Testing the potential antifungal activity of *Origanum vulgare* against *Aspergillus fumigatus, Aspergillus niger* and *Talaromyces marneffei* isolated from pets

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Abstract

Origanum vulgare (oregano) is an aromatic herb commonly used in the Mediterranean, widely known for its medicinal properties. In this experiment, we tested its antifungal properties against two species of *Aspergillus (A. niger and A. fumigatus)* and the emerging *Talaromyces marneffei.* We tested the plant's antifungal activity in a range of concentrations (5, 10, 20 and 30 mg/mL) in which the plant was dissolved and mixed with growth medium (PDA) and then inoculated with the fungi. The percentage of inhibition was measured over 7 days with data being collected on the 3rd, 5th and 7th days. Fungal activity inhibition ranging from 30 to 100% was achieved against *Aspergillus niger* and *Aspergillus fumigatus* and from 50 to 100% against *Talaromyces marneffei.* This multi-disciplinary study brought the use of plants into the veterinary sciences by using samples isolated from pets, with the aim of researching potential alternatives to traditional antifungal treatments and the ultimate goal of increasing animal wellbeing.

Key words: *microbiology; Origanum vulgare; Aspergillus fumigatus; Aspergillus niger; Talaromyces marneffei*

Introduction

The first time that the term *Aspergillus* was used to refer to this group of fungi was around 300 years ago by a priest and botanist named Antonio Micheli. He chose this name due to the morphological similarities of the fungus to the *aspergillum*, an instrument used to disperse holy water during certain religious celebrations. This name represents the asexual phase of the fungus when it most resembles the aforementioned *aspergillum* (Gibbons et al., 2013).

Mostly saprophytic fungi, they are commonly present in soil and other organic and inorganic substrates. The asexual spores known as conidia are hydrophobic

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and usually airborne. Usually very resilient, they have the ability to germinate in a myriad of conditions. They are thermotolerant, meaning they can survive in a wide range of temperatures, ranging from 12ºC to 50ºC, making them greatly able to infect vertebrates in an opportunistic way (Foley et al., 2014; Bassetti et al., 2015).

The *Aspergillus* genus is frequently associated with *hyalohyphomicosis*, this referring to term used to describe infections caused by fungi of hylane sptate branched hyphae that are colourless (without pigment), were this condition normally affects pets and humans (Galimberti et al., 2012).

Aspergillus are known for their ability to produce a variety of secondary metabolites, including mycotoxins, which can have harmful effects on humans and animals. Some species of *Aspergillus* are also important opportunistic pathogens that can cause infections in immunocompromised individuals. The rate of infections by fungi of the *Aspergillus* genus is increasing, mostly due to the introduction of drugs and therapies with an immunosuppressive effect. These are considered nosocomial diseases that have the potential to cause serious fungal infections and have the potential to be fatal, depending on the immune status of the host (Dagenais et al., 2009).

Aspergillus fumigatus is considered the most pathogenic species of the *Aspergillus* genus, and it is able to grow at temperatures between 15°C and 55°C (thermotolerant). It has an simple life cycle, and one of the main features is its enormous capacity for sporulation, resulting in the ubiquitous presence of high spore concentrations in the environment (Latgé et al., 2001; Nierman et al., 2005).

They form flat and compact colonies with a rapid growth rate. The macroscopic appearance is white in the early stages of development, proceeding to turn a bluish-green colour with a velvety texture. The surface possesses a few kinks and white tufts and acquires a yellowish tint as it develops into further stages. Its ideal growth temperature hovers around 37°C (Latgé et al., 2009).

Aspergillus fumigatus is an opportunistic pathogen and a major allergen. Its conidia production is prolific, and so human respiratory tract exposure is almost constant. In immunocompromised individuals, the incidence of invasive infection can be as high as 50% and the mortality rate is often about 50% (Nierman et al., 2005).

