pela destruição de cerca de 1/3 da produção anual de alimento. Extratos de plantas têm mostrado atividade contra diversos organismos devido à composição diversa em compostos bioativos, podendo ser uma alternativa no biocontrolo de doenças fúngicas. O objetivo deste trabalho foi avaliar a atividade antifúngica e antimicrobiana de extratos de *Juglans regia* e *Magnolia grandiflora:* extrato aquoso, etanólico (50%) (v/v) e etanólico (100%) obtidos da casca verde da noz; extrato aquoso de folhas de nogueira; e extrato aquoso e etanólico (80%) de sementes de magnólia. Em ensaios de viabilidade em *Saccharomyces cerevisiae* os extratos de sementes de magnólia foram os mais promissores. Os ensaios de avaliação da atividade antifúngica e antimicrobiana *in vitro* contra os microrganismos fitopatogénicos: *Diplodia corticola, Colletotrichum acutatum e Phytophthora cinnamomi* revelaram que o extrato etanólico de sementes de magnólia foi o mais eficiente, inibindo o crescimento em 64% (*D. corticola*), 47% (*P. cinnamomi*) e 55% (*C. acutatum*). Este extrato foi selecionado para se investigar o seu mecanismo de ação tendo-se verificado que danificou a membrana dos esporos, o que sugere que um dos possíveis mecanismos seja a desestabilização dessa membrana. Ativo contra microrganismos fitopatogénicos devastadores de plantas para espécies de alto valor económico em Portugal e no mundo- *Quercus suber* e *Vitis vinifera* (afetadas por *D. corticola*), *Fragaria* spp. (afetada por *C. acutatum*) e *Castanea sativa* (afetada por *P. cinnamomi*) - este extrato, utilizado em formulações de fungicidas naturais poderá ajudar no desenvolvimento de um setor agrícola mais seguro e sustentável.

Palavras-chave: agricultura sustentável, extratos de plantas, fungicidas naturais, *Magnolia grandiflora*, *Juglans regia.*

Natural antifungals from plants and the potential application in sustainable farming

Abstract

The growth of the world population and awareness of the importance of product quality for consumer health and ecosystems has made it necessary to increase agricultural production in a sustainable way. Synthetic pesticides have been effective in controlling diseases of cultivated plants, reducing food and economic losses, but have negative impacts on the environment and health. Phytopathogenic fungi are responsible for the destruction of about 1/3 of annual food production. Plant extracts have shown activity against several organisms due to the diverse composition in bioactive compounds and can be an alternative in the biocontrol of fungal diseases. The aim of this work was to evaluate the antifungal and antimicrobial activity of *Juglans regia* and *Magnolia grandiflora* extracts: aqueous, ethanolic (50%) (v/v) and ethanolic (100%) extracts obtained from the green walnut husk; aqueous extract from walnut leaves; and aqueous and ethanolic (80%) extract from magnolia seeds. In viability assays in *Saccharomyces cerevisiae* the magnolia seed extracts were the most promising. *In vitro* antifungal and antimicrobial activity evaluation assays against the phytopathogenic microorganisms: *Diplodia corticola*, *Colletotrichum acutatum* and *Phytophthora cinnamomi* revealed that the ethanolic extract of magnolia seeds was the most efficient, inhibiting growth by 64% (*D. corticola*), 47% (*P. cinnamomi*) and 55% (*C. acutatum*). This extract was selected to investigate its mechanism of action and it was found to damage the spore membrane, which suggests that one of the possible mechanisms is the destabilization of this membrane. Active against devastating plant pathogenic microorganisms for species of high economic value in Portugal and worldwide- *Quercus suber* and *Vitis vinifera* (affected by *D. corticola*), *Fragaria* spp. (affected by *C. acutatum*) and *Castanea sativa* (affected by *P. cinnamomi*)- this extract, used in natural fungicide formulations could help in the development of a safer and sustainable agricultural sector.

Keywords: *Juglans regia, Magnolia grandiflora*, natural fungicides, plant extracts, sustainable farming.

INDEX

FIGURES LIST

TABLES LIST

1. INTRODUCTION

1.1. Challenges of modern agriculture for sustainability

Agriculture is an important sector related to the production of crops for food, feed, and ornamental purposes (Zulfiqar et al., 2019). The history of agriculture began about 10 000 years ago, where today is Iraq, Turkey, Syria, and Jordan. Later, already in farms, people started to cultivate wheat, barley, peas, lentils, chickpeas, bitter vetch, and flax (Tudi et al., 2021).

The continuous growth of the human population raises the potential problem of food scarcity. FAO forecasts that till 2050 the global food production will need to increase by 70% to fulfil the necessities of the estimated 9.1 billion people, to avoid more poverty and hunger, problems that already affected 25.9% of the global population in 2019 (Brennan & Browne, 2021). This need of increasing global food production can be controlled with the enhancement of crop yield, because the land that can be used for agriculture is not unlimited, and with the decrease of food loss and food waste, once approximately one-third of the annual food production is lost worldwide (Habashy et al., 2021; Mahanty et al., 2017). The food supply chain can be divided into five stages: production, post-harvest, processing, retail, and consumption. Food loss occurs at the first 3 stages, before the food is ready to human consumption and food waste occurs at the last 2 stages (Brennan & Browne, 2021).

To overtake the challenge of being able to produce food, for humans and animals, at a large-scale production and at a low price, synthetic pesticides and mineral fertilizers had been used for decades. This type of agriculture is named intensive agriculture, which can be recognized as conventional agriculture (Mie et al., 2017). The use of these synthetic products affects the environment polluting air, water, and soil and, directly or indirectly, and causing harm to living beings (Tilman, 2020). Because of this, one of the main goals of several intergovernmental organizations is to develop a sustainable agriculture, avoiding high inputs of chemicals, and capable of keeping the high levels of required food production (Mie et al., 2017). The European Union defined objectives to accomplish, named “EU Green Deal Targets”, such as to “reduce by 50% the overall use and risk of chemical pesticides and reduce use by 50% of more hazardous pesticides by 2030” and to “achieve at least 25% of the EU’s agricultural land under organic

farming" (European Comission, 2021), in order to create a sustainable food system, defined as a system that delivers quality food and nutrition for all, without affecting the economic, social and environmental bases (Brennan & Browne, 2021; European Commission, 2021). In addition to waste in the agricultural sector, there are also plant diseases that reduce production and lead to damage or even destruction of plants and plant-based products. Phytopathogenic fungi are one of the major plant devastators agents, destroying one third of the annual food production, making it urgent to create effective methods to combat them (Almeida et al., 2019).

1.2. Phytopathogenic fungi and oomycetes

The kingdom fungi is one of the most widespread throughout the world, with a vast diversity of taxa and about 18,000 species already described (Aguilar-Marcelino et al., 2020). There are studies suggesting that fungi evolved over a billion years ago and that the interaction with plants began around 425 million years ago when plants started to occupy terrestrial ecosystems, leading to a process of co-evolution with fungi that acquired the ability to associate with plants through the root system and aerial parts as well (Yan et al., 2019).

Fungal plant pathogens have different lifestyles depending on the strategy they use to interact with the host. They are divided into 3 large groups: i) necrotrophic, when the fungi kill the host plant cells and feeds on the dead tissues; ii) biotrophic, when it colonizes the living tissues of the plant and uses them as a source of nutrients either as a unique source or as an alternative, and iii) the hemibiotrophs that initially establish a biotrophic relationship with the host plant and later kill the plant tissues and feed on them (Doehlemann et al., 2017). The biotrophic group includes endophytes, which are fungi that establish biotrophic interactions without causing visible symptoms in plants. Mycorrhizas are an example of this, they are fungi that establish mutualistic interactions with host plants and that represent the most important symbiosis, at an ecological and economical level, in terrestrial ecosystems (Lo Presti et al., 2015). Independently of the group, any part of the plant can be infected by phytopathogenic fungi. Many fungi develop in the aerial parts, where symptoms are easily noticed, but there are also several fungi that infect the plant from the root system, which makes it difficult to detect the first symptoms (Coque et al., 2020). Phytopathogenic fungi have a great impact, agronomically and economically, because they threaten agricultural production at all stages of growth and can even cause post-harvest diseases (Lo Presti et al., 2015).

In this work we decided to choose phytopathogenic fungi and also an oomycete, which although not belonging to the kingdom of fungi will be included together with the phytopathogenic fungi for reasons of practicality and writing. These phytopathogenic microorganisms affect crops present in Portugal, or crops that have high relevance worldwide, such as *Diplodia corticola* that affects *Quercus suber*, *Colletotrichum acutatum* that is one of the major causers of losses in *Fragaria* spp. and *Phytophthora cinnamomi* that affects *Castanea sativa*, a major tree in Portugal.

1.2.1. *Diplodia corticola*

Diplodia corticola is an ascomycete (Muñoz-Adalia et al., 2022), belonging to the *Botryosphaeriaceae* family (Fernandes et al., 2014) that has a hemibiotrophic lifestyle, changing from biotrophy to necrotrophy after stress caused in the plant (Fernandes, 2015). This fungus can produce secondary metabolites with phytotoxic, antibacterial, and even antifungal capacity (Masi et al., 2016). Although the mechanism of infection is not fully known, studies suggest that phytotoxins, degradative and oxidative enzymes, and cytotoxic proteins play a role in this process (Félix et al., 2017). This fungus affects several plant species, such as grapevines (*Vitis vinifera* L.), eucalypts (e.g. *Eucalyptus globulus* L.), and oak trees (from the *Quercus* genus) (Linaldeddu et al., 2013; Félix et al., 2017). As the cork oak (*Quercus suber* L.) is an important crop in Portugal - in 2015, it occupied almost 25% of the Portuguese forest, corresponding to 720,000 hectares (APCOR, 2020)-, with an enormous economic value and being threatened, this work will focus on this species.

The decline of cork oak forests in the Iberian Peninsula (Fernandes et al., 2014), represents major problems at an ecological level, because Montados are agro-forestry ecosystems rich in animals and plants and also contribute to high CO_2 fixation: a cork oak forest with 30% cork oak coverage is able to fix about 3.2 tons of CO_2/ hectare/ year (APCOR, 2020). It also represents major problems at the economic level, since the cork stopper sector, in 2010, represented a turnover of around 1 billion euros in Portugal (Félix et al., 2017). *Diplodia corticola*, being one of the most aggressive fungi in several species of the genus *Quercus*, is particularly associated with the decline of cork oak. The infection may cause the canker disease (Linaldeddu et al., 2013), with symptoms ranging from mortality in seedlings, to necrosis, wilting, dieback of branches, and strong reduction in phellem regeneration after the extraction of cork (Muñoz-Adalia et al., 2022).

1.2.2. *Colletotrichum acutatum*

Colletotrichum acutatum is an ascomycete included in *Phyllachoraceae* family (Peres et al., 2005). This fungus was first identified by Simmonds (1966) and verified in 1968. Regarding morphology, it initially presents white colonies that are later covered by pink to orange coloured conidia, but its most known morphological characteristic is the shape of its conidia, which have acute ends, although it also has conidia with other shapes (Damm et al., 2012; Peres et al., 2005). This ascomycete has a hemibiotrophic lifestyle that is dependent on several factors, such as the host, the affected tissue, and the environment in which it is located. Baroncelli (2017)address the fact that several authors have shown that, for example, on apples (*Malus domestica*) species belonging to the *C. acutatum* species complex cause necrotrophic lesions on fruit but do not cause any type of damage on leaves; and that on orange trees they show a necrotrophic lifestyle on flowers and biotrophic on leaves (Baroncelli et al., 2017). Due to its economic importance, this fungus appears in the Top 10 most important fungal pathogens (Tomas-Grau et al., 2019), affecting hosts like strawberries (Freeman et al., 2001), apples (Nekoduka et al., 2018), peaches and grapes (Biggs & Miller, 2003), almonds, citrus, lupin and olive trees (Sreenivasaprasad & Talhinhas, 2005). Anthracnose, also called bitter rot (Børve & Stensvand, 2006), is one of the main diseases cause by *C. acutatum* and is widely associated with strawberries, apples, olive fruit and also chilli pepper. Studies with species of the genus *Capsicum* have revealed that the fungus penetrates the cuticular layers of the fruits forming highly branched and differentiated hyphae and that after entering the cells, the fungus colonises and multiplies producing conidia (Liao et al., 2012). In the case of strawberries (*Fragaria* x *ananassa* Duch.) this disease affects all the plant tissues and manifests itself with symptoms such as irregular and black leaf spot, flower blight, and fruit and crown rot, leading to great losses (Freeman et al., 2001).

1.2.3. *Phytophthora cinnamomi*

Phytophthora cinnamomi is an oomycete that affects natural ecosystems and horticultural crops worldwide (Engelbrecht et al., 2021). This oomycete belongs to the kingdom Chromista, the phylum Oomycota or pseudofungi, the class Oomycetes, the order Peronosporales, the family Peronosporaceae and the genus *Phytophthora* and assumes a hemibiotrophic lifestyle, initially with a biotrophic phase and later with the necrotic phase (Boughanmi et al., 2022; Joubert et al., 2021). It was first identified on cinnamon trees in Sumatra in 1922 and it is believed to have originated in Papua New Guinea but now is widespread throughout the world (Hardham, 2005). Initially this

species was thought to belong to the kingdom of fungi because of the similarities in growth but unlike true fungi, this plant pathogen spends most of its life cycle as a diploid, the cell wall is composed of cellulose and β-glucans instead of chitin, it produces biflagellate zoospores and is unable to synthesise sterols (Boughanmi et al., 2022).

Due to its economic and scientific importance, this oomycete is one of the top 10 oomycete plant pathogens causing enormous losses in more than 76 countries (Chen et al., 2022). As one of the most devastating plant pathogens in the world, this species infects about 5000 plant species, such as chestnut trees (*Castanea sativa*), avocado trees (*Persea americana* Miller), plane trees (*Platanus acerifolia*), chive (*Allium fistulosum* L.), lettuce (*Lactuca sativa* L.), pineapple (*Ananas* Mill spp.) and peach (*Prunus* L. spp.) (de Andrade Lourenço et al., 2020; Engelbrecht et al., 2021; Hardham, 2005).

Regarding the method of infection, normally *Phytophthora cinnamomi* infects feeder roots although it can also invade woody stems, in this case through wounds or natural openings in the peridermal layer. Its growth in the root system causes root rot and interferes with water uptake and transport to the shoot, causing wilting and chlorosis of the leaves. Plants can either die quickly or remain intact without symptoms for many years (Hardham & Blackman, 2018).

One of the species most affected is the chestnut tree, a species that occupies around 2 million hectares and makes a major contribution to European agriculture. Since the 20th century, chestnut production has fallen considerably, representing losses of around 300 million euros. These losses are associated with the ink disease caused by this pathogen which represents a problem mainly in Portugal (Boughanmi et al., 2022; de Andrade Lourenço et al., 2022).

1.3. Fungicides

The agricultural sector uses pesticides, chemicals (natural or synthetic) which are applied to control pests, weeds, and plant diseases. These products include herbicides, insecticides, rodenticides, nematicides and fungicides (Sharma et al., 2019). The latter account for around 35% of the pesticides market worldwide (García-Machado et al., 2022). Fungicides are used to destroy or inhibit the growth of fungi or fungal spores. They play a key role in the protection of crops, fruits, vegetables, trees and ornamental plants (Singh et al., 2019), since fungi account for about 70-80% of plant diseases (Ray et al., 2017). Since the appearance of the first synthetic fungicide, phenylmercury acetate, in 1913, numerous products have emerged that have helped increase food

production and made agriculture dependent on these types of fungicides (Ons et al., 2020). Synthetic fungicides have advantages such as the already mentioned increase in productivity in the agricultural sector, associated with the ease of use and the relatively low price at which they are sold (Dias, 2012). Their application and their recurrent use also brings disadvantages such as: i) they are not biodegradable (Singh et al., 2019); ii) some plant pathogens are becoming resistant (Ons et al., 2020); iii) they contaminate the soil and exert their effect against beneficial fungi that exist there, such as mycorrhizae (Buysens et al., 2015); and iv) they pollute underground water and can enter the food chain and have toxic effects on animals, including humans (Dias, 2012; Edwards et al., 2019; Moreno-González et al., 2013). The fact that synthetic fungicides have toxic effects on non-target organisms has raised environmental and human health concerns that have led some governments to restrict the use of some fungicides, such as vinclozolin, which has been totally banned (García-Machado et al., 2022; Singh et al., 2019; Yoon et al., 2013).

The effectiveness of these synthetic products has been declining due to the emergence of resistance by some plant pathogens. The evolution of resistance can be divided into two phases: the emergence phase and the selection phase. In the emergence phase, the mutated strain is able to invade the pathogen population. The emergence phase lasts until the resistant strain creates a sub-population that is unlikely to disappear. At this point it enters the selection phase where fungicide application increases the frequency of the resistant strains (Hobbelen et al., 2014).

Current selective fungicides act on certain cellular processes and bind to specific protein targets, called single-site; in contrast to earlier fungicides that acted across a spectrum of cellular processes and are considered multi-site. The development of resistance in single-site fungicides happens by four main mechanisms: i) alteration of the target protein which prevents the fungicide from binding; ii) overexpression of the target protein; iii) pumping, from the intracellular to the extracellular medium, of the fungicide via the efflux pump; and iv) degradation of the fungicide by metabolic enzymes (Lucas et al., 2015).

Although these fungicide products have helped raise food production since their development, they have several disadvantages associated with them, which makes it necessary to have more sustainable alternatives.

