

## Research Article

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# *Cinnamomum cassia* and *Origanum compactum* Essential Oils as Antifungal Candidates for the Treatment of Aspergillosis

## Abstract

Aspergillosis is a disease that usually affects the respiratory system. While most patients develop mild symptoms, aspergillosis can become a serious health threat in immunocompromised populations and patients with pre-existing respiratory conditions. Aspergillosis is caused by fungi of the *Aspergillus* genus. Existing treatments include drugs such as azoles, ergosterol interfering agents (Amphotericin B), and echinocandins (Caspofungin), which usually come with various drawbacks. Numerous studies have shown the antifungal properties of certain essential oils (EOs), which represent potential alternative treatments against fungal infections. Here, we screened the antifungal properties of five EOs against *Aspergillus oryzae*: *Melaleuca alternifolia*, *Mentha x piperita*, *Thymus zygis*, *Origanum compactum*, and *Cinnamomum cassia*. Of the five EOs, two demonstrated antifungal activity: *Origanum compactum* acted as a fungistatic, while *Cinnamomum cassia* showed both fungistatic and fungicidal effects against *A. oryzae*. Therefore, both EOs represent potential alternative treatments against Aspergillosis.

## Introduction

Aspergillosis is an infection of the respiratory tract caused by members of the genus *Aspergillus*. Their infectious abilities reside in their asexual spores, also known as conidia (singular: conidium). *Aspergillus* conidia are found everywhere in the environment (such as air, soil, plants, and water) as well as inside houses and hospitals. Patients become infected by inhaling fungi, which then grow inside the lungs<sup>1,2</sup>.

Depending on the state of the host's immune system, aspergillosis may cause various complications. For instance, in immunocompromised patients, it usually leads to a lung disease named invasive pulmonary aspergillosis, which is the most common cause of death by aspergillosis. Other aspergillosis related pathologies exist, such as allergic bronchopulmonary aspergillosis (ABPA), which occurs in atopic patients<sup>2,3</sup>.

While many species of the genus *Aspergillus* may cause aspergillosis, one of the most reported pathogens is *Aspergillus fumigatus*<sup>1,2</sup>. *A. fumigatus* is a Class 2 pathogenic agent<sup>4</sup>. Therefore, scientists who wish to study it

must gain access to research facilities of an equivalent biosafety level. While *A. fumigatus* is the main cause of aspergillosis, other fungi of the genus *Aspergillus* can also cause it. For example, the presence of *Aspergillus oryzae* is often reported in cases of ABPA<sup>5,6</sup>. *A. oryzae* is classified as a Class 1 pathogenic agent<sup>7</sup>, which means it may be handled in any research facility of a minimal biosafety level.

Various *Aspergillus* genomes, including that of *A. oryzae*, share a high degree of similarity with *A. fumigatus*. This suggests that they are phylogenetically very close to each other<sup>8</sup>. In addition, the similarity between *A. fumigatus* and other *Aspergillus* species such as *A. flavus*, *A. niger*, *A. oryzae* and *A. nidulans* has been studied at the amino acid level. In brief, researchers calculated their percentage of similarity from an alignment of 2753 orthologous proteins. Based on this alignment, researchers found a 78% similarity between *A. fumigatus* and *A. oryzae* — a result on par with the other reported *Aspergillus* species<sup>9</sup> (Table 1). It is worth noting that some studies suggest that both *A. fumigatus* and *A. oryzae* may be able to reproduce asexually<sup>10</sup>, although this last point is subject to debate<sup>11</sup>. Finally, many studies have shown the antifungal effect of our EO panel against other members

of the genus *Aspergillus*, including *A. fumigatus*<sup>12–18</sup>, which suggests shared mechanisms of action across many *Aspergillus* species. Therefore, *A. oryzae* represents a good alternative model to *A. fumigatus* for scientists who wish to study aspergillosis agents with no access to high biosafety level facilities.

**Table 1.** Percentage of similarity from an alignment of 2753 orthologous proteins across *Aspergillus* genomes. *A. oryzae* and *A. fumigatus* show the highest percentage of alignment. Adapted from Rokas and Galagan, 2007<sup>9</sup>.

	<i>A. flavus