In late 2022, the World Health organization released a list entitled "WHO fungal priority pathogens list to guide research, development and public health action". The criteria used to define the groups were: deaths, annual incidence, current global distribution, trends in the last 10 years, impatient care, complications and sequelae, antifungal resistance, presentability, access to diagnostic test and evidence-based treatments. According, *Aspergillus fumigatus* was placed in the "critical group" for research, as the third most important overall on the list (WHO, 2022).

Aspergillus niger is an opportunistic filamentous fungus that has the ability to fully develop colonies in less than seven days. Macroscopically, the initial colonies appear white or yellow before turning black (*niger*). *Aspergillus niger* is a secondary etiologic agent of bacterial ear infections it is also common in lung diseases in immunocompromised patients. It is associated with otomycosis, invasive pulmonary *aspergillosis* and pulmonary *aspergilloma* among others. It is one of most widely used microorganisms in biotechnology, generally used in the production of enzymes and citric acid, making it a valuable asset for biotechnology (Schuster et

al., 2002; Krull et al., 2010; Chao-Lan et al., 2011; Bassetti et al., 2015; King et al., 2016).

One of the most well-known fungi genera, *Aspergillus* contains roughly 345 species which are prevalent in a plethora of environments, from the soil to the air, indoors and even in certain food products (e.g., cheeses like Camembert and Roquefort) (Galimberti et al., 2012; Visagie et al., 2014).

It has a global presence and contaminates a series of substrates, and is able to produce mycotoxins. Its main role in nature is the decomposition of organic materials, making its presence unwelcome or even devastating in certain situations, such as in the harvesting process. They are considered food crop pathogens, leading to the loss of huge crops and resulting in monetary and agricultural impoverishment (Zhang et al., 2006).

There are certain pathogenic species for humans and animals in this genus. Opportunistic infections caused by species of this genus may lead to death, depending on the host immune state (Samson et al., 2011).

Talaromyces marneffei was formerly known as *Penicillium marneffei,* and was recently transferred from the genus *Penicillium* to *Talaromyces* based on phylogenetic analysis. However, the name of the disease caused by this fungus still sometimes appears as *penicilliosis*, and the fungus may appear as *Penicillium marneffei* or *Talaromyces marneffei* (Samson et el., 2011; Yilmaz et al., 2014).

Talaromyces marneffei is a dimorphic pathogen in which temperature influences its growth. At temperatures near 25ºC, it grows as a mycelium that presents vegetative conidia in the saprophytic form. If these are inhaled by animals with internal temperatures near 37ºC (e.g., humans and warm-blooded animals), this fungus undergoes a dimorphic switch to produce yeast cells that represent the parasitic stage. Pathogenicity is associated with this dimorphic switch, though the mechanisms involved in this process are still unknown (Liu et al., 2007; Galimberti et al., 2012; Chang et al., 2015).

Talaromyces marneffei is a pathogenic fungus thought to be mostly prevalent in China and Southeast Asia. This fungus can cause fatal systemic mycoses in both immunocompetent and immunocompromised patients, with or without HIV infection. The *penicilliosis* pathology in different organs varies depending on host immunity, while early diagnosis and prompt initiation of treatment are crucial for patient survival (Samson et al., 2011; Tam et al., 2015; Qiu et al., 2015).

Due to human migration and travel, this fungal infection has been diagnosed and become more prevalent in individuals in Europe. In the "WHO fungal priority pathogens list to guide research, development and public health action", the World health Organization ranks this fungus within the "medium" priority group (Galimberti et al., 2012; WHO 2022).

In general, the study of these fungi is imperative, as they may have malignant effects in humans, other mammals and avians (Krautwald-Junghanns et al., 2015).

Presently, there is a series of antifungal drugs used in human/animal medicine, where the most common are those of the allylamine groups (Antimetabolite, Imidazoles, Triazoles, Echinocandins and Polyenes to name a few). Their pathway of application is oral, topical or intravenous (Bassetti et al., 2015; Murray et al., 2016).