1.4. Plant extracts

Plants have been used since ancient times in folk medicine and agri-food science to fight diseases and preserve food (Nazzaro et al., 2017). Natural products from plants have various biological activities that are of great importance, including anti-inflammatory, anticarcinogenic, antibacterial, antiviral, antimutagenic, antiallergenic and antifungal (Mahlo et al., 2016). In the case of antifungal activity, many plants can protect themselves against various pathogens by having the capacity, precisely, to produce compounds with antimicrobial activity (Koval et al., 2020). These compounds differ in molecular weight, structure, functionality, and major classes include alkaloids, flavonoids, terpenoids, phenolics and tannins (Shuping & Eloff, 2017). They are called secondary metabolites, as they are not considered essential for sustaining plant growth but play a key role in their survival (Ribera & Zuñiga, 2012). Therefore, plants secondary metabolites can play an important role in the agricultural sector in the fight against phytopathogenic fungi (Khan et al., 2020). One of the advantages of plant extracts over single site synthetic fungicides is that a plant extract may contain several compounds that work synergistically to inhibit or impair the growth of phytopathogenic fungi, reducing the probability of resistance by these pathogens (Shuping & Eloff, 2017). Plant extracts and natural products are also inherently biodegradable, tend to have low mammalian toxicity and simultaneously low toxicity to non-target organisms, all advantages that synthetic products do not have (Al-Samarrai et al., 2012; Miastkowska et al., 2020). However, there are some limitations that still do not allow the use of these products to be fully adopted, such as the high cost of refined commercial products, the limited capacity to satisfy market needs and also the susceptibility of the products to variations due to environmental factors (Fenibo et al., 2021). There are already several studies showing the ability of plant extracts to inhibit their growth, for example Tripathi and Dubey (2004) showed that an aqueous extract of *Acacia nilotica* was active against *Penicillium italicum* and increased the shelf life of oranges by 6 days and Eloff and his co-workers (2007) showed that an extract of *Melianthus comosus* leaves, provided more antifungal activity against phytopathogens than six commercial fungicides (Eloff et al., 2007; Shuping & Eloff, 2017; Tripathi & Dubey, 2004). In the work here presented, two species were chosen to study the possibility of their antifungal activity against phytopathogenic fungi. Below I will present them and show how promising they are as a sustainable antifungal agents against some pests in agriculture.

1.4.1. *Juglans regia*

Juglans regia, known as English walnut, Persian walnut, or simply as walnut tree, is a long-lived, wind-pollinated, diploid (2n=32), monoecious and heterodichogamous tree belonging to the family Juglandaceae of the order Fagales. About 500 varieties and cultivars of this species have been registered worldwide. Studies suggest that *Juglans regia* appeared as an ancestral hybrid between American and Asian walnut strains about 3.45 million years ago (Luo et al., 2022). The walnut tree has been cultivated since 4000 B.C. for its nutritional and therapeutic properties and is now spread all over the world due to its wood and fruit, which have remained in the human diet since preagricultural times, as a source of macro and micronutrients and also of bioactive compounds (Amaral et al., 2003; Catanzaro et al., 2018). Folk medicine uses the different parts of the walnut tree as therapy, for example, walnut leaves are used in Turkey to relieve fever, in Iran they use to treat diabetes, heart disease and inflammatory conditions and in Mexico for protection against liver damage (Panth et al., 2016). This species, widely spread in Portugal, is a crop with high economic importance for the food industry due to the commercialisation of the seed (or kernel) but the remaining parts of the tree can also be used in the cosmetic and pharmaceutical industry (Martínez et al., 2010; Oliveira et al., 2008). The use of the walnuts give rise to large quantities of walnut green husk (the fruit that covers the seed), which are discarded. The walnut green husk is a source of bioactive compounds with antioxidant and antimicrobial properties (Jahanban-Esfahlan et al., 2019). The use of this by-product as a source of phytochemicals would increase the value of walnut production by increasing interest in different parts of the tree (Oliveira et al., 2008).

Juglone is the main active compound in walnut green husk extracts, representing 30% of the phenolic compounds present in green husk. Juglone is a naphthoquinone (5-hydroxy-1,4-naphthoquinone) that is found in different parts such as the green husk, leaves, roots, root hairs, and pericarp along with other phenolic compounds. Thirty phenolic compounds have been identified in walnut including gallic acid, vinyl acid, ellagic acid, protocatechuic acid, catechin, epicatechin, myricetin and juglone (Soto-Maldonado et al., 2019). Dorota Wianowska and co-workers (2020) showed that juglone was primarily responsible for inhibiting fungal growth, but that there were also other components in walnut husk extract that had antifungal potential, although they did not show effects on their own. So, it can be said that there is a synergy between the components and juglone to exert antifungal activity. The same author showed in 2016 that walnut green husk extracts from different cultivars showed activity against several phytopathogenic fungi: *Alternaria alternata, Rhizoctonia solani, Botrytis cinerea, Fusarium culmorum* and *Phytophthora*

infestans (Wianowska et al., 2016). Due to their demonstrated antifungal capacity against some phytopathogenic fungi, the presence of many phenolic compounds and the possibility of adding value to a product that otherwise would be wasted, we decided to use some walnut parts to test their antifungal capacity.

1.4.2. *Magnolia grandiflora*

Magnolia is a genus comprising around 210 flowering species belonging to family Magnoliaceae and to the order Magnoliales (Lee, 2011). Several species of the genus *Magnolia* are used in traditional Chinese medicine to treat various diseases. For example, the flowers of *Magnolia biondii*, *Magnolia denudate* and *Magnolia sprengeri* are used to treat respiratory diseases, the bark of *Magnolia officinalis* is used to treat edema, lung diseases, asthma, and intestinal problems and *Magnolia grandiflora* is used to treat headaches and stomach ache (Abdelgaleil & Ahmed, 2005; Ding et al., 2018). *Magnolia grandiflora*, commonly known as southern magnolia or bull bay, is a tree native to North America and Mexico (Lee, 2011). They are medium to large evergreen trees, the leaves are dark green above and yellow-brown below and after flowering, it produces fibrous follicles, the see compartments, which later open to reveal the red coloured seeds that have been shown to have effects on human diseases (Lee, 2011; Lim, 2015; Lovisetto et al., 2015). The species is used for medicinal and ornamental purposes (Ali et al., 2020). The medicinal effects of this species may be due to the presence of alkaloids, tyramine, flavonoids, terpenes, phenolic alcohols, glycosides and sesquiterpene lactone (Li et al., 2015). Honokiol and magnolol are the major bioactive constituents of magnolia species (Jacobo-Salcedo et al., 2011). Honokiol, a polyphenol that can be found in bark, leaves, seeds, and cones has been shown to have therapeutic properties such as antidepressant, antimicrobial, antitumorigenic, anxiolytic, analgesic and neuroprotective, among others (Rauf et al., 2021). These compounds, honokiol and magnolol, were isolated from an extract of *M. grandiflora* seeds that showed cytotoxic effects on hepatocellular carcinoma cell lines in human liver (Li et al., 2015). Also, Clark, El-feraly, and his co-workers, (1981) showed antimicrobial activity of these compounds on gram-positive bacteria, acid-fast bacteria, yeast, and filamentous fungi.

To the best of our knowledge there are only few studies reporting the effect of *Magnolia grandiflora* extracts on phytopathogenic fungi, but Y. F. Yan (2020) and his co-workers (2020) showed activity of extracts, with different solvents, of Magnolia officinalis against 7 phytopathogenic fungi:

Rhizoctonia solani, *Botrytis cinerea*, *Fusarium graminearum*, *Fusarium oxysporum f. sp. vasinfectum*, *Sclerotinia sclerotiorum*, *Magnaporthe oryzae* and *Phytophthora capsica*. There is also a report showing that dichloromethane extracts of the bark were able to exert an inhibitory effect on *Helminthosporium* spp. and *Rhizoctonia solani* with EC_{50} of 21.72 and 24.37 µg/mL, respectively. In the same work it was shown that the leaf extract, with the same solvent, had antifungal effect against *Alternaria alternata* and *Helminthosporium* spp. with EC_{50} of 21.50 and 23.97 µg/mL, respectively (Abdelgaleil & Ahmed, 2005). Due to the possibility of similarities between species with respect to bioactive compounds, we decided to study the antifungal potential of *Magnolia grandiflora* seeds extract against phytopathogenic fungi.

1.5. Scientific problem and objectives

Phytopathogenic fungi are a real problem and a permanent threat to agriculture and synthetic fungicides are vital in preventing major losses. As previously mentioned, besides the fact that these products are losing effectiveness, they are also very harmful to human health and to the environment, promoting the emergence of resistant strains and being toxic when entering the food chain. Plant extracts appear as a possible safer alternative that is less likely to cause resistance in phytopathogens. In this work we intend to study the antifungal activity and possible mechanism of action of the extracts of the selected plants. The plants were chosen with resource to literature, for having diverse compounds with associated biological activities and we tried to choose extracts that were the result of the exploitation of a by-product. And to study the capacity of the chosen plant extracts, fungi that have great relevance worldwide and fungi that affect crops present in Portugal were selected.

The aim of this work is to find one or more extracts that have sufficient antifungal capacity to replace conventional fungicides in the future. This replacement would make agricultural practices more sustainable and less susceptible to the emergence of resistant fungal strains, reducing the toxicity associated with the use of conventional products, which affects non-target organisms, including humans.

To study the possible activity of the selected extracts, we started the work with a screening using *Saccharomyces cerevisiae* as model organism. Afterwards, the capacity of the extracts to inhibit fungal growth was analysed. The remaining tasks were performed only for the magnolia seed extract, as it presented more promising results, where we evaluated a possible mechanism of action on *Colletotrichum acutatum* spores.

2. MATERIALS AND METHODS

2.1. Preparation of plant extracts

Two plants species were used in this study: *Juglans regia* (walnut tree) and *Magnolia grandiflora* (southern magnolia and from now on just referred as magnolia). Fresh green husks and leaves from walnut and magnolia seeds were collected from mature trees in Braga, North of Portugal, in October 2021 (Table 1.). The fresh green husks were washed, stored in plastic bags and frozen at -20 °C until the time of use. Before the extraction procedure, the green husks were freeze-dried and ground to powder. The walnut leaves and the seeds were washed, air-dried in the dark at room temperature, ground to powder and stored until the time of use.

Table 1. Plant organs collected from different plant species used in this work.

Plant	Plant organ
Juglans regia	Husk
	Leaves
Magnolia grandiflora	Seeds

To prepare the aqueous extract, walnut husks powder (0.1 g of plant material per mL of solvent) were immersed in boiling deionized water for 45 min, and the walnut leaves and the magnolia seeds powders (0.1 g/mL) were immersed in deionized water, at room temperature, with occasional shaking, for 3 days. The solution from the walnut husk extraction was filtered using a Whatman's filter paper n° 1 followed by a 0.2 μm syringe filter to obtain a sterile extract, which was then stored at -20 °C until use. The solutions from the walnut leaves and the magnolia seeds were filtered using a kitasato (with a Whatman's filter paper n° 1) coupled to a vacuum pump followed by a 0.2 μm syringe filter and stored at -20 °C. For the ethanolic extracts, walnut husks were extracted overnight with 100% (v/v) or 50% (v/v) ethanol (EtOH) and the walnut leaves and magnolia seeds powders were extracted, also overnight, with 80% (v/v) EtOH, all at room temperature and with occasional agitation. The ethanolic extracts were filtered as described above, for each type of tissue, and then, to obtain the dry extract, the solvent was evaporated at 35 °C,

60 rpm, under reduced pressure in a rotavapor. The dried extracts were lyophilised. After the lyophilization, stock solutions were made (Table 2.) for studies with yeast and filamentous fungi.

Table 2. Solvents used and stock solutions of the different extracts.

Species/ Organ	Solvent	Stock solutions (mg/mL)
Walnut husk	H_2O	50
	EtOH 50%	50
	EtOH 100%	50
Walnut leaves	H_2O	50
	EtOH 80%	50
Magnolia seeds	H_2O	50
	EtOH 80%	200

2.2. Viability test - CFUs (colony forming units)

Viability tests were performed to study the effect of the extracts using yeast (*Saccharomyces cerevisiae*) as model. A pre-inoculum of *S. cerevisiae* (BY4741 strain) with Yeast extract- Peptone-Dextrose medium (YPD; 1% w/v yeast extract (FisherBioreagents), 2% w/v peptone (Bacto™) and 2% w/v dextrose (Labchem)) was inoculated and incubated overnight at 30 °C and 200 rpm. The optical density at 600 nm (OD_{600}) was measured and then a dilution with fresh YPD medium was made to a OD_{600} of 0.1. After another incubation of 4 h under the same conditions, generally the necessary to reach an OD_{600} of 0.4, the suspension was divided into 5 aliquots: 3 treatments corresponding to different concentrations of the extracts (500, 1000 and 2000 µg/mL), a negative control (where the extract volume was replaced with YPD medium) and a solvent control (where the extract volume was replaced with the solvent of the respective extract). All suspensions were incubated again under the same conditions described above, and samples were harvested at 0, 30, 60 and 90 min. After each sampling time, 100 µL of each sample was serially diluted, from 10^{-1} to 10^{-4} and four 40 µL drops of the 10^{-4} dilution were placed onto solid YPDA medium (YPD with 2% w/v Labchem agar agar) and incubated for 48 h at 30 °C. The number of colonies was counted, and the percentage of viability was calculated as percentage of colony forming units (CFUs) assuming 0 min as 100% viability.

2.3. Evaluation of antifungal activity *in vitro*

Antifungal effects of the plant extracts were evaluated on different species of filamentous fungi: *Colletotrichum acutatum, Diplodia corticola* and on the oomycete *Phytophthora cinnamomi* (Table 3). The extracts, at final concentrations of 500, 1000 and 2000 µg/mL, were incorporated in 15 mL of melted Potato Dextrose Agar (PDA; Biolife) medium at ~50 °C before plating. For the negative control, the volume of extract used for the highest concentration was replaced by the solvent of the extract. A small portion (7 mm diameter) of fungal mycelium was obtained from the margins of a fully covered culture in a Petri dish, placed in the middle of the prepared Petri dishes, and incubated at 25 °C protected from light. The assay was based on comparing the mycelium growth of the filamentous fungi under different conditions, with the extract incorporated in the medium and without extract (control). The mycelium diameter was measured daily, and the formula used to determine the growth inhibition was $\frac{DC-DE}{DC} \times 100$, where DC is the mycelial diameter of the controls and DE is the mycelial diameter of the treatment measured on the same day.

Table 3. Phytopathogenic fungi and oomycete used and their suppliers.

Species and strain	Suppliers
Colletotrichum acutatum 15-015	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)
Phytophthora cinnamomi PH107	Helena Machado (National Institute of Agrarian and Veterinary Research)

2.4. Membrane integrity assay

In order to understand the mechanism of action of the magnolia seed extract, a spore suspension was prepared from a culture of *C. acutatum*. Sterile deionized water was placed in a Petri dish containing the fungal colony previously prepared on PDA medium until it filled the plate and, shaken vigorously to detach the spores. After stirring, the suspension was filtered through a sterilised gauze, to eliminate mycelia. The concentration of the spore suspension was determined using a Neubauer chamber and adjusted to a titre of 6.2×10^6 spores/mL and then they were incubated with the extract (2000 µg/mL) for 2 h (2 µL extract + 48 µL spores suspension). Spores were labelled with 10 µg/mL propidium iodide (PI), a DNA-binding fluorochrome that is unable to cross

a fully functional plasma membrane, for 15 min before observation under brightfield and fluorescence microscopy (Leica DM5000B) (Riccardi & Nicoletti, 2006). The negative control was prepared with deionized sterile water and PI, where the extract was replaced by deionized sterile water, and the solvent control where the extract volume was replaced by the solvent used (EtOH 80%). The percentage of fluorescence was calculated after counting the positive spores according to the following formula:

$$\frac{number\ of\ fluorescent\ spores}{total\ number\ of\ spores} x100$$

, where the number of fluorescing spores corresponds to the spores affected by the extract and the total number of spores corresponds to the spores marked and unmarked by propidium iodide.

2.5. Phytotoxicity evaluation

Before preparing formulations with fungicide activity based on plant extracts, it is important to prior evaluate the potential phytotoxicity of those extracts at antifungal effective concentrations. With this purpose, magnolia seed extract was incorporated into melted MS medium (2% w/v sucrose, 0.8% w/v agar; Murashige & Skoog, 1962) at different concentrations, 500, 1000 and 2000 µg/mL and placed in glass culture flasks with polypropylene caps. As EtOH is inhibitory of seed germination (Miyoshi & Sato, 1997) for each concentration of extract tested a control was prepared using the same volume of solvent (80% w/v EtOH) as that used for the corresponding treatment. Also, a negative control (0 mg/mL) was prepared where the maximum volume of extract was replaced by sterile deionized water. Seeds of lettuce (*Lactuca sativa*), variety May Queen, were disinfected with 5% v/v bleach (Moderna) for 20 min under agitation, and then washed 3 times with sterile deionized water. In a flow chamber, the seeds were inoculated on the culture media, 4 per flask and 4 flasks per condition. Germination and early lettuce growth were monitored for 6 days, after which the number of leaves per plantlet, root length and largest leaf length were measured.

2.6. Statistical analysis

All the results are presented as the mean ± standard deviation (SD) of 3 independent assays in the cell viability, mycelium growth and membrane integrity assays and at least 4 replicates in the phytotoxicity assay. The software used was GraphPad Prism 8.0 version 8.0 for Windows, GraphPad Software (San Diego, California USA, www.graphpad.com) and the results were analysed

by one-way ANOVA ,Tukey and Dunnett tests for multiple comparisons. To indicate the level of significance the asterisk notation (*)(GP style) was used, with $*p < 0.05$, $** p < 0.01$, $***p < 0.001$ and $**** p < 0.0001$.

3. RESULTS AND DISCUSSION

3.1. Antifungal activity of walnut leaves

3.1.1. Effects of walnut leaves aqueous extract on *Saccharomyces cerevisiae*

To evaluate possible effects of walnut leaves extract as antifungal, cell viability assays were performed with *Saccharomyces cerevisiae* strain BY4741 as a fungus experimental model. Different concentrations of the extract were incubated with the yeast culture and the viability was calculated after 30, 60 and 90 min. As we can see in Figure 1 the extract, at all concentrations, showed similar behaviour when compared to the negative control, with cell viability increasing over time. Statistical analysis showed that none of the concentrations managed to a significantly decrease of cell viability after 30, 60 or 90 min of incubation with the walnut leaves aqueous extract (Figure A1). This result suggests that there is no presence of either antifungal or fungistatic activity against this *S. cerevisiae* strain. These results are in line with the results obtained by (Pereira et al., 2007) that showed that there was no activity by an aqueous extract of walnut leaves against fungi such as *Candida albicans* and *Cryptococcus neoformans*.