In this day and age, there is increasing research in new antifungal solutions, since there is evidences of fungi acquiring resistance to drugs that are currently in use (e.g. *Aspergillus* spp.). Meanwhile, some antifungals currently used are highly hepatotoxic, leading to side effects such as

nausea, vomiting, diarrhoea, and ulcers in different parts of the digestive tract, which is further driving new areas of research (Linden et al., 2013; Seyedmousavi et al., 2014; Groll et al., 2014; Bassetti et al., 2015; Kumari et al., 2015).

The complexity associated with fungal infection is in the interaction between the fungi and the host and furthermore with the antifungal agents, making antifungal agents prone to fail. The immune status of the host, infection site and severity are the most prevalent factors when it comes to the success/fail ratio of antifungal agents. Therefore, the study of multifaceted antifungal agents is imperative (Muray et al., 2016).

The use of plants in antifungal research seems promising since plants are capable of producing different bioactive compounds to defend themselves from potentially detrimental microorganisms present in the environment (Zhang et al., 2006; Castillo et al., 2012; Thippeswamy et al., 2014; Kumari et al., 2015).

Certain secondary metabolites produced by the plants possess antifungal activity, in which they can inhibit or even stop fungal proliferation, this is achieved through the inhibition of mycelium growth, inhibition of germination and the reduction of sporulation of the pathogens (Castillo et al., 2012; Đorđević et al., 2014).

Origanum vulgare, commonly known as oregano, is an herb known worldwide for its flavouring properties. It has also been used in traditional medicine in a variety of cultures, due to its health benefits. It is most commonly used to aid in respiratory tract maladies, a plethora of digestive issues, the treatment of colds, and as an antiseptic. The medically active components are found in the aboveground parts of the plant. These include phenolic glycosides, sterols, flavonoids, and terpenoids, though the concentration of each component can differ in the same species as a function of harvest time, plant maturity, and geographical location (Pezzani et al., 2017).

Material and methods

Plant material

For the *preparation* of plant extracts used in this experiment we applied two different approaches, dried *Origanum Vulgare* was used in two rounds of testing and fresh plants were purchased and dehydrated for the third testing round. All plant material used in this experiment was certified organic. The plant material was weighed and mixed with hot water to obtain the following final concentrations, 5 mg/mL, 10 mg/mL, 20 mg/mL and 30 mg /mL (5, 10, 20, 30). This procedure was performed in triplicate for each fungal species.

Fungal material

Fungal isolates were obtained from the collection of pathogenic fungi maintained in the University of Trás-os-Montes and Alto Douro, Vila Real, Portugal that were originally isolated from the skin of pets. The fungi used in the assays were *Talaromyces* (*Penicillium*) *marneffei, Aspergillus fumigatus* and *Aspergillus niger*. The mould was grown on potato dextrose agar medium (PDA).

Plate preparation

The medium used in this assay was PDA at a concentration of 39 mg/mL. The medium was prepared and autoclaved in conjunction to the flacons containing the aqueous plant extracts.

The medium was plated using a 6 mL PDA to 4 mL aqueous extract ratio. Each of the assays also contained two control plates for each of the fungi, one containing only the PDA (10 mL) and

the second containing PDA and water in the same ratio as the test groups. After solidification, a mycelial disk of 4 mm diameter of each of the corresponding fungi, taken from 24-hour old fungal subculture, was placed at the centre of the plates. After incubation, the plates were kept in darkness at around 25ºC, and fungal growth was analysed on the third, fifth and seventh day after inoculation. The average between the largest diameter of the fungal disk and its perpendicular measurement were used to survey the rate of fungal development. Growth was compared using the following formula: [(C -T) x 100/C] in which T stands for the average growth on the treatment plate and C for the average growth on the control plus water plates. Statistical analysis was performed using the SPSS® program (Statistical Package for the Social Sciences, version 22.0). One-way ANOVA analysis of variance followed by Tukey's test with a significance of $P<0.05$ was used to detect differences in the percent inhibition between the mean aqueous extract concentrations.

Results

The effect of the four different concentrations (5, 10, 20 and 30 mg/mL) of *Origanum vulgare* aqueous plant extracts was tested against *A. niger, A. fumigatus* and *T. marneffei* in in triplicate. The antifungal activity was assayed using the above formula and the data are presented in Tables 1 to 3.

Assay 1

During the first assay, we achieved an average of 79% inhibition across all days and all concentrations against *Aspergillus niger*, (F=6.629; P=0.015). Notably, we achieved a 100% inhibition rate for the concentration 30mg/mL across all days, and we also achieved 100% inhibition rates on the third day with 10mg/mL, and on the third and fifth days with 20 mg/mL (Figure 1).

For *Aspergillus fumigatus,* we achieved an average of 75% inhibition across all days/concentrations (F=42.431; P=0.000) and most notably, we achieved a 100% inhibition rate with 20 and 30 mg/mL for each day of the assay (Figure 2).

For *Talaromyces marneffei*, we achieved an average of 96% inhibition across all concentrations/days (F=1; P=0.442). In this assay, we achieved 100% rates of inhibition across all the days and concentrations, with the exception of the 7th day with the 5 mg/mL concentration, in which the inhibition rate dropped to 47% (Figure 3).

Assay 2

In the second assay, we observed promising inhibitory effects against *Aspergillus niger*, achieving an average inhibition rate

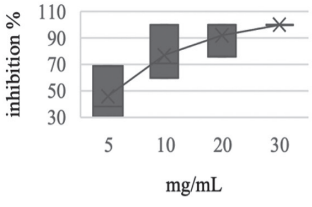

Figure 1. Boxplot of inhibition % by aqueous extracts of *Origanum vulgare* on *Aspergillus niger*

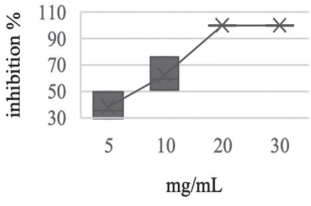

Figure 2. Boxplot of inhibition % by aqueous extracts of *Origanum vulgare* on *Aspergillus fumigatus*

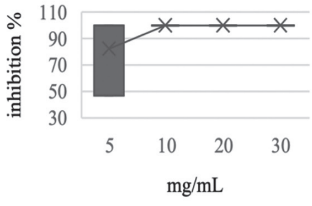

Figure 3. Boxplot of inhibition % by aqueous extracts of *Origanum vulgare* on *Talaromyces marneffei*

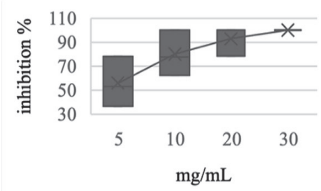

Figure 4. Boxplot of inhibion % by aqueous extracts of *Origanum vulgare* on *Aspergillus niger*

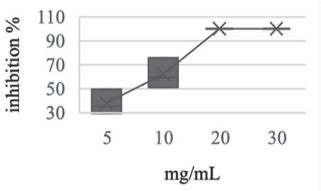

Figure 5. Boxplot of inhibion % by aqueous extracts of *Origanum vulgare* on *Aspergillus fumigatus*

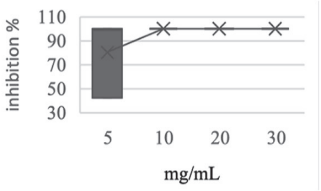

Figure 6. Boxplot of inhibion % by aqueous extracts of *Origanum vulgare* on *Talaromyces marneffei*

of 66% across all days and concentrations (F=42.428; *P*=0.000). Particularly noteworthy was the 100% inhibition rate observed for the concentrations of 20 and 30 mg/mL throughout the assay (Figure 4).