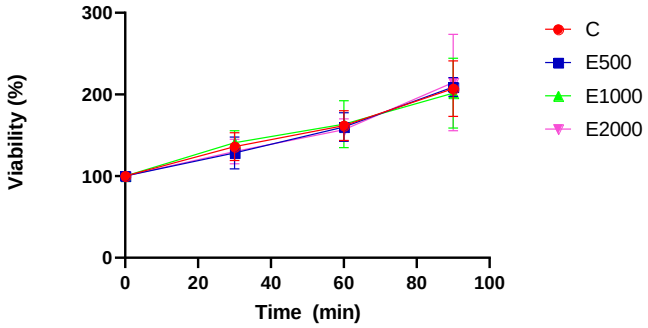


Figure 1. Viability of *Saccharomyces cerevisiae* BY4741 in the presence of aqueous extract of walnut leaves (*Juglans regia*). Cells from a culture in exponential phase were incubated with the extract at different concentrations: 500 µg/ mL (E500), 1000 µg/ mL (E1000) or 2000 µg/ mL (E2000). After 0, 30, 60 and 90 min, aliquots were harvested, serially diluted, and spread on YPDA plates. After 48 h incubation at 30 °C, 200 rpm, colonies were counted, and cell viability was calculated assuming 0 min as 100% viability. Control (C) was prepared by replacing the largest volume of extract by sterile deionized water. Data is

presented as the mean of three experiments ± SD. The variance analysis was performed using one-way ANOVA and Tukey test for multiple comparisons.

3.1.2. Antifungal activity of walnut leaves aqueous extract on phytopathogenic fungi

Although the viability assay did not show antifungal or fungistatic activity against the fungal model *S. cerevisiae*, the possibility of the extract to have activity on filamentous fungi was tested. Only the concentration of 2000 μg/mL was used, and the extract was incorporated into the PDA medium and then a mycelium disk of *Diplodia corticola* was placed in the middle of the Petri dishes. The antifungal activity was evaluated through measurements of the mycelium, comparison between the control and treatment plates and calculating the percentage of inhibition of fungal growth. After 3 days of incubation there were already significant differences between the control and the treatment (Figure 2) with the extract being able to inhibit about 18% of the fungus growth, which represented the highest percentage during the trial. Over the days the percentage of inhibition gradually decreased, reaching approximately 12% on day 6 and on day 7, the day the control plate was filled, it reached approximately 8.5% (Figure 2 and Figure 3). The results suggest that the extract was able to act against the fungus but the decrease in inhibition over time suggests that somehow, possibly, the fungus adapted to the presence of the extract, or the extract can easily degrade. These results are in line with those obtained by (Kocacaliskan et al., 2018) when they showed that aqueous extracts of walnut leaves from different cultivars were able to inhibit the growth of fungi such as *Botrytis cinerea* and *Candida albicans*.

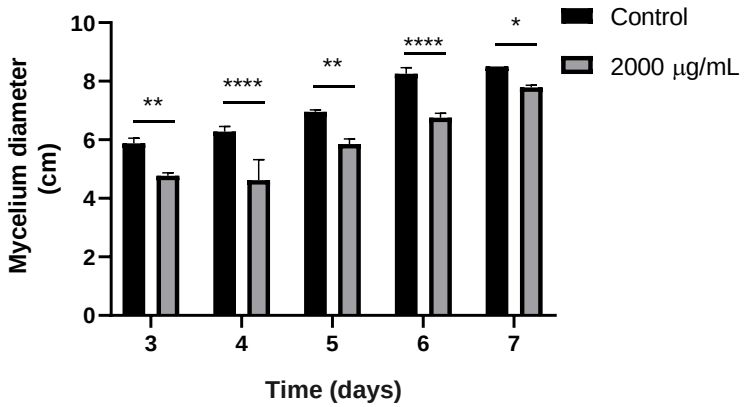


Figure 2. Antifungal effect of walnut (*Juglans regia*) leaves extract on *Diplodia corticola*. A mycelium disk of the fungus was placed in the middle of Petri dishes with PDA with or without 2000 μg/mL extract (control). In the control the volume of extract used was replaced by deionized sterile water. The diameter of the colonies was measured along time. Data is presented as the mean of three experiments ± SD. The variance analysis was performed using two-way ANOVA and Sidak's test for multiple comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

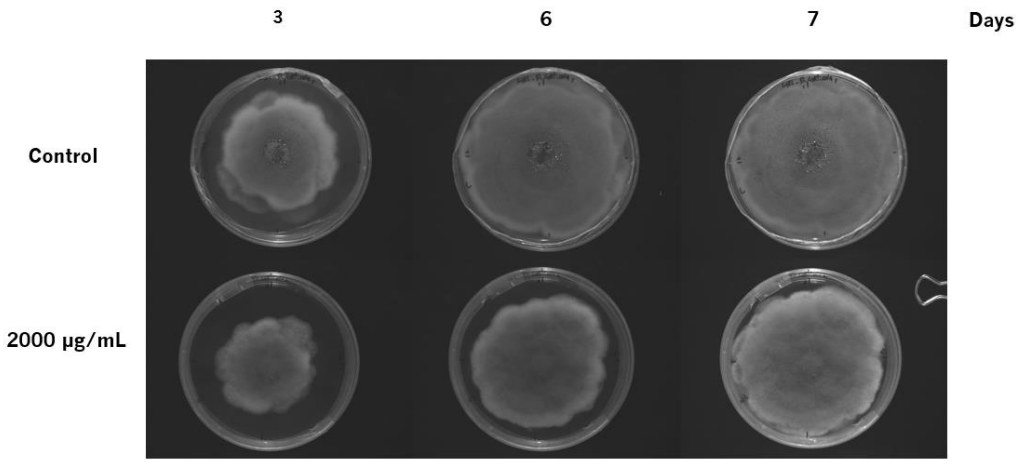


Figure 3. Photographs of *Diplodia corticola* plates throughout the antifungal assay of walnut (*Juglans regia*) leaves extract. In the first row are images of the control after 3, 6 and 7 days, respectively, and in the second row are images of the treatment plates after the same time points.

3.2. Antifungal activity of walnut husk aqueous extract

3.2.1. Effects of walnut husk aqueous extract on *Saccharomyces cerevisiae*

The effect of aqueous extract was also tested on yeast, using cell viability assays as described above for walnut leaves extract. The control behaved similarly to the walnut leaves extract, with the control reaching about 200% viability after 90 min (Figure 4). After 30 min, viability of the control (141%) was slightly higher than in the two lowest concentrations, 500 µg/mL (131%) and 1000 µg/mL (132.5%), while the highest concentration 2000 µg/mL (104%) maintained approximately the same number of colonies compared to 0 min and statistically there are significant differences between treatment (2000 µg/mL) and control ($p < 0.01$). After 60 min all concentrations showed a lower percentage of viability than the control, with the highest concentration (2000 µg/mL) showing the major increase in %, from approximately 104% at 30 min to 147% at 60 min, however none of the differences in viability, when compared with the control, were significant, according to the statistical analysis. At 90 min none of treatments showed significant differences with the control. These results suggest that only the higher concentration may have some toxic effect on yeast, but this effect is transient.

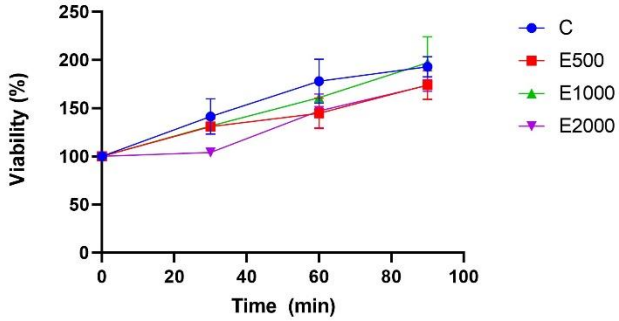


Figure 4. % Viability of *Saccharomyces cerevisiae* BY4741 strain in the presence of aqueous extract of walnut (*Juglans regia*) husk. Cells from an exponential phase were incubated with the extract at different concentrations: 500 µg/mL (E500), 1000 µg/mL (E1000) and 2000 µg/mL (E2000). After 0, 30, 60 and 90 min cell viability was calculated assuming 0 min as 100% viability. Control (C) was prepared by replacing the largest volume of extract by sterile deionized water. Data presented as the mean of three experiments ± SD. The variance analysis was performed using one-way ANOVA and Dunnett's test for multiple comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The result of the statistical analysis can be found in the annexes (Figure A2).

3.2.2. Antifungal activity of walnut husk aqueous extract on phytopathogenic fungi

3.2.2.1. *Diplodia corticola*

As it happened for the walnut leaves extract, even though there were no promising results with yeast, the possibility of the aqueous walnut husk extract having antifungal activity against some fungal species was tested. Three concentrations of the extract 250 µg/ mL, 500 µg/ mL and 1000 µg/ mL were used, and the incorporation of the extract and the evaluation of fungal growth was carried out as described above. During the assay, and according to the statistical analysis, there were no significant differences in the growth of fungi in contact with the extract when compared to the control plate (Figure 5). This result suggests that the aqueous walnut husk extract has no influence in the growth of *Diplodia corticola* (Figure 6). Oliveira et al (2008) showed that an aqueous walnut husk extract also did not exhibit antimicrobial activity against fungi such as *Candida albicans* and *Cryptococcus neoformans*, which match with the results obtained with *Diplodia corticola*.

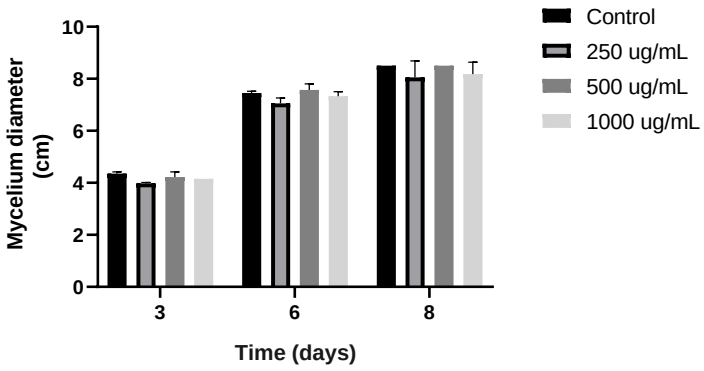


Figure 5. Antifungal effect of aqueous walnut (*Juglans regia*) husk extract on *Diplodia corticola*. A mycelium disk was placed in the middle of petri dishes, with extract (250 µg/mL, 500 µg/mL, and 1000 µg/mL) and without extract (control). In the control the higher volume of extract used was replaced by deionized sterile water. The mycelium of treatment plates was measured and compared with control. Data presented as the mean of three experiments ± SD. The variance analysis was performed using two-way ANOVA and Sidak's test for multiple comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

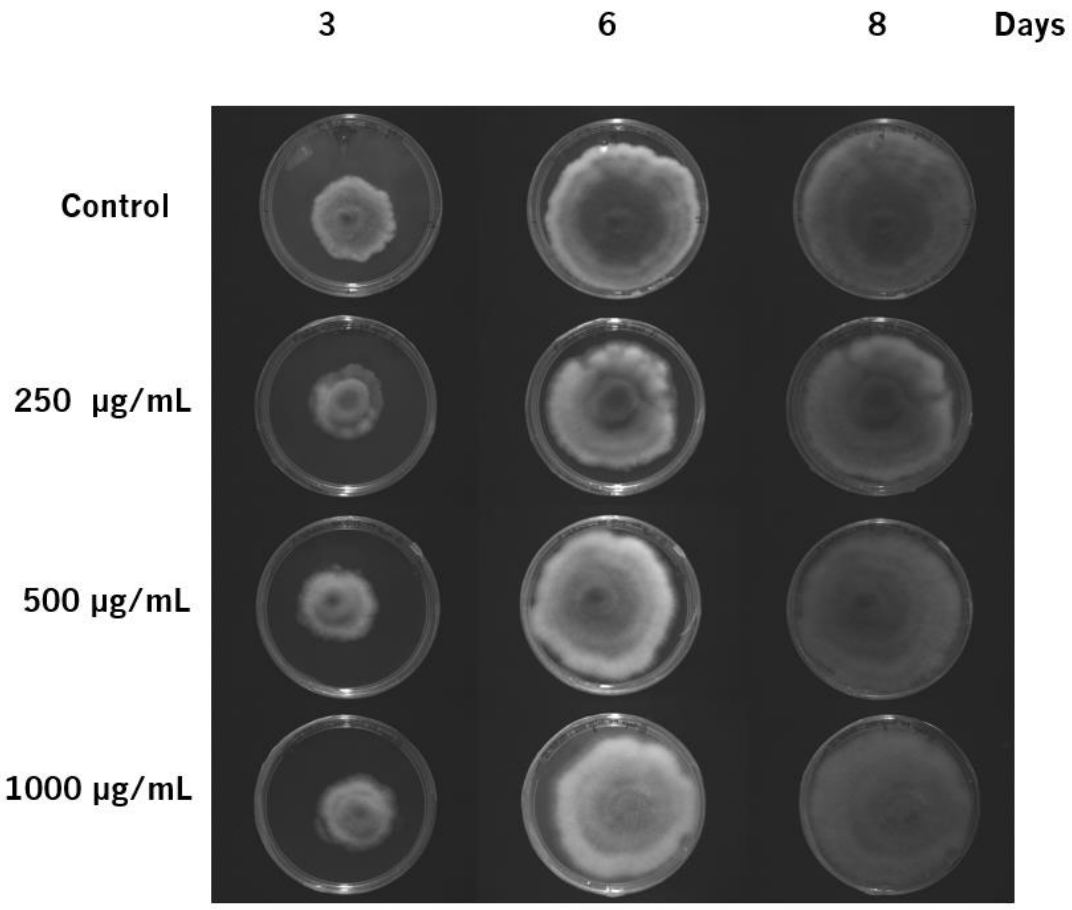


Figure 6. Images of *Diplodia corticola* plates throughout the assay. In the first row are images of the control and in the others are the different treatments, ordered from the lowest to the highest concentration. Throughout the assay the highest % inhibition (13%) was recorded at day 4 at the lowest concentration of extract (250 µg/mL).

3.2.2.2. *Colletotrichum acutatum*

The assay for the evaluation of the antifungal potential of walnut husk aqueous extract in *Colletotrichum acutatum* was designed in the same way as for *D. corticola*. Over 13 days, the diameters of the control plates and the different extract concentrations were measured (Figure 7 and Figure 8). Throughout the assay, there were significant differences ($p < 0.05$): on the sixth day at the concentration of 250 μg/mL with a growth inhibition of approximately 9%, on the tenth day at the concentration of 1000 μg/ mL with an inhibition of approximately 6% and on the thirteenth day the concentration of 500 μg/mL managed to inhibit growth by approximately 5%. To our knowledge, there are no reports of tests with this extract against *C. acutatum*. However, as stated above, when the same extract interacted with *Diplodia corticola*, Oliveira et al., (2008b) showed that this extract had no antimicrobial activity against 2 fungal species (*C. albicans* and *C. neoformans*) which is in line with our results

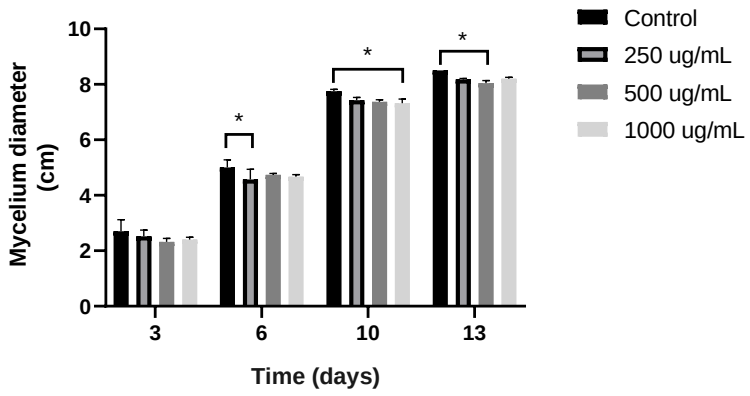


Figure 7. Antifungal effect of aqueous walnut (*Juglans regia*) husk extract on *Colletotrichum acutatum*. A mycelium disk was placed in the middle of petri dishes, with extract (250 μg/mL, 500 μg/mL, and 1000 μg/mL) and without extract (control). In the control the higher volume of extract used was replaced by deionized sterile water. The mycelium of treatment plates was measured and compared with control. On day 6 the 250 μg/mL extract showed a significant difference to the control with a ≈ 9% growth inhibition; on day 10 the 1000 μg/mL concentration also showed a significant difference to the control, with a ≈ 6% inhibition; and on day 13 the 500 μg/ mL concentration showed a ≈ 5% inhibition. Data presented as the mean of three experiments ± SD. The variance analysis was performed using two-way ANOVA and Sidak's test for multiple comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

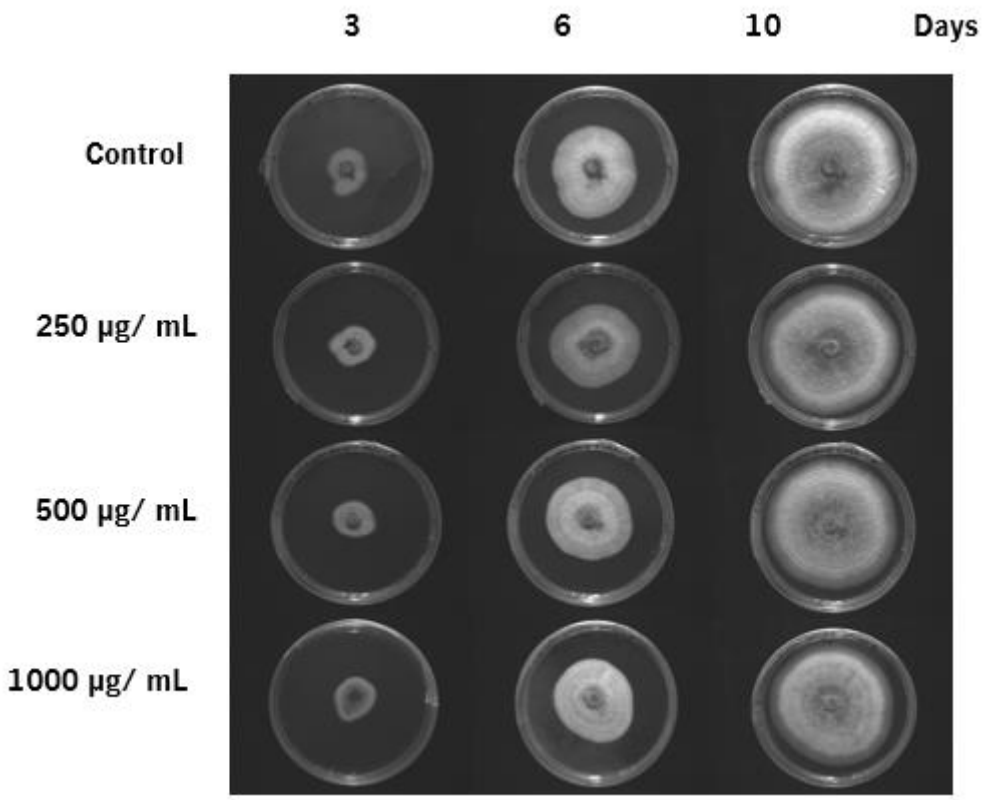


Figure 8. Images of *Colletotrichum acutatum* plates throughout the assay. In the first row are images of the control and in the others are the different treatments, ordered from the lowest to the highest concentration (250, 500 and 1000 µg/mL)

3.3. Antifungal activity of walnut husk (ethanolic extracts: 50 % EtOH and 100% EtOH)

3.3.1. Effects of walnut husk ethanolic extracts (50% and 100%) on *Saccharomyces cerevisiae*

The activity of ethanolic extracts of walnut husk was also tested against *S. cerevisiae* BY4741 strain using cell viability assays. The assay was performed in the same way as for the other extracts, already described above. Two types of extract were tested, a 50% ethanolic extract (hydroethanolic) that was dissolved in water after freeze-drying and a 100% ethanolic extract, dissolved in ethanol 100% (v/v). In the assay with the extract prepared with 100% EtOH, for the solvent control, the larger volume of extract used was replaced with absolute ethanol in order to ensure that no effects could be derived from the use of ethanol. For the hydroethanolic extract that was resuspended in water after freeze-drying, the larger volume of extract was replaced with sterile deionised water for the control. In both assays the behaviour of the negative control was very similar, reaching about 200% viability after 90 min (Figure 9 and Figure 10) Wianowska et al., 2016) reported that hydroethanolic extracts of walnut husk had activity against different fungi: *Botrytis cinerea,*

Rhizoctonia solani, Alternaria alternata, Fusarium culmorum, Phytopthora infestans and Ascosphaera apis. They showed, by obtaining different biological activities, that different walnut cultivars resulted in different extracts. All cultivars showed antifungal activity with inhibitions ranging from 15% to 65%. According to Wianowska et al., (2016) results, it was expected that the extract would have effect on yeast which did not happen. This may not have happened due to differences in extraction methods like, percentage of ethanol used and time of the year when the walnut husk was harvested, because all these variables affect the composition of the extract.

3.3.1.1. Walnut husk extract- ethanolic extract

During the assay the only significant differences occurred at time 60 min where the extract with the highest concentration caused a significantly lower viability than the negative control ($p < 0.05$) but despite these differences, when comparing the extract (2000 µg/mL) and the solvent control, there was no significance, suggesting that the effect may be due to the use of ethanol at a dose that affects the yeast cells. The solvent control exposes cells to more ethanol than the 500 µg/mL concentration, which might explain why this extract shows a significant increase in viability over the control. However, the extract at this concentration appears to have no activity. When compared with the negative control, the same extract showed significantly different results only at time 60 ($p < 0.05$).

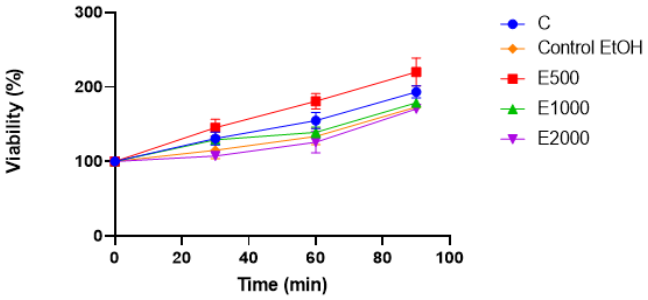


Figure 9. % Viability of *Saccharomyces cerevisiae* BY4741 strain in the presence of ethanolic extract of walnut (*Juglans regia*) husk (EtOH 100%). Cells from an exponential phase were incubated with the extract at different concentrations: 500 µg/mL (E500), 1000 µg/mL (E1000) and 2000 µg/mL (E2000). After 0, 30, 60 and 90 min cell viability was calculated assuming 0 min as 100% viability. Control (C) was prepared by replacing the largest volume of extract by sterile deionized water and the solvent control (Control EtOH) was prepared by replacing the volume of extract by the solvent used (EtOH 100%). Data presented as the mean of three experiments ± SD. The variance analysis was performed using one-way ANOVA and Dunnett's test for multiple comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The result of the statistical analysis can be found in the annexes (Figure A3 and Figure A4)

3.3.1.2. Walnut husk extract - hydroethanolic extract

In the cell viability assay with ethanolic extract (extraction with 50% EtOH and resuspension of the dried extract in sterile deionised water) none of the treatments presented a different profile from the negative control except for the concentration of 1000 µg/mL where a delay in the increase of viability was observed. A slower increase in viability was observed until 60 min where a significant difference was observed in relation to the control ($p < 0.05$). The oscillations that occurred in the 1000 µg/mL extract, may suggest that the extract has a small effect, but this effect disappeared 30 min later, with the yeast being able to recover.

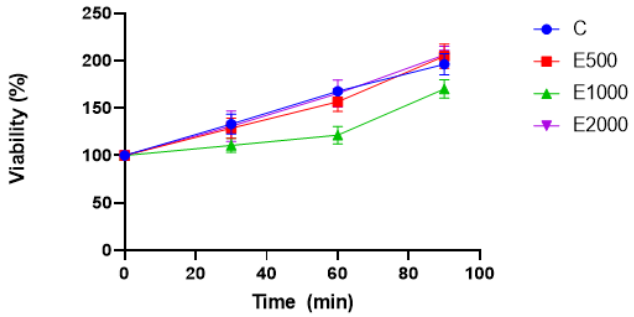


Figure 10. % Viability of *Saccharomyces cerevisiae* BY4741 strain in the presence of ethanolic extract of walnut (*Juglans regia*) husk (EtOH 50% resuspended in deionized sterile water). Cells from an exponential phase were incubated with the extract at different concentrations: 500 µg/mL (E500), 1000 µg/mL (E1000) and 2000 µg/mL (E2000). After 0, 30, 60 and 90 min cell viability was calculated assuming 0 min as 100% viability. Control (C) was prepared by replacing the largest volume of extract by sterile deionized water. Data presented as the mean of three experiments ± SD. The variance analysis was performed using one-way ANOVA and Dunnett's test for multiple comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The result of the statistical analysis can be found in the annexes (Figure A5).

3.3.2. Antifungal activity of walnut husk hydroethanolic extract on phytopathogenic fungi- *Diplodia corticola*

Because it was the only type of ethanolic walnut husk extract that showed results that could indicate some effect on yeast, the walnut husk hydroethanolic extract (EtOH 50%) was also tested against the phytopathogenic fungi (*Diplodia corticola*). The test was performed in the same way as described above with the concentration of 2000 µg/mL of extract. The fungus that was tested showed no significant differences (Figure 11 andFigure 12), with the extract being unable to cause changes in the normal growth of the fungi. As also noted above these results are not in line with Wianowska et al., (2016) results, which showed antifungal activity of an extract similar to this one against several different fungi which suggests that the extract may have activity against some fungi and not others.

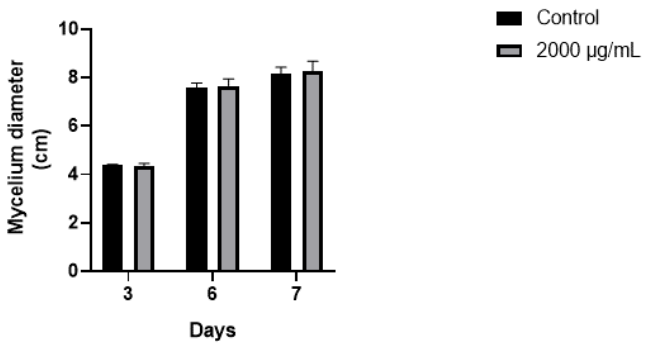


Figure 11. Antifungal effect of hydroethanolic walnut (*Juglans regia*) husk extract on *Diplodia corticola*. A mycelium disk was placed in the middle of petri dishes, with extract (2000 µg/mL) and without extract (control). In the control the higher volume of extract used was replaced by deionized sterile water. The mycelium of treatment plates was measured and compared with control. Throughout the assay there were no significant differences between the control and the extract. Data presented as the mean of three experiments ± SD. The variance analysis was performed using two-way ANOVA and Sidak's test for multiple comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

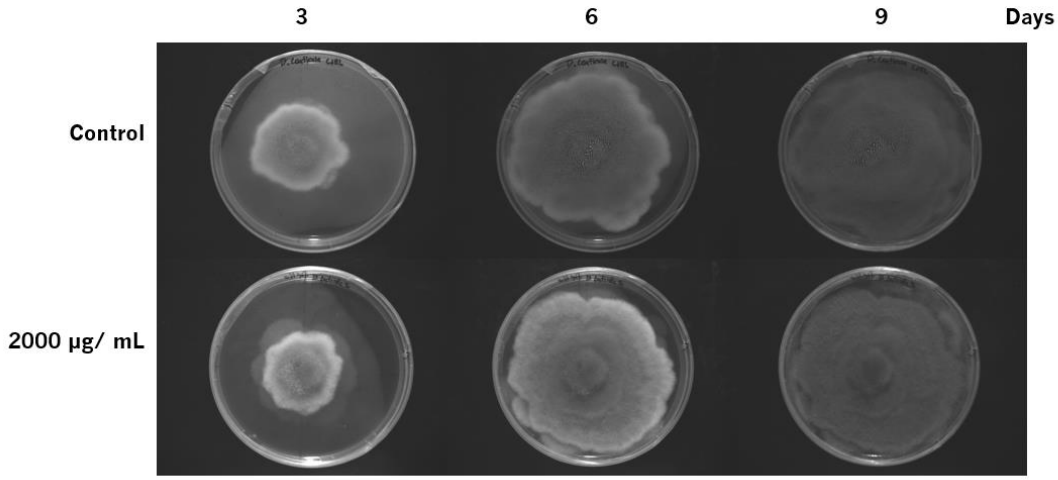


Figure 12. Images of *Diplodia corticola* plates throughout the assay. In the first row are images of the control and in the other are images of the treatment (2000 µg/mL).

3.4. Antifungal activity of magnolia seeds (aqueous extract)

3.4.1. Effects of magnolia seeds aqueous extract on *Saccharomyces cerevisiae*

One of the extracts also tested was the aqueous extract made from magnolia (*Magnolia grandiflora*) seeds. As with the walnut extracts, viability assays were first performed on *S. cerevisiae* as fungal model. Viability in the negative control increased over time and reached 200% after 90 min (Figure 13). Regarding the treatments (500, 1000 and 2000 µg/mL) all had quite different behaviours from each other with the lowest concentration (500 µg/mL), after 30 min, viability presenting higher

viability percentages than all the other conditions, including the control. Regarding the extract at the concentration of 1000 µg/ml, it behaviour was very similar to the control throughout the whole assay, without significant differences between both. Finally, the extract at the highest concentration tested (2000 µg/ml) accentuated the difference in percentage viability only in the last timepoint (90 min), where viability was significantly lower than the control ($p < 0.01$). In general terms the viability values decreased along with increasing concentration, being only significant for the higher concentration, which may suggest that there was a dose/response effect. To the best of our knowledge, no antifungal tests were performed with aqueous extracts of *Magnolia grandiflora* seeds, but experiments were performed by Clark, El-feraly et al (1981), who reported that phenolic compounds present in magnolia seeds (magnolol, honokiol and 3,5'-diallyl-2'-hydroxy-4-methoxy-biphenyl) showed antimicrobial activity against yeast, fungi and bacteria. In this assay the MICs of the phenolic compounds were calculated where, for example the 3 compounds presented MICs of 10 mg/mL with *S. cerevisiae* and two of them (magnolol and honokiol) MICs of 30 mg/mL with *Aspergillus niger*, a phytopathogenic fungus. The results obtained in this work show that there is an ability of the extract, at the highest concentration, to lower viability after 90 min, which is in line with the results described above, obtained by Clark, El-feraly and his co-workers (1981) where it is shown that compounds present in magnolia seeds are able to have effects on yeast. To ensure that the same compounds are responsible for the decrease in viability, a chemical analysis should be performed.

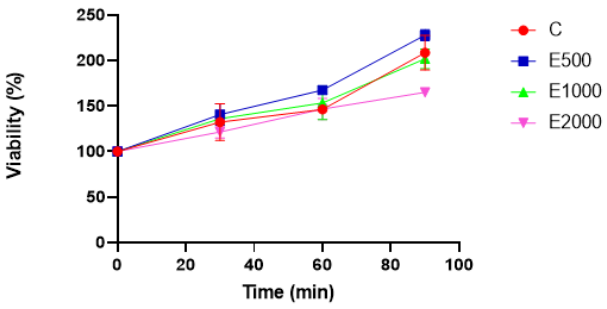


Figure 13. % Viability of *Saccharomyces cerevisiae* BY4741 strain in the presence of aqueous extract of magnolia (*Magnolia grandiflora*) seeds. Cells from an exponential phase were incubated with the extract at different concentrations: 500 µg/ mL (E500), 1000 µg/mL (E1000) and 2000 µg/mL (E2000). After 0, 30, 60 and 90 min cell viability was calculated assuming 0 min as 100% viability. Control (C) was prepared by replacing the largest volume of extract by sterile deionized water. Data presented as the mean of three experiments ± SD. The variance analysis was performed using one-way ANOVA and Dunnett's test for multiple comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The result of the statistical analysis can be found in the annexes (Figure A6).

3.4.2. Antifungal activity of magnolia seeds aqueous extract on phytopathogenic fungi

Due to the results obtained in yeast, with the significant differences shown by 2000 µg/ml extract, it was decided that possible effects of the extract on different fungal species should also be tested. The assays occurred in a similar way and only the highest concentration was tested, the one that showed promising results on yeast. The fungi tested was *Diplodia corticola.* The extract slowed the fungus growth throughout the assay, reaching approximately 20% inhibition on days 3 and 6 (Figure 14 and Figure 15). The results obtained in this work show that the aqueous extract of magnolia seeds was able to inhibit the growth of the fungus, achieving 20% inhibition of the growth of *Diplodia corticola*. Yan and his co-workers (2020) tested the ability of aqueous extracts (250 and 500 µg/mL) of a part of *Magnolia officinalis*, not revealed by the authors, to inhibit the growth of 7 phytopathogenic fungi. The aqueous extracts only showed some percentage inhibition, at the highest concentration and on 2 fungi only, on *Phytophthora capsica* with 5.53 % inhibition and on *Sclerotinia sclerotiorum* with 2.42 % inhibition. Although these are different extracts from different species, the results obtained with *Magnolia grandiflora* seeds seem to indicate that there is an antifungal potential of this extract.

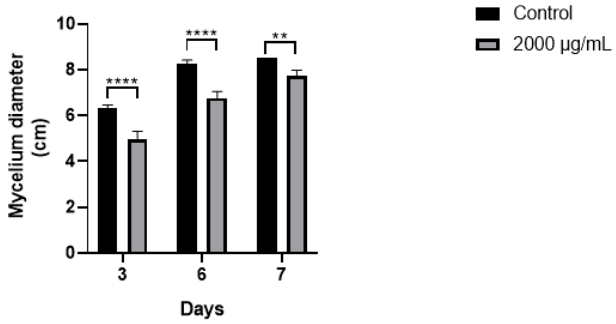


Figure 14. Antifungal effect of magnolia seeds aqueous extract on *Diplodia corticola*. A mycelium disk was placed in the middle of petri dishes, with extract (2000 µg/mL) and without extract (control). In the control the higher volume of extract used was replaced by deionized sterile water. The mycelium of treatment plates was measured and compared with control. Throughout the assay there were no significant differences between the control and the extract. Data presented as the mean of three experiments ± SD. The variance analysis was performed using two-way ANOVA and Sidak's test for multiple comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

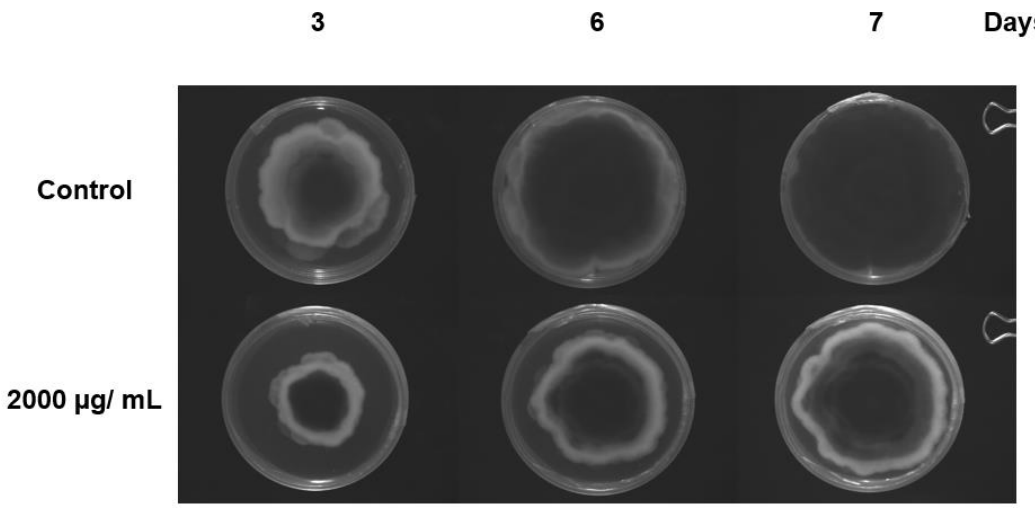


Figure 15. Images of *Diplodia corticola* plates throughout the assay. In the first row are images of the control and in the other are images of the treatment (2000 µg/mL).