As for *Aspergillus fumigatus*, we obtained an average inhibition rate of 74% across all days and concentrations (F=31.583; *P*=0.000). Remarkably, a 100% inhibition rate was consistently achieved at 20 and 30 mg/mL on each day of the assay (Figure 5).

As for *Aspergillus fumigatus*, we obtained an average inhibition rate of 74% across all days and concentrations (F=31.583; *P*=0.000). Remarkably, a 100% inhibition rate was consistently achieved at 20 mg/mL and 30 mg/mL on each day of the assay (Figure 5).

Regarding *Talaromyces marneffei*, we observed an impressive average inhibition rate of 96% across all concentrations and days (F=1; *P*=0.437). Throughout this assay, we consistently achieved 100% inhibition rates for all days and concentrations, except on the 5th and 7th day with the 5 mg/mL concentration, which resulted in a drop to 99.8% and 47% inhibition rate, respectively (Figure 6).

Assay 3

In the third assay, we observed the best inhibitory effects against *Aspergillus niger*, with an average inhibition rate of 82% across all days and concentrations (*P*=0.035). Particularly remarkable was the consistent 100% inhibition rate observed at the 30 mg/mL concentration throughout the entire assay and at 20 mg/mL on the third and fifth day (Figure 7).

As for *Aspergillus fumigatus*, we obtained an average inhibition rate of 75% across all days and concentrations (F=42.5431; *P*=0.000). Notably, a consistent

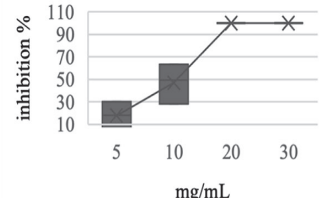

Figure 7. Boxplot of inhibion % by aqueous extracts of *Origanum vulgare* on *Aspergillus niger*

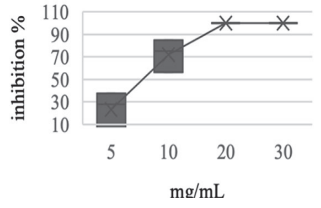

Figure 8. Boxplot of inhibion % by aqueous extracts of *Origanum vulgare* on *Aspergillus fumigatus*

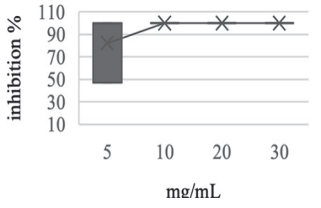

Figure 9. Boxplot of inhibion % by aqueous extracts of *Origanum vulgare* on *Talaromyces marneffei*

100% inhibition rate was achieved at both 20 and 30 mg/mL on each day of the assay (Figure 8).

Regarding *Talaromyces marneffei*, we observed an impressive average inhibition rate of 96% across all concentrations and days ($F=1$; $P=0.441$). Throughout this assay, we consistently achieved 100% inhibition rates for all days and concentrations, except on the 5th and 7th day when using the 5 mg/mL concentration, resulting in inhibition rates of 98.8% and 42%, respectively (Figure 9).

Discussion

In this study, we gathered data about the antifungal activity of *Origanum vulgare* to provide insight into its potential use as a natural antifungal agent. The three assays performed showed that *Origanum vulgare* had significant inhibitory action on the growth rates of all tested fungal species, furthermore enhancing the importance of this herb as a promising antifungal agent.

The observed inhibitory effects of oregano against *Aspergillus niger*, *Aspergillus fumigatus*, and *Talaromyces marneffei* are consistent with previous research on the antimicrobial properties of this herb. The main bioactive compounds in oregano, such as carvacrol and thymol, are believed to be responsible for its antifungal activity. These compounds have been shown to disrupt the fungal cell membrane, interfere with ergosterol biosynthesis (an essential component of fungal cell membranes), and induce oxidative stress, ultimately leading to fungal cell death (Carmo et al., 2008; Mota et al., 2012).