3.5. Activity of magnolia seeds (ethanolic extract) against phytopathogenic microorganisms

3.5.1. Effects of magnolia seeds ethanolic extract on *Saccharomyces cerevisiae*

The magnolia seeds extract was again tested as described above, but using a different solvent, EtOH 80%. In the assay, the viability of the negative control increased over time, reaching approximately 200% viability after 90 min. For the solvent control, the largest volume of extract used was replaced with EtOH 80% to ensure that there were no effects arising from the use of ethanol. During the assay the negative control and the solvent control behaved similarly and although the solvent control had lower percentage viability values, this difference was not significant (Figure 16). The lowest concentration extract (500 µg/mL) did not show, during the whole assay, significant differences when compared with the negative control. The intermediate concentration (1000 µg/mL) showed significant differences at time 30 min and at time 60 min (p<0.05) and presented at time 90 min a more significant difference (p<0.01) when compared with the negative control. As the volume of ethanol added to the solvent control was equal to the maximum volume of extract added in the treatments, comparing the viability values between the solvent control and 500 and 1000 µg/mL would not translate into reliable conclusions. At the highest concentration (2000 µg/ml) there were, relative to the solvent control, significant differences at 60 min and 90 min (p<0.01), with the percentage viability decreasing to close to 10% at 60 min and standing at 45% approximately at the end of 90 min. The 2000 µg/mL extract appears to exhibit activity against

S. cerevisiae BY4741. As previously shown, there are studies that show the antifungal capacity of compounds present in *Magnolia grandiflora* seeds, such as honokiol and magnolol, which like the extract used in this trial, were able to inhibit the growth of yeast *(S. cerevisiae* ATCC 9763; Clark, El-Feraly et al., 1981).

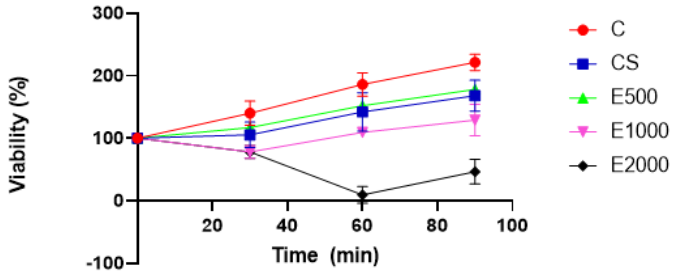


Figure 16. % Viability of *Saccharomyces cerevisiae* BY4741 strain in the presence of ethanolic extract of magnolia (*Magnolia grandiflora*) seeds. Cells from an exponential phase were incubated with the extract at different concentrations: 500 µg/mL (E500), 1000 µg/mL (E1000) and 2000 µg/mL (E2000). After 0, 30, 60 and 90 min cell viability was calculated assuming 0 min as 100% viability. Control (C) was prepared by replacing the largest volume of extract by sterile deionized water and solvent control (CS) was prepared by replacing the largest volume of extract by ethanol 80%. Data presented as the mean of three experiments ± SD. The variance analysis was performed using one-way ANOVA and Dunnett's test for multiple comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The result of the statistical analysis can be found in the annexes (Figure A7).

3.5.2. Activity of magnolia seeds ethanolic extract on phytopathogenic microorganisms

3.5.2.1. *Diplodia corticola*

The ethanolic extract of magnolia seeds showed great potential in yeast. Since the concentration that showed the higher activity was the highest concentration (2000 µg/mL), we decided to test only this concentration in the evaluation of the antifungal activity on *Diplodia corticola*. After 3 days of incubation of the extract with the fungus, a 45% inhibition was detected when comparing the diameter of the control and treatment plates (

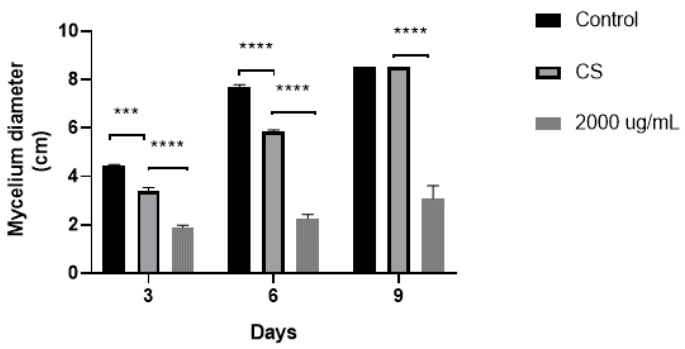


Figure 17 and Figure 18). On day 6 the inhibition increased to an average of approximately 62%, a value that was surpassed on day 9, when the control plate filled the Petri dish, showing the highest percentage of inhibition with an average of approximately 64%. These results seem promising since, to the best of our knowledge, magnolia seed extracts have not yet been tested against phytopathogenic fungi and there are only reports of the antifungal activity of different parts of *Magnolia officinalis*. In that study, Yan et al., (2020) evaluated the antifungal capacity of ethanolic extracts (250 and 500 μg/mL) of a part, not revealed by the author, of *Magnolia officinalis* against 7 phytopathogenic fungi and all showed inhibitions greater than 65%, which is in line with the results obtained with *Magnolia grandiflora* seeds against *Diplodia corticola*, not tested by them.

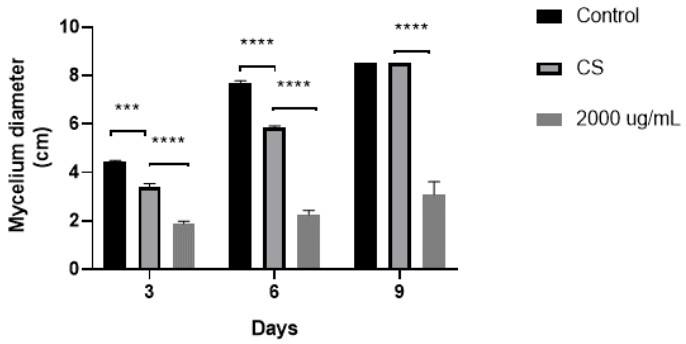


Figure 17. Antifungal effect of magnolia seeds ethanolic extract on *Diplodia corticola*. A mycelium disk was placed in the middle of petri dishes, with extract (2000 μg/mL) and without extract (control). In the control the higher volume of extract used was replaced by ethanol 80%. The mycelium of treatment plates was measured and compared with control. Throughout the assay there were no significant differences between the control and the extract. Data presented as the mean of three experiments ± SD. The variance analysis was performed using two-way ANOVA and Dunnett's test for multiple comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

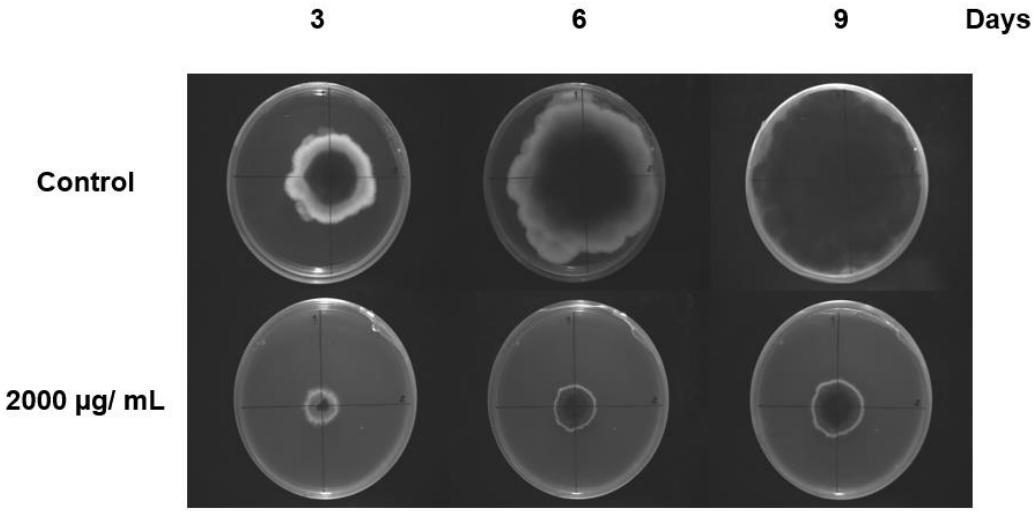


Figure 18. Images of *Diplodia corticola* plates throughout the assay. In the first row are images of the control and in the other are images of the treatment (2000 µg/mL).

3.5.2.2. *Phytophthora cinnamomi*

As for *D. corticola*, the ability of magnolia seed extract to inhibit the growth of *Phytophthora cinnamomi* was also tested. The assay was carried out in the same way as described above and significant differences ($p < 0.0001$) were found on day 3 where the growth of the control plates was twice that of the treatment plates, with inhibitions of approximately 47% (Figure 19 and Figure 20). On day 6, with the control plates already filled, with the oomycete *P. cinnamomi*, the significant differences remained ($p < 0.0001$) although the percentage of inhibition had decreased to 35%. To the best of our knowledge, there are no studies addressing the antimicrobial capacity of *Magnolia grandiflora* seed extracts against phytopathogens but Yan et al., (2020) with extracts of Magnolia officinalis, a species of the same genus, was able to show inhibitions of 77.79% ± 0.31 and 74.96% ± 0.59, with extract concentrations of 500 and 250 µg/mL, respectively, against the oomycete of the same genus, *Phytophthora capsica*. Again, the ethanolic extract of magnolia seeds was shown to have potential against phytopathogens and that it may have a role in controlling infections caused by *Phytophthora cinnamomi*.

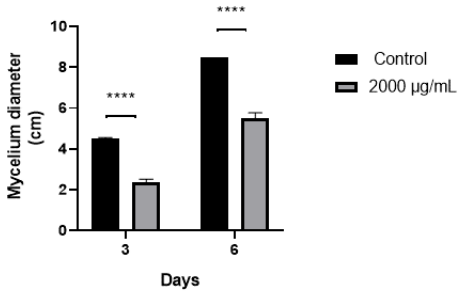


Figure 19. Antimicrobial effect of magnolia seeds ethanolic extract on *Phytophthora cinnamomi*. A mycelium disk was placed in the middle of petri dishes, with extract (2000 µg/mL) and without extract (control). In the control the higher volume of extract used was replaced by ethanol 80% (v/v). The mycelium of treatment plates was measured and compared with control. Throughout the assay there were no significant differences between the control and the extract. Data presented as the mean of three experiments ± SD. The variance analysis was performed using two-way ANOVA and Dunnett's test for multiple comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

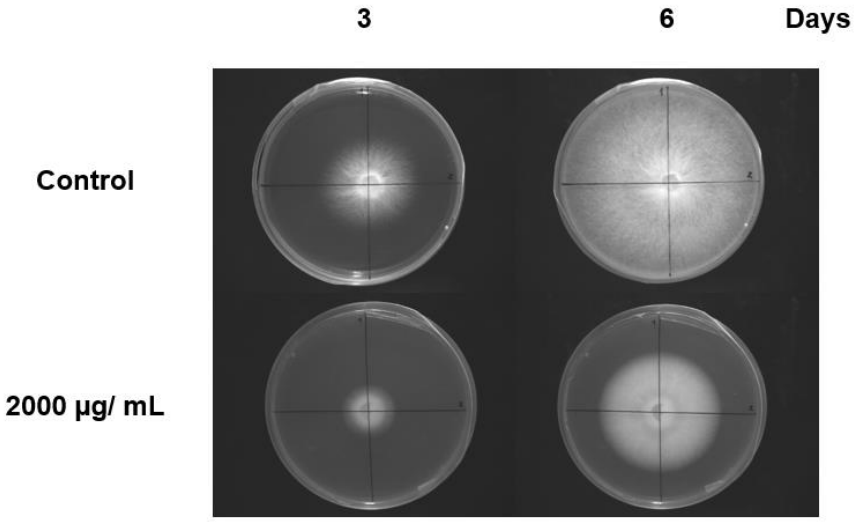


Figure 20. Images of *Phytophthora cinnamomi* plates throughout the assay. In the first row are images of the control and in the other are images of the treatment (2000 µg/mL).

3.5.2.3. *Colletotrichum acutatum*

As the ethanolic extract of magnolia seeds proved to be effective against *D. corticola* and *P. cinnamomi* we decided to also evaluate the inhibitory potential of the extract against *Colletotrichum acutatum*. The assay proceeded in the same way as previously described but instead of using only the highest concentration (2000 µg/mL), concentrations of 500 and 1000 µg/mL were also tested. The solvent control was shown to have some negative effect on fungal growth by showing significant differences (p<0.0001; p<0.001) from the control on all days measured except day 3 (Figure 20). The tested 3 concentrations of the extract showed ability to inhibit the fungus in a dose response

effect, with increasing concentration being reflected in increased inhibition of the fungus. The 500 µg/mL extract showed inhibitions around 30%, reaching its maximum inhibition level of 36% on day 9. The extract of 1000 µg/mL showed inhibitions very close to the extract of 2000 µg/mL, but lower, with the highest % inhibition corresponding to 49%, on day 9. The extract with the highest concentration (2000 µg/mL) managed to inhibit the growth of the fungus by 55% on day 9, representing the highest inhibition during the assay (Figure 20 and Figure 21). As mentioned in the other topics, from what we know there are no studies using this type of extract against phytopathogenic fungi but there are studies showing activity of extracts of other parts of *Magnolia grandiflora*. Abdelgaleil & Ahmed (2005) tested with bark and leaf extracts of *Magnolia grandiflora* and the higher activity was obtained using dichloromethane as solvent, in both extracts. The absence of studies on antifungal assays with *Magnolia grandiflora* extracts may mean that these results are innovative and that in the future this type of extract may serve as a control for diseases in plants.

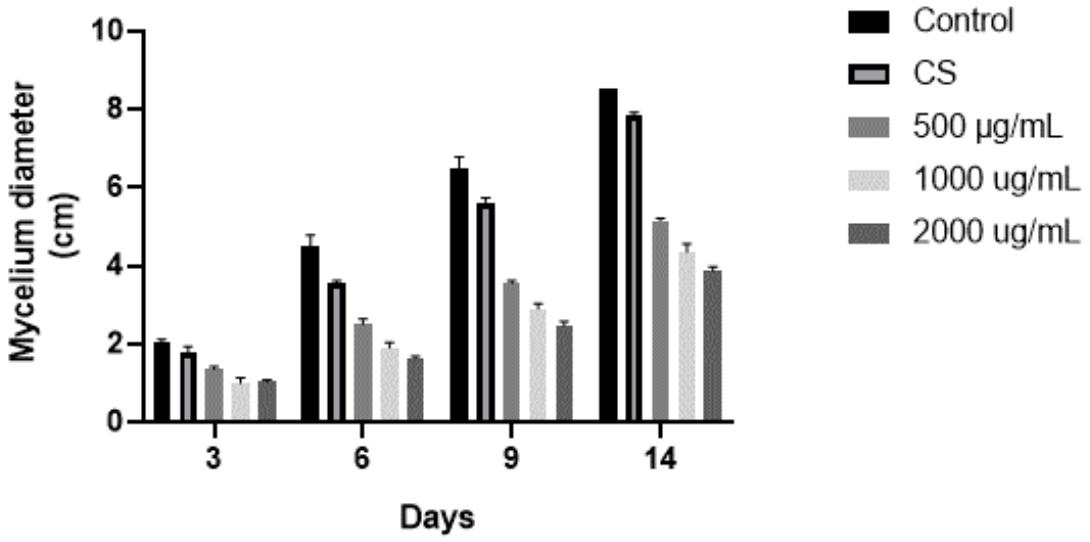


Figure 21. Antifungal effect of magnolia seeds ethanolic extract on *Colletotrichum acutatum*. A mycelium disk was placed in the middle of petri dishes, with extract (2000 µg/mL) and without extract (control). In the control the higher volume of extract used was replaced by ethanol 80% (v/v). The mycelium of treatment plates was measured and compared with control. Throughout the assay there were no significant differences between the control and the extract. Data presented as the mean of three experiments ± SD. The variance analysis was performed using two-way ANOVA and Dunnett's test for multiple comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Figure A8).

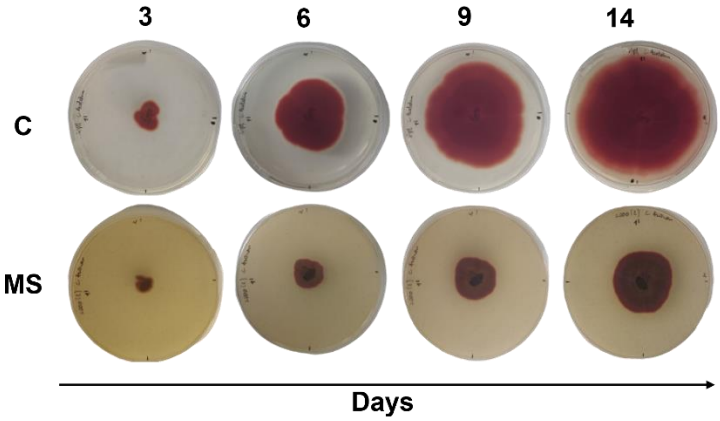


Figure 22. Images of *Colletotrichum acutatum* plates throughout the assay. In the first row are images of the control and in the other are images of the treatment (2000 μg/mL).

3.5.3. Membrane integrity assay - mechanism of action of magnolia seeds ethanolic extract

After showing potential activity against phytopathogenic fungi it was important to understand how the magnolia seed ethanolic extract acted on the fungi. To try to understand the mechanism of action of the extract, spores of *Colletotrichum acutatum* were used and incubated with the ethanolic extract of magnolia seeds only in the concentration 2000 μg/mL for 2 h. After this incubation period, propidium iodide was added and incubated for 15 min, and then observed under fluorescence microscopy. Fluorescence would only be seen under the microscope if propidium iodide had entered the spores. Propidium iodide, a DNA-binding fluorochrome, is not able to cross fully functional plasma membranes and so only if the extract had managed to affect the stability of the membrane in some way could fluorescence be seen in cells (Riccardi & Nicoletti, 2006). The extract, as shown in Figure 23**Figure *23.*** Membrane integrity assay with Colletotrichum acutatum spores to understand the mechanism of action of ethanolic extract of magnolia seeds. A suspension of spores was incubated with the extract (2000 μg/mL) and propidium iodide, a DNA-binding fluorochrome which is unable to cross fully functional plasma membranes. The control was prepared with the solvent used in the preparation of the extract, EtOH 80%. Spores were observed under brightfield microscopy (A and D), fluorescence microscopy (B and E). Images C and F result from the superposition of image pairs A-B and D-E. Magnification: 40x, was able to destabilise the membrane by showing an average percentage of fluorescent spores of approximately 64%, whereas the control, showed percentage of fluorescent spores of approximately 12% (Figure 24).