The significant and consistent inhibition of the *Aspergillus* genus in all three assays is particularly noteworthy. *Aspergillus* species are known to cause infections in humans, and the emergence of antifungal resistance is a challenge that has become more and more emergent. The ability of oregano to achieve 100% inhibition rates at certain concentrations and sustain substantial inhibition rates across all days of the assays suggests its potential as an effective antifungal agent against these clinically relevant fungi (Krishnan et al., 2009).

Furthermore, the remarkable inhibitory effects against *Talaromyces marneffei* are of particular interest. *Talaromyces marneffei* is an opportunistic pathogen that can cause severe infections, especially in immunocompromised individuals. The high inhibition rate across all concentrations and days highlights the potency of *Origanum vulgare*, as a potential treatment option for *T. marneffei* infections (Limper et al., 2017).

The results also point to a concentration-dependent relationship between oregano's antifungal activity and the fungal pathogens tested. Higher concentrations of oregano resulted in more pronounced inhibition rates, with 100% inhibition achieved at 20 mg/mL and 30 mg/mL concentrations for both *Aspergillus* species in all assays. This concentration-dependent effect suggests that the potency of oregano as an antifungal agent that could be optimised through proper dosage.

While the results are promising, it is essential to acknowledge the limitations of this study. The research was conducted *in vitro*, and further studies are needed to validate these findings in vivo using animal models and, eventually, in human clinical trials. Additionally, more comprehensive investigations are required to elucidate the precise mechanisms of *Origanum vulgare* antifungal activity and explore potential coordination with existing antifungal agents.

In conclusion, the research on the antifungal activity of oregano presents compelling evidence of its potential as a nat-

ural alternative for combating fungal infections caused by *Aspergillus niger*, *Aspergillus fumigatus*, and *Talaromyces marneffei*. The consistent inhibitory effects demonstrated in the three assays, along with the concentration-dependent relationship, suggest that oregano's bioactive compounds play a pivotal role in its antifungal activity. These findings open new avenues for further research and hold promise for the development of new, effective, and safer antifungal therapies with the final goal of improving animal wellbeing.

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Ispitivanje potencijalnog antifungalnog djelovanja *Origanum vulgare* na *Aspergillus fumigatus, Aspergillus niger* i *Talaromyces marneffei* izolate od kućnih ljubimaca

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Origanum vulgare (*tj.* origano) je aromatična biljka koja se rabi na Mediteranu, a osim toga poznata je poznata i zbog svojih medicinskih svojstava. U ovom eksperimentu ispitali smo antifungalno djelovanje na 2 različite vrste *Aspergillus (Niger* i *fumigatus)* i novu *Talaromyces marneffei.* Ispitali smo antifungalno djelovanje biljaka u sljedećim koncentracijama (5, 10, 20 i 30 mg /mL) pri čemu je biljka otopljena i pomiješana s rabljenom hranjivom podlogom (PDA) nakon čega smo cijepili plitice s gljivicama, postotak inhibicije je mjeren tijekom 7 dana, a podaci su prikupljeni 3., 5. i 7. dana, postignuti su visoki postotci inhibicije fungalne aktivnosti u rasponu od 30 do 100 % za *Aspergillus niger* i *Aspergillus fumigatus* te od 50 do 100 % za *Talaromyces marneffei.* Ova multidisciplinarna studija uvela je uporabu biljaka u veterinarske znanosti uporabom uzoraka izoliranih od kućnih ljubimaca s ciljem istraživanja potencijalnih alternativa tradicionalnim antifungalnim terapijama s tim da je krajnji cilj općenito povećati dobrobit životinja.

Ključne riječi: *mikrobiologija, Origanum vulgare, Aspergillus fumigatus, Aspergillus niger, Talaromyces marneffei*