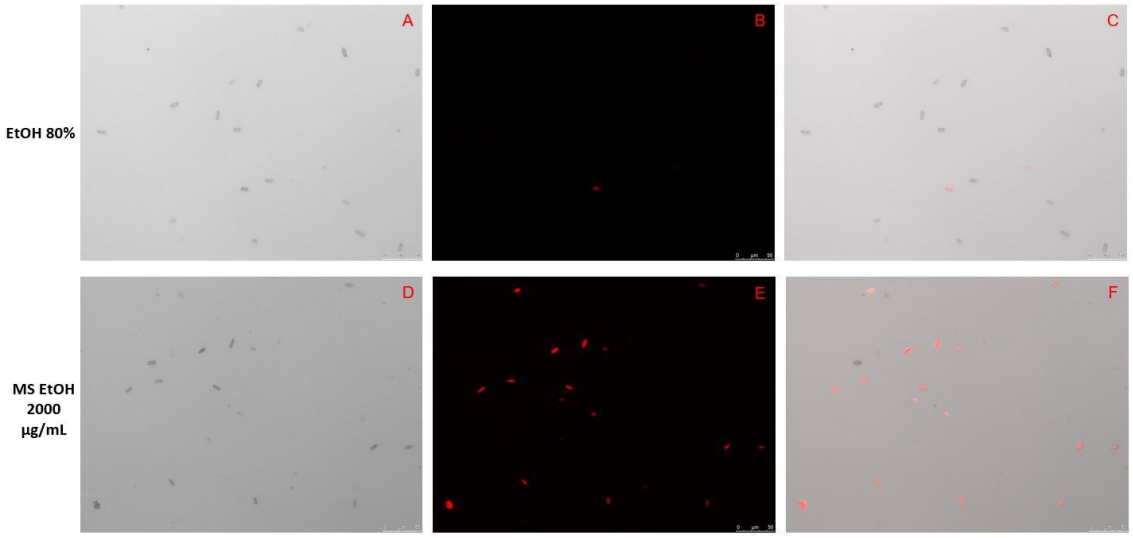


Figure 23. Membrane integrity assay with *Colletotrichum acutatum* spores to understand the mechanism of action of ethanolic extract of magnolia seeds. A suspension of spores was incubated with the extract (2000 µg/mL) and propidium iodide, a DNA-binding fluorochrome which is unable to cross fully functional plasma membranes. The control was prepared with the solvent used in the preparation of the extract, EtOH 80%. Spores were observed under brightfield microscopy (A and D), fluorescence microscopy (B and E). Images C and F result from the superposition of image pairs A-B and D-E. Magnification: 40x

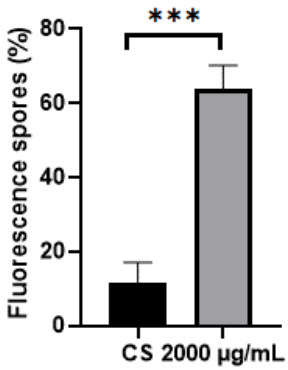


Figure 24. Percentage of fluorescent spores that had been in contact with the extract (2000 µg/mL) and by spores that had been in contact with the solvent (CS). The two conditions were compared and there were significant differences implying that the extract was able to damage the plasma membrane and allow propidium iodide to enter. The percentage of fluorescent spores was calculated after counting the positive spores according to the following formula: (number of fluorescing spores/ total number of spores) *100 where the number of fluorescent spores corresponds to the spores affected by the extract and the total number of spores corresponds to the spores marked and unmarked by propidium iodide. Data presented as the mean of three experiments ± SD. The variance analysis was performed using t-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

These percentage of fluorescent spores values reflect the ability of the extract to affect the stability of the spore membrane. The results obtained are in line with *in vitro* tests with phytopathogenic fungi. The percentage of fluorescent spores can then be associated with the percentage of spores

that were affected. To the best of our knowledge there are no studies addressing the ability of plant extracts of this genus to affect the plasma membrane of spores, but Zhang and his co-workers (2022) carried out a study similar to this one where they tested the effect of chloroxaloterpin, a diterpenoid that can be found in *Streptomyces* spp. bacteria, on *Botrytis cinerea* spores showing that it also had the ability to damage the membrane.

3.5.4. Phytotoxicity evaluation

Before considering formulations of plant extracts with antifungal capacity, we must first assess the phytotoxic potential of extracts at concentrations that show antifungal activity. For this, we tested the phytotoxicity of the ethanolic extract of magnolia seeds on seeds of lettuce (*Lactuca sativa)*, variety "May Queen", using 3 concentrations of extract 500, 1000 and 2000 µg/mL, also preparing the respective solvent controls, where the volume of added extract was replaced by 80% (v/v) ethanol, and the negative control, with only the culture medium. The assay lasted for 6 days and was divided into 2 parts, i) observation phase of percentage of germinated seeds over the 6 days (**Figure 25**) and ii) a destructive phase on the last day of the assay where root length, number of leaves and largest leaf length were measured (Figure 26). Regarding the percentage of germination in the evaluation of the solvent controls it is possible to verify that the increase in the amount of ethanol leads to a decrease in the number of germinated seeds, because ethanol, after a certain volume is an inhibitor of seed germination (Figure 25). In the case of the 500 µg/mL concentration, there are no significant differences between the solvent control (C500) and the negative control (control), which may indicate that the amount of ethanol used does not harm the germination process. When comparing the solvent control (C500) with the treatment (500), there are also no significant differences, which may mean that the extract at these concentrations does not affect the germination process either. At the 1000 µg/ mL concentration there are significant differences on the second day of the assay between the negative control (control) and the solvent control (C1000). These differences fade and are not replicated on days 4 and 6, meaning that ethanol in these quantities was able to delay germination but not prevent it from happening. As for the comparison between the controls and the treatment (1000), there are no significant differences, indicating that this concentration of extract does not harm the percentage of germinated seeds either. At the concentration of 2000 µg/mL there are significant differences between the solvent control (C2000) and the negative control (Control), which indicates that the amount of ethanol

used has toxic effects on the seeds, which leads to a decrease in the percentage of germination of seeds. When comparing the treatment (2000) and the solvent control (C2000) no significant differences are found which suggests that the adverse effects presented by this condition are due to the presence of ethanol and not the toxicity of the compounds present in the extract.

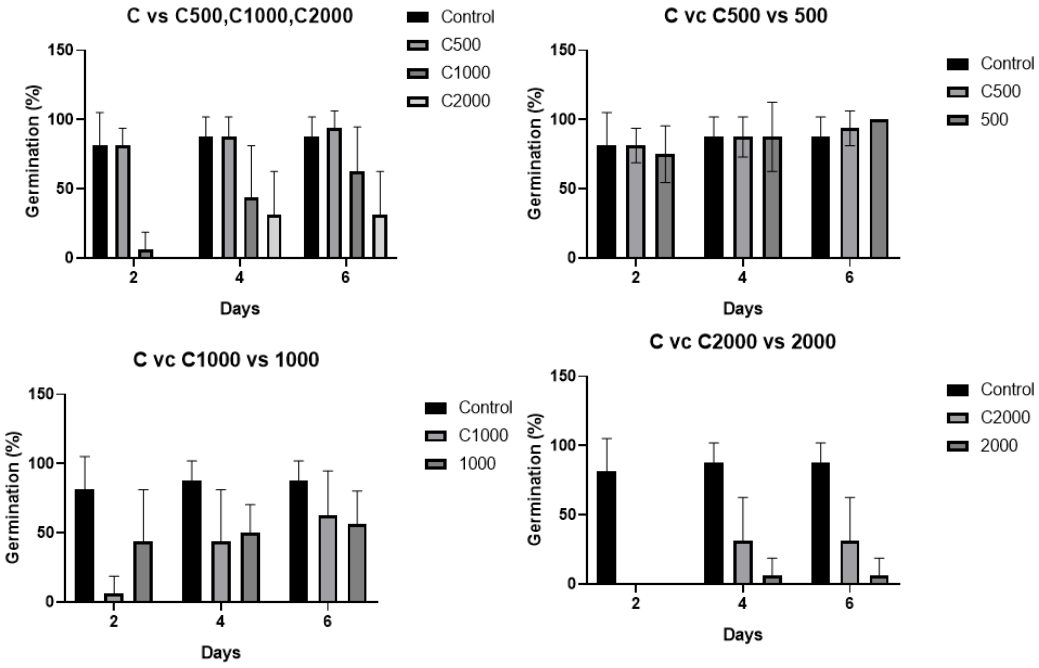


Figure 25. Observation phase of the germination capacity of lettuce (*Lactuca sativa*) seeds. The graph C vs C500 vs C1000 vs C2000 compares the germination capacity of the respective solvent controls for each extract concentration against the negative control. In the remaining graphs the influence of the extracts on seed development is evaluated and compared with the respective controls. Data presented as the mean of three experiments ± SD. The variance analysis was performed using two-way ANOVA and Tukey's test for multiple comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In the destructive phase, the only comparison that did not show significant differences was the negative control (Control) vs. the solvent control (C500), in the evaluation of the "number of leaves" parameter. The extract of 500 µg/mL (500) showed to be able to increase the length of the roots, in a significant way, in relation to both controls it somehow manages to promote the growth of the roots. The rest of the parameters showed no differences in relation to the other conditions, which once again indicates, together with the results of the percentage of germination, that this amount of extract does not present phytotoxicity. The concentration of 1000 µg/mL (1000) also showed to be able to promote the growth of the roots in relation to the controls. In the remaining parameters it did not show significant differences in relation to the solvent control (C1000) and the differences that exist in relation to the negative control (Control) seem to indicate that the possible decrease

in the number of leaves and the length of the largest leaf is due to the presence of ethanol and not to the compounds that are part of the extract. At the concentration of 2000 µg/mL (2000) the amount of ethanol used is highly toxic in the early development of the plant. It drastically decreased the values of root length, number of leaves and length of the largest leaf. Despite this there are no significant differences between the extract (2000) and solvent control (C2000) which suggests that the adverse effects are derived from the excessive use of 80% (v/v) ethanol and not the compounds present in the extract.

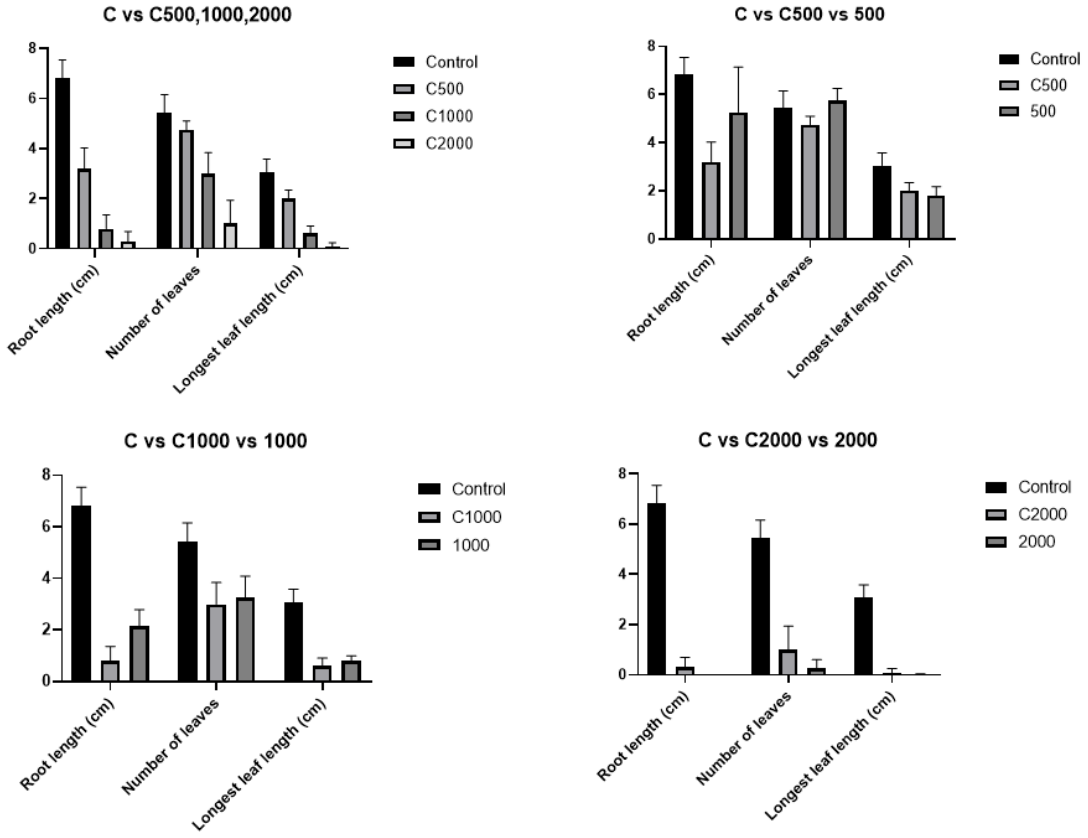


Figure 26. Destructive phase of the assay where the influence of the extracts and their solvent controls on root length, leaf number and largest leaf length were evaluated. In the graph C vs C500 vs C1000 vs C2000 it was evaluated if the solvent controls (C500,C1000 and C2000) would have effects on these parameters. In the remaining graphs, the influence of the extracts at different concentrations compared to the respective controls was tested. Data presented as the mean of three experiments ± SD. The variance analysis was performed using two-way ANOVA and Tukey's test for multiple comparisons: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. FINAL REMARKS AND FUTURE PERSPECTIVES

4.1. Final remarks

Although synthetic fungicides play an important role in agriculture by preventing the spread of disease and consequent loss of many important crops, they carry many associated risks that urgently need to be reduced. For this reason, the need arises to look for safer, more effective, and less dangerous alternatives for the environment and human health. Plants, due to the secondary metabolites present in their constitution, appear as an alternative to this type of products.

Of the extracts tested, the most promising was the ethanolic extract of magnolia seeds. This extract showed antifungal capacity in all tested phytopathogenic fungi: *Diplodia corticola*, *Colletotrichum acutatum* and *Phytophthora cinnamomi*. Later, to understand the mechanism of action of the extract, the results suggested that it acts on the spore membrane (in *C. acutatum*). In order to understand if the extract would have toxic effects on non-target organisms, we tested its phytotoxicity on lettuce seeds (*Lactuca sativa*) and it was possible to show that the adverse effects were due to the excessive presence of ethanol, indicating that in future formulations, the use of ethanol should be reduced, or alternatives found so that it is not so aggressive to the seeds.

The results obtained in this work show that the ethanolic extract of magnolia seeds has the potential to be used in formulations of natural fungicides, fighting against fungi that cause diseases in important species (e.g., *Castanea sativa*, *Quercus suber*, *Fragaria* spp. and *Vitis vinifera*)

4.2. Future perspectives

Applied to all the tested extracts, it would be interesting to test different extraction methods, different solvents, and different harvesting periods, since all these factors influence the compounds present in the extracts.

The magnolia seed extract showed potential to become part of natural fungicide formulations. However, to know which compounds are present in the extract, a chemical analysis is required, which is currently in progress. After knowing the constitution of the extract, it would be important to understand which compounds are responsible for the antifungal activity by testing the pure compounds.

The study of resistance induction in fungi by the extract would be very important to highlight one of the advantages that natural products have over synthetic fungicides. Conducting studies of the

doses necessary for the emergence of resistance, after application of synthetic fungicides and after application of the extract. This would allow to understand if the lower probability for the emergence of resistance actually applies.

Tests addressing the phytotoxicity of the extract, on adult plants, would allow to understand if the field application would affect the crops in a positive or negative way, since the lettuce seeds that were studied in this work are more sensitive than the adult plants.

In the future, *ex situ* assays on strawberry leaves or apples, for example, will play a fundamental role in determining whether the extract can be applied in the field. Extending the action zone of this extract by testing it on more phytopathogenic fungi would also enrich the product.

In the future, if the commercialisation of magnolia seed extract were to be considered, toxicity to non-target organisms, including humans, would need to be tested using animal models.

5. BIBLIOGRAPHY

Abdelgaleil, S. A. M., & Ahmed, S. M. (2005). Antifungal Activity of Extracts and Sesquiterpene Lactones from *Magnolia grandiflora* L . (Magnoliaceae). *International Journal of Agriculture & Biology*, *3*, 638–642. https://www.researchgate.net/publication/242293459%0Asamir1969us@yahoo.com

Aguilar-Marcelino, L., Mendoza-de-Gives, P., Al-Ani, L. K. T., López-Arellano, M. E., Gómez-Rodríguez, O., Villar-Luna, E., & Reyes-Guerrero, D. E. (2020). Using molecular techniques applied to beneficial microorganisms as biotechnological tools for controlling agricultural plant pathogens and pest. *Molecular Aspects of Plant Beneficial Microbes in Agriculture*, 333–349. https://doi.org/10.1016/b978-0-12-818469-1.00027-4

Al-Samarrai, G., Singh, H., & Syarhabil, M. (2012). Evaluating eco-friendly botanicals (natural plant extracts) as alternatives to synthetic fungicides. *Annals of Agricultural and Environmental Medicine*, *19*(4), 673–676.

Ali, A., Tabanca, N., Demirci, B., Raman, V., Budel, J. M., Baser, K. H. C., & Khan, I. A. (2020). Insecticidal and biting deterrent activities of *Magnolia grandiflora* essential oils and selected pure compounds against aedes aegypti. *Molecules*, *25*(6). https://doi.org/10.3390/molecules25061359

Almeida, F., Rodrigues, M. L., & Coelho, C. (2019). *The Still Underestimated Problem of Fungal Diseases Worldwide*. *10*(February), 1–5. https://doi.org/10.3389/fmicb.2019.00214

Amaral, J. S., Casal, S., Pereira, J. A., Seabra, R. M., & Oliveira, B. P. P. (2003). Determination of Sterol and Fatty Acid Compositions, Oxidative Stability, and Nutritional Value of Six Walnut (*Juglans regia* L.) Cultivars Grown in Portugal. *Journal of Agricultural and Food Chemistry*, *51*(26), 7698–7702. https://doi.org/10.1021/jf030451d

APCOR. (2020). *A fileira da cortiça: da floresta ao consumidor*. 1–128. https://www.apcor.pt/wp-content/uploads/2020/12/APCOR_-estudo_caraterizacao_2020.pdf

Baroncelli, R., Talhinhas, P., Pensec, F., Sukno, S. A., Floch, G. Le, & Thon, M. R. (2017). The *Colletotrichum acutatum* species complex as a model system to study evolution and host specialization in plant pathogens. *Frontiers in Microbiology*, *8*(OCT), 1–7. https://doi.org/10.3389/fmicb.2017.02001

Biggs, A. R., & Miller, S. S. (2003). Relative susceptibility of selected apple cultivars to Botryosphaeria dothidea. *HortScience*, *38*(3), 400–403. https://doi.org/10.21273/hortsci.38.3.400

Børve, J., & Stensvand, A. (2006). *Colletotrichum acutatum* overwinters on sweet cherry buds. *Plant Disease*, *90*(11), 1452–1456. https://doi.org/10.1094/PD-90-1452

Boughanmi, M. B., Branco, I., & Choupina, A. (2022). In silico characterization of molecular factors involved in metabolism and pathogenicity of *Phytophthora cinnamomi*. *Molecular Biology Reports*, *49*(2), 1463–1473. https://doi.org/10.1007/s11033-021-06901-0

Buysens, C., Dupré de Boulois, H., & Declerck, S. (2015). Do fungicides used to control Rhizoctonia solani impact the non-target arbuscular mycorrhizal fungus Rhizophagus irregularis? *Mycorrhiza*, *25*(4), 277–288. https://doi.org/10.1007/s00572-014-0610-7

Catanzaro, E., Greco, G., Potenza, L., Calcabrini, C., & Fimognari, C. (2018). Natural products to fight cancer: A focus on *Juglans regia*. *Toxins*, *10*(11). https://doi.org/10.3390/toxins10110469

Chen, Z., Jiao, B., Zhou, J., He, H., & Dai, T. (2022). Rapid detection of *Phytophthora cinnamomi* based on a new target gene Pcinn13739. *Frontiers in Cellular and Infection Microbiology*, *12*(August), 1–15. https://doi.org/10.3389/fcimb.2022.923700

Clark, A. M., El-Feraly, A. S., & Li, W. -S. (1981). Antimicrobial activity of phenolic constituents of *Magnolia grandiflora* L. *Journal of Pharmaceutical Sciences*, *70*(8), 951–952. https://doi.org/10.1002/jps.2600700833

Coque, J. J. R., Álvarez-Pérez, J. M., Cobos, R., González-García, S., Ibáñez, A. M., Diez Galán, A., & Calvo-Peña, C. (2020). Advances in the control of phytopathogenic fungi that infect crops through their root system. *Advances in Applied Microbiology*, *111*, 123–170. https://doi.org/10.1016/bs.aambs.2020.01.003

Damm, U., Cannon, P. F., Woudenberg, J. H. C., & Crous, P. W. (2012). The *Colletotrichum acutatum* species complex. *Studies in Mycology*, *73*, 37–113. https://doi.org/10.3114/sim0010

de Andrade Lourenço, D., Branco, I., & Choupina, A. (2020). Phytopathogenic oomycetes: a review focusing on *Phytophthora cinnamomi* and biotechnological approaches. *Molecular Biology Reports*, *47*(11), 9179–9188. https://doi.org/10.1007/s11033-020-05911-8

de Andrade Lourenço, D., Branco, I., & Choupina, A. (2022). A systematic review about biological control of phytopathogenic *Phytophthora cinnamomi*. *Molecular Biology Reports*, *49*(10), 9947–9962. https://doi.org/10.1007/s11033-022-07547-2

Dias, M. C. (2012). Phytotoxicity: An Overview of the Physiological Responses of Plants Exposed to

Fungicides. *Journal of Botany*, *2012*, 1–4. https://doi.org/10.1155/2012/135479

Ding, L. F., Su, J., Pan, Z. H., Zhang, Z. J., Li, X. N., Song, L. D., Wu, X. De, & Zhao, Q. S. (2018). Cytotoxic sesquiterpenoids from the leaves of *Magnolia grandiflora*. *Phytochemistry*, *155*(March), 182–190. https://doi.org/10.1016/j.phytochem.2018.08.006

Doehlemann, G., Ökmen, B., Zhu, W., & Sharon, A. (2017). Plant pathogenic fungi. *The Fungal Kingdom*, 703–726. https://doi.org/10.1128/9781555819583.ch34

Edwards, Q. A., Sultana, T., Kulikov, S. M., Garner-O'Neale, L. D., & Metcalfe, C. D. (2019). Micropollutants related to human activity in groundwater resources in Barbados, West Indies. *Science of the Total Environment*, *671*, 76–82. https://doi.org/10.1016/j.scitotenv.2019.03.314

Eloff, J. N., Angeh, I., & McGaw, L. J. (2007). A potentised leaf extract of Melianthus comosus has higher activity than six commercial products used against plant fungal pathogens. *South African Journal of Botany*, *73*(2), 285–286. https://doi.org/10.1016/j.sajb.2007.02.040

Engelbrecht, J., Duong, T. A., Prabhu, S. A., Seedat, M., & van den Berg, N. (2021). Genome of the destructive oomycete *Phytophthora cinnamomi* provides insights into its pathogenicity and adaptive potential. *BMC Genomics*, *22*(1), 1–15. https://doi.org/10.1186/s12864-021-07552-y

European Comission. (2021). *List of potential agricultural practices that eco-schemes could support*. *January*, 5. https://ec.europa.eu/info/sites/default/files/food-farming-fisheries/key_policies/documents/factsheet-agri-practices-under-ecoscheme_en.pdf

Félix, C., Pinto, G., Amaral, J., Fernandes, I., Alves, A., & Esteves, A. C. (2017). Strain-related pathogenicity in *Diplodia corticola*. *Forest Pathology*, *47*(6), 1–9. https://doi.org/10.1111/efp.12366

Fenibo, E. O., Ijoma, G. N., & Matambo, T. (2021). Biopesticides in Sustainable Agriculture: A Critical Sustainable Development Driver Governed by Green Chemistry Principles. *Frontiers in Sustainable Food Systems*, *5*(June), 1–6. https://doi.org/10.3389/fsufs.2021.619058

Fernandes, I., Alves, A., Correia, A., Devreese, B., & Esteves, A. C. (2014). Secretome analysis identifies potential virulence factors of *Diplodia corticola*, a fungal pathogen involved in cork oak (Quercus suber) decline. *Fungal Biology*, *118*(5–6), 516–523. https://doi.org/10.1016/j.funbio.2014.04.006

Fernandes, I. O. (2015). *Infection mechanism of Diplodia corticola*. 69–129.

Freeman, S., Horowitz, S., & Sharon, A. (2001). Pathogenic and nonpathogenic lifestyles in *Colletotrichum acutatum* from strawberry and other plants. *Phytopathology*, *91*(10), 986–992. https://doi.org/10.1094/PHYTO.2001.91.10.986

García-Machado, F. J., García-García, A. L., Borges, A. A., & Jiménez-Arias, D. (2022). Root treatment with a vitamin K3 derivative: a promising alternative to synthetic fungicides against Botrytis cinerea in tomato plants. *Pest Management Science*, *78*(3), 974–981. https://doi.org/10.1002/ps.6707

Hardham, A. R. (2005). *Phytophthora cinnamomi*. *Molecular Plant Pathology*, *6*(6), 589–604. https://doi.org/10.1111/j.1364-3703.2005.00308.x

Hardham, A. R., & Blackman, L. M. (2018). *Phytophthora cinnamomi*. *Molecular Plant Pathology*, *19*(2), 260–285. https://doi.org/10.1111/mpp.12568

Hobbelen, P. H. F., Paveley, N. D., & Van Den Bosch, F. (2014). The emergence of resistance to fungicides. *PLoS ONE*, *9*(3), e91910. https://doi.org/10.1371/journal.pone.0091910

Jacobo-Salcedo, M. D. R., Gonzalez-Espindola, L. A., Alonso-Castro, A. J., Gonzalez-Martinez, M. D. R., Domínguez, F., & Garcia-Carranca, A. (2011). Antimicrobial activity and cytotoxic effects of Magnolia dealbata and its active compounds. *Natural Product Communications*, *6*(8), 1121–1124. https://doi.org/10.1177/1934578x1100600818

Jahanban-Esfahlan, A., Ostadrahimi, A., Tabibiazar, M., & Amarowicz, R. (2019). A comprehensive review on the chemical constituents and functional uses of walnut (Juglans spp.) husk. *International Journal of Molecular Sciences*, *20*(16). https://doi.org/10.3390/ijms20163920

Joubert, M., Backer, R., Engelbrecht, J., & Van Den Berg, N. (2021). Expression of several *Phytophthora cinnamomi* putative RxLRs provides evidence for virulence roles in avocado. *PLoS ONE*, *16*(7 July). https://doi.org/10.1371/journal.pone.0254645

Khan, R. A. A., Najeeb, S., Hussain, S., Xie, B., & Li, Y. (2020). Bioactive secondary metabolites from Trichoderma spp. Against phytopathogenic fungi. *Microorganisms*, *8*(6), 1–22. https://doi.org/10.3390/microorganisms8060817

Kocacaliskan, I., Albayrakb, A., & Ilhan, S. (2018). Varietal differences in antimicrobial activities of walnut (*Juglans regia* L.) leaf extracts. *Gaziosmanpasa Journal of Scientific Research*, *7*(3), 173–180.

Koval, D., Plocková, M., Kyselka, J., Skřivan, P., Sluková, M., & Horáčková, Š. (2020). Buckwheat Secondary Metabolites: Potential Antifungal Agents. *Journal of Agricultural and Food Chemistry*, *68*(42), 11631–11643. https://doi.org/10.1021/acs.jafc.0c04538

Lee, Y. D. (2011). Use of Magnolia (*Magnolia grandiflora*) Seeds in Medicine, and Possible Mechanisms of Action. *Nuts and Seeds in Health and Disease Prevention*, 727–732. https://doi.org/10.1016/B978-0-12-375688-6.10086-6

Li, H. M., Zhao, S. R., Huo, Q., Ma, T., Liu, H., Lee, J. K., Hong, Y. S., & Wu, C. Z. (2015). A new dimeric

neolignan from *Magnolia grandiflora* L. seeds. *Archives of Pharmacal Research*, *38*(6), 1066–1071. https://doi.org/10.1007/s12272-014-0476-4

Liao, C. Y., Chen, M. Y., Chen, Y. K., Wang, T. C., Sheu, Z. M., Kuo, K. C., Chang, P. F. L., Chung, K. R., & Lee, M. H. (2012). Characterization of three *Colletotrichum acutatum* isolates from Capsicum spp. *European Journal of Plant Pathology*, *133*(3), 599–608. https://doi.org/10.1007/s10658-011-9935-7

Lim, T. K. (2015). Edible Medicinal and Non Medicinal Plants. In *Edible Medicinal and Non Medicinal Plants* (Vol. 8). https://doi.org/10.1007/978-94-017-9511-1

Linaldeddu, B. T., Franceschini, A., Alves, A., & Phillips, A. J. L. (2013). Diplodia quercivora sp. nov.: A new species of Diplodia found on declining Quercus canariensis trees in Tunisia. *Mycologia*, *105*(5), 1266–1274. https://doi.org/10.3852/12-370

Lo Presti, L., Lanver, D., Schweizer, G., Tanaka, S., Liang, L., Tollot, M., Zuccaro, A., Reissmann, S., & Kahmann, R. (2015). Fungal effectors and plant susceptibility. *Annual Review of Plant Biology*, *66*, 513–545. https://doi.org/10.1146/annurev-arplant-043014-114623

Lovisetto, A., Masiero, S., Rahim, M. A., Mendes, M. A. M., & Casadoro, G. (2015). Fleshy seeds form in the basal Angiosperm *Magnolia grandiflora* and several MADS-box genes are expressed as fleshy seed tissues develop. *Evolution and Development*, *17*(1), 82–91. https://doi.org/10.1111/ede.12106

Lucas, J. A., Hawkins, N. J., & Fraaije, B. A. (2015). The Evolution of Fungicide Resistance. In *Advances in Applied Microbiology* (Vol. 90). Elsevier. https://doi.org/10.1016/bs.aambs.2014.09.001

Luo, X., Zhou, H., Cao, D., Yan, F., Chen, P., Wang, J., Woeste, K., Chen, X., Fei, Z., An, H., Malvolti, M., Ma, K., Liu, C., Ebrahimi, A., Qiao, C., Ye, H., Li, M., Lu, Z., Xu, J., … Zhao, P. (2022). Domestication and selection footprints in Persian walnuts (*Juglans regia*). *PLoS Genetics*, *18*(12), e1010513. https://doi.org/10.1371/journal.pgen.1010513

Mahlo, S. M., Chauke, H. R., McGaw, L., & Eloff, J. (2016). Antioxidant and antifungal activity of selected medicinal plant extracts against phytopathogenic fungi. *African Journal of Traditional, Complementary and Alternative Medicines*, *13*(4), 216–222. https://doi.org/10.21010/ajtcam.v13i4.28

Martínez, M. L., Labuckas, D. O., Lamarque, A. L., & Maestri, D. M. (2010). Walnut (*Juglans regia* L.): Genetic resources, chemistry, by-products. In *Journal of the Science of Food and Agriculture* (Vol. 90, Issue 12, pp. 1959–1967). https://doi.org/10.1002/jsfa.4059

Masi, M., Maddau, L., Linaldeddu, B. T., Cimmino, A., D'Amico, W., Scanu, B., Evidente, M., Tuzi, A., & Evidente, A. (2016). Bioactive Secondary Metabolites Produced by the Oak Pathogen *Diplodia*

corticola. *Journal of Agricultural and Food Chemistry*, *64*(1), 217–225. https://doi.org/10.1021/acs.jafc.5b05170

Miastkowska, M., Michalczyk, A., Figacz, K., & Sikora, E. (2020). Nanoformulations as a modern form of biofungicide. *Journal of Environmental Health Science and Engineering*, *18*(1), 119–128. https://doi.org/10.1007/s40201-020-00445-4

Miyoshi, K., & Sato, T. (1997). The Effects of Ethanol on the Germination of Seeds of Japonica and Indica Rice (Oryza sativa L .) under Anaerobic and Aerobic Conditions. *Annals of Botany*, *79*(1990), 391–395. https://academic.oup.com/aob/article-abstract/79/4/391/2587593

Moreno-González, R., Campillo, J. A., & León, V. M. (2013). Influence of an intensive agricultural drainage basin on the seasonal distribution of organic pollutants in seawater from a Mediterranean coastal lagoon (Mar Menor, SE Spain). *Marine Pollution Bulletin*, *77*(1–2), 400–411. https://doi.org/10.1016/j.marpolbul.2013.09.040

Muñoz-Adalia, E. J., Meijer, A., & Colinas, C. (2022). New qPCR protocol to detect *Diplodia corticola* shows phoretic association with the oak pinhole borer Platypus cylindrus. *Pest Management Science*, *78*(8), 3534–3539. https://doi.org/10.1002/ps.6994

Murashige, T., & Skoog, F. (1962). Murashige1962Revised.Pdf. *Physiologia Plantarum*, *15*, 474–497.

Nazzaro, F., Fratianni, F., Coppola, R., & De Feo, V. (2017). Essential oils and antifungal activity. *Pharmaceuticals*, *10*(4), 1–20. https://doi.org/10.3390/ph10040086

Nekoduka, S., Tanaka, K., & Sano, T. (2018). Epidemiology of apple bitter rot caused by *Colletotrichum acutatum* sensu lato. *Journal of General Plant Pathology*, *84*(4), 262–271. https://doi.org/10.1007/s10327-018-0789-0

Oliveira, I., Sousa, A., Ferreira, I. C. F. R., Bento, A., Estevinho, L., & Pereira, J. A. (2008a). Total phenols, antioxidant potential and antimicrobial activity of walnut (*Juglans regia* L.) green husks. *Food and Chemical Toxicology*, *46*(7), 2326–2331. https://doi.org/10.1016/j.fct.2008.03.017

Oliveira, I., Sousa, A., Ferreira, I. C. F. R., Bento, A., Estevinho, L., & Pereira, J. A. (2008b). *Total phenols , antioxidant potential and antimicrobial activity of walnut (Juglans regia L .) green husks*. *46*, 2326–2331. https://doi.org/10.1016/j.fct.2008.03.017

Ons, L., Bylemans, D., Thevissen, K., & Cammue, B. P. A. (2020). Combining biocontrol agents with chemical fungicides for integrated plant fungal disease control. *Microorganisms*, *8*(12), 1–19. https://doi.org/10.3390/microorganisms8121930

Panth, N., Paudel, K. R., & Karki, R. (2016). Phytochemical profile and biological activity of *Juglans regia*.

Journal of Integrative Medicine, *14*(5), 359–373. https://doi.org/10.1016/S2095-4964(16)60274-1

Pereira, J. A., Oliveira, I., Sousa, A., Valentão, P., Andrade, P. B., Ferreira, I. C. F. R., Ferreres, F., Bento, A., Seabra, R., & Estevinho, L. (2007). Walnut (*Juglans regia* L.) leaves: Phenolic compounds, antibacterial activity and antioxidant potential of different cultivars. *Food and Chemical Toxicology*, *45*(11), 2287–2295. https://doi.org/10.1016/j.fct.2007.06.004

Peres, N. A., Timmer, L. W., Adaskaveg, J. E., & Correll, J. C. (2005). Lifestyles of *Colletotrichum acutatum*. In *Plant Disease* (Vol. 89, Issue 8, pp. 784–796). https://doi.org/10.1094/PD-89-0784

Rauf, A., Olatunde, A., Imran, M., Alhumaydhi, F. A., Aljohani, A. S. M., Khan, S. A., Uddin, M. S., Mitra, S., Emran, T. Bin, Khayrullin, M., Rebezov, M., Kamal, M. A., & Shariati, M. A. (2021). Honokiol: A review of its pharmacological potential and therapeutic insights. *Phytomedicine*, *90*(July), 153647. https://doi.org/10.1016/j.phymed.2021.153647

Ray, M., Ray, A., Dash, S., Mishra, A., Achary, K. G., Nayak, S., & Singh, S. (2017). Fungal disease detection in plants: Traditional assays, novel diagnostic techniques and biosensors. In *Biosensors and Bioelectronics* (Vol. 87, pp. 708–723). Elsevier Ltd. https://doi.org/10.1016/j.bios.2016.09.032

Ribera, A. E., & Zuñiga, G. (2012). Induced plant secondary metabolites for phytopatogenic fungi control: a review. *Journal of Soil Science and Plant Nutrition*, *12*(4), 893–911.

Riccardi, C., & Nicoletti, I. (2006). Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nature Protocols*, *1*(3), 1458–1461. https://doi.org/10.1038/nprot.2006.238

Sharma, A., Kumar, V., Shahzad, B., Tanveer, M., Sidhu, G. P. S., Handa, N., Kohli, S. K., Yadav, P., Bali, A. S., Parihar, R. D., Dar, O. I., Singh, K., Jasrotia, S., Bakshi, P., Ramakrishnan, M., Kumar, S., Bhardwaj, R., & Thukral, A. K. (2019). Worldwide pesticide usage and its impacts on ecosystem. *SN Applied Sciences*, *1*(11). https://doi.org/10.1007/s42452-019-1485-1

Shuping, D. S. S., & Eloff, J. N. (2017). the Use of Plants To Protect Plants and Food Against Fungal Pathogens: a Review. *African Journal of Traditional, Complementary, and Alternative Medicines : AJTCAM*, *14*(4), 120–127. https://doi.org/10.21010/ajtcam.v14i4.14

Simmonds, J. H. (1966). A study of the species of Colletotrichum causing ripe fruit rots in Queensland. *Queensland Journal of Agricultural and Animal Sciences*, *22*(4), 437–459.

Singh, P. K., Singh, R. P., Singh, P., & Singh, R. L. (2019). Food hazards: Physical, chemical, and biological. In *Food Safety and Human Health*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-816333-7.00002-3

Soto-Maldonado, C., Vergara-Castro, M., Jara-Quezada, J., Caballero-Valdés, E., Müller-Pavez, A., Zúñiga-Hansen, M. E., & Altamirano, C. (2019). Polyphenolic extracts of walnut (*Juglans regia*) green husk containing juglone inhibit the growth of HL-60 cells and induce apoptosis. *Electronic Journal of Biotechnology*, *39*, 1–7. https://doi.org/10.1016/j.ejbt.2019.02.001

Sreenivasaprasad, S., & Talhinhas, P. (2005). Genotypic and phenotypic diversity in *Colletotrichum acutatum*, a cosmopolitan pathogen causing anthracnose on a wide range of hosts. *Molecular Plant Pathology*, *6*(4), 361–378. https://doi.org/10.1111/j.1364-3703.2005.00291.x

Tomas-Grau, R. H., Di Peto, P., Chalfoun, N. R., Grellet-Bournonville, C. F., Martos, G. G., Debes, M., Arias, M. E., & Díaz-Ricci, J. C. (2019). *Colletotrichum acutatum* M11 can suppress the defence response in strawberry plants. *Planta*, *250*(4), 1131–1145. https://doi.org/10.1007/s00425-019-03203-5

Tripathi, P., & Dubey, N. K. (2004). Exploitation of natural products as an alternative strategy to control postharvest fungal rotting of fruit and vegetables. *Postharvest Biology and Technology*, *32*(3), 235–245. https://doi.org/10.1016/j.postharvbio.2003.11.005

Wianowska, D., Garbaczewska, S., Cieniecka-Roslonkiewicz, A., Dawidowicz, A. L., & Jankowska, A. (2016). Comparison of antifungal activity of extracts from different *Juglans regia* cultivars and juglone. *Microbial Pathogenesis*, *100*, 263–267. https://doi.org/10.1016/j.micpath.2016.10.009

Wianowska, Dorota, Garbaczewska, S., Cieniecka-Roslonkiewicz, A., Dawidowicz, A. L., Typek, R., & Kielczewska, A. (2020). Influence of the Extraction Conditions on the Antifungal Properties of Walnut Green Husk Isolates. *Analytical Letters*, *53*(12), 1970–1981. https://doi.org/10.1080/00032719.2020.1725889

Yan, L., Zhu, J., Zhao, X., Shi, J., Jiang, C., & Shao, D. (2019). Beneficial effects of endophytic fungi colonization on plants. *Applied Microbiology and Biotechnology*, *103*(8), 3327–3340. https://doi.org/10.1007/s00253-019-09713-2

Yan, Y. F., Yang, C. J., Shang, X. F., Zhao, Z. M., Liu, Y. Q., Zhou, R., Liu, H., Wu, T. L., Zhao, W. Bin, Wang, Y. L., Hu, G. F., Qin, F., He, Y. H., Li, H. X., & Du, S. S. (2020). Bioassay-guided isolation of two antifungal compounds from Magnolia officinalis, and the mechanism of action of honokiol. *Pesticide Biochemistry and Physiology*, *170*(September), 104705. https://doi.org/10.1016/j.pestbp.2020.104705

Yoon, M. Y., Cha, B., & Kim, J. C. (2013). Recent trends in studies on botanical fungicides in agriculture. *Plant Pathology Journal*, *29*(1), 1–9. https://doi.org/10.5423/PPJ.RW.05.2012.0072

Zhang, L., Wang, X., Bi, Y., & Yu, Z. (2022). Semi-Synthesis of Chloroxaloterpin A and B and Their Antifungal Activity against Botrytis cinerea. *Journal of Agricultural and Food Chemistry*, *70*(23), 7070–7076.

https://doi.org/10.1021/acs.jafc.2c01242

Annexes

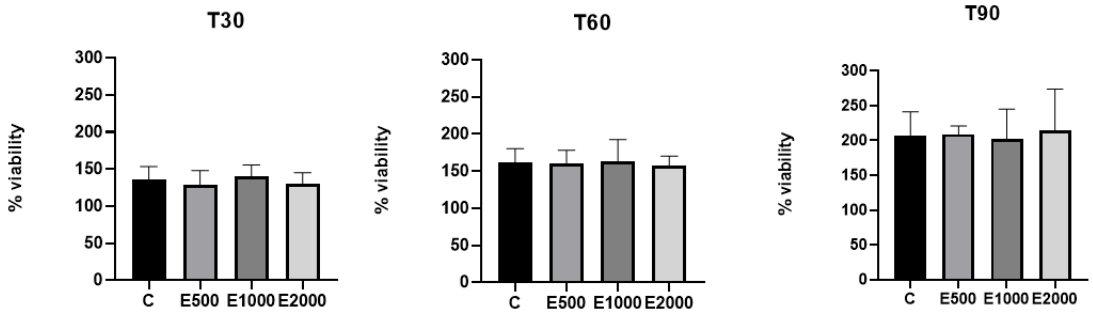


Figure A1. Statistical analysis of the data from

Figure *1* after 30 (T30), 60 (T60) and 90 (T90) min of incubation with the walnut leaves aqueous extract. A one-way ANOVA and Tukey test for multiple comparisons was used and the *p*-value shows the significance of each sample tested. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

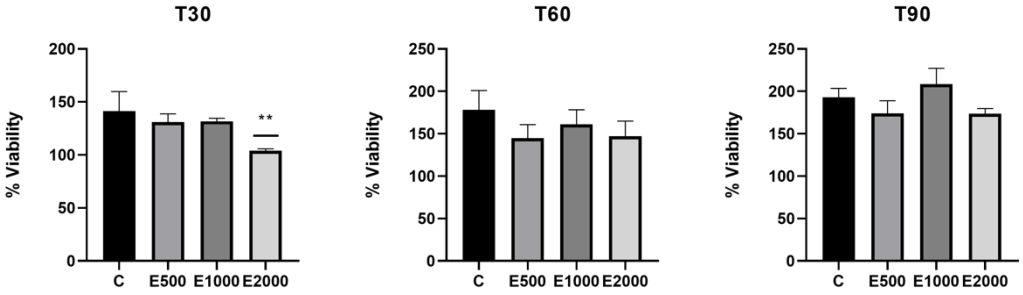


Figure A2. Statistical analysis of the data from **Figure 4** after 30 (T30), 60 (T60) and 90 (T90) min of incubation with the walnut husk aqueous extract. A one-way ANOVA and Dunnett's test for multiple comparisons was used and the *p*-value shows the significance of each sample tested. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

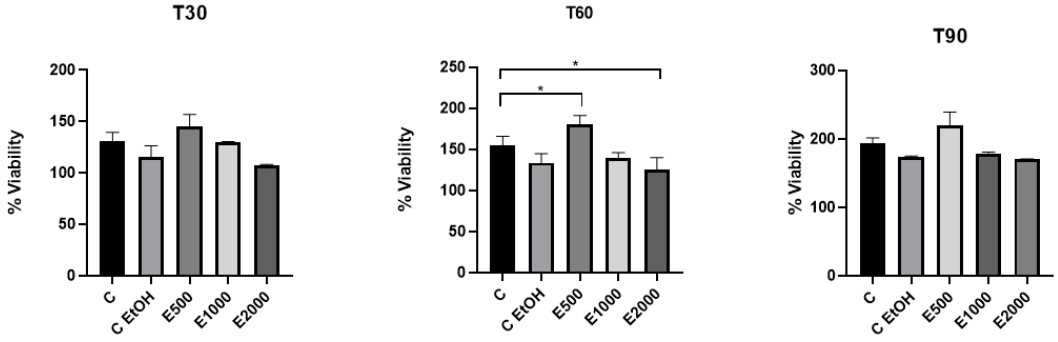


Figure A3. Statistical analysis of the data from **Figure 9** comparison between negative control and the different conditions after 30 (T30), 60 (T60) and 90 (T90) min of incubation with the walnut husk ethanolic extract (EtOH 100%). A one-way ANOVA and Dunnett's test for multiple comparisons was used and the p-value shows the significance of each sample tested. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

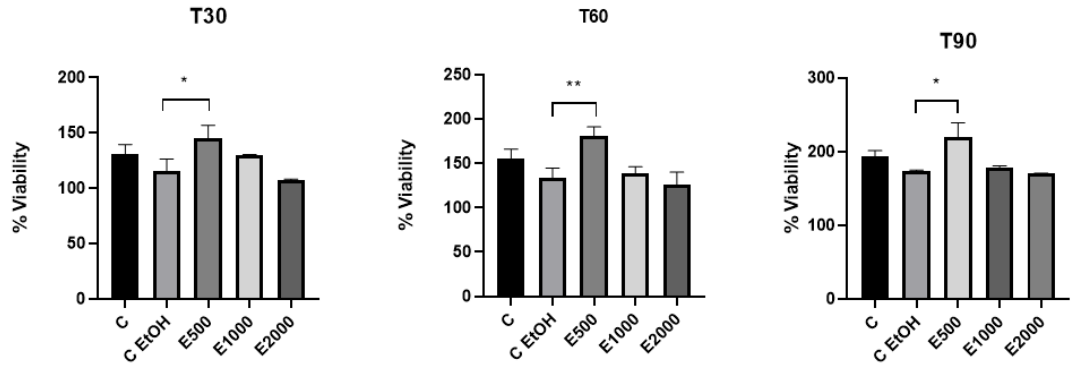


Figure A4. Statistical analysis of the data from **Figure 9**, comparison between solvent control (EtOH 100%) and the different conditions after 30 (T30), 60 (T60) and 90 (T90) min of incubation with the walnut husk ethanolic extract (EtOH 100%). A one-way ANOVA and Dunnett's test for multiple comparisons was used and the p-value shows the significance of each sample tested. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

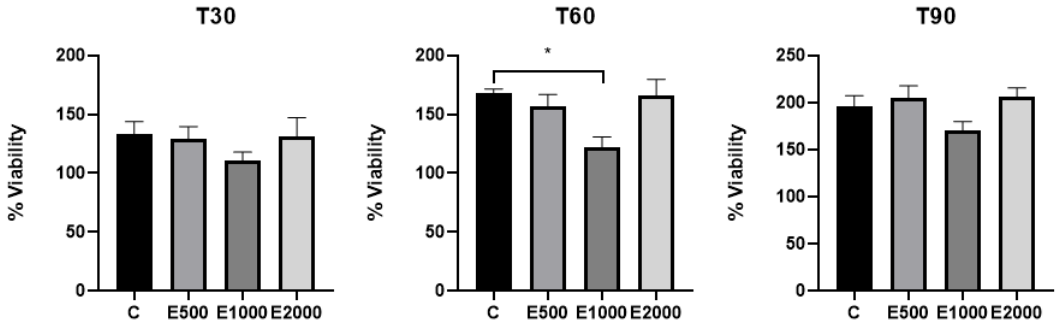


Figure A5. Statistical analysis of the data from **Figure 10Figure 9**., comparison between control (deionized sterile water) and the different conditions after 30 (T30), 60 (T60) and 90 (T90) min of incubation with the walnut husk ethanolic extract (EtOH 50 %. resuspended in water). A one-way ANOVA and Dunnett's test for multiple comparisons was used and the p-value shows the significance of each sample tested. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

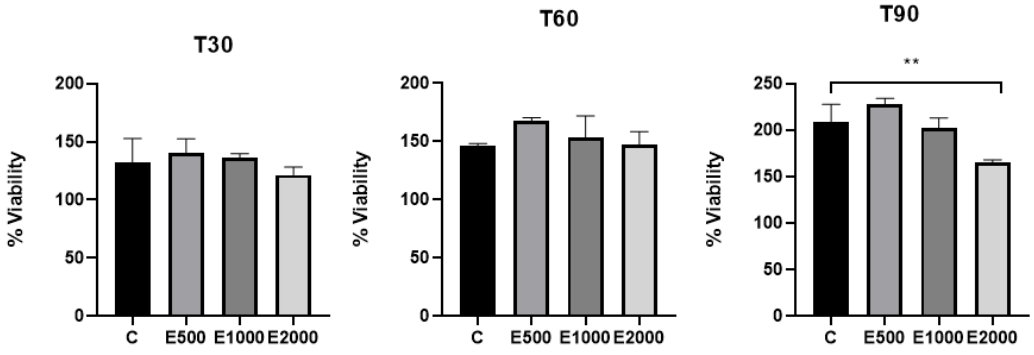


Figure A6. Statistical analysis of the data from **Figure 13** after 30 (T30), 60 (T60) and 90 (T90) min of incubation with the magnolia seeds aqueous extract. A one-way ANOVA and Tukey test for multiple comparisons was used and the p-value shows the significance of each sample tested. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

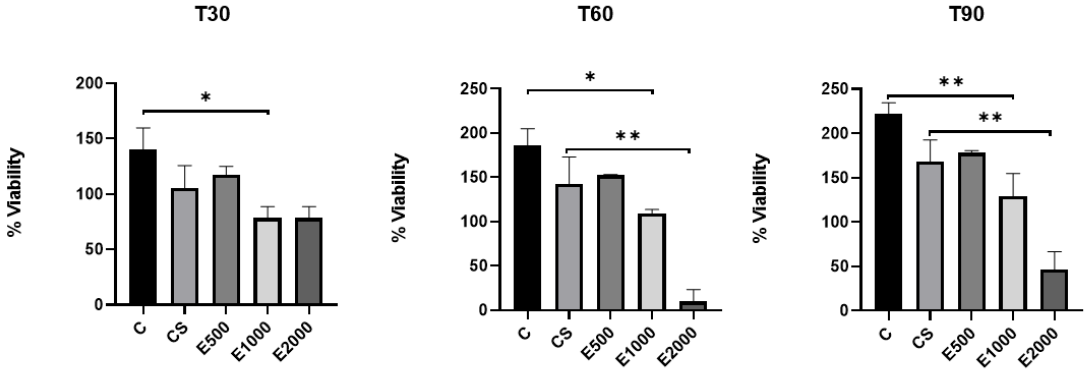


Figure A7. Statistical analysis of the data from **Figure 16** after 30 (T30), 60 (T60) and 90 (T90) min of incubation with the magnolia seeds ethanolic extract. A one-way ANOVA and Tukey test for multiple comparisons was used and the p-value shows the significance of each sample tested. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

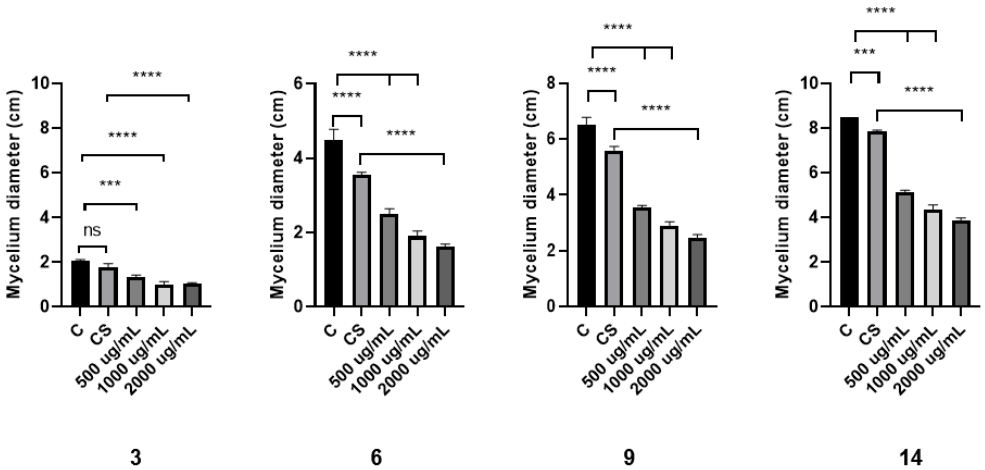


Figure A8. Statistical analysis of the data from **Figure 21** after 3,6,9 and 14 days of inoculation of the fungus with the extract. In solvent control, the maximum amount of extract, used at the concentration of 2000 µg/mL was replaced by solvent (EtOH 80%). Therefore, this condition can only be compared with the highest concentration. The remaining concentrations, in this case, were compared with the negative control. On day 3 the negative control and the solvent control showed no significant differences (ns), however during the rest of the assay the differences were marked (p<0.0001). All treatments showed significant differences when compared to the respective controls. A one-way ANOVA and Tukey test for multiple comparisons was used and the p-value shows the significance of each sample tested. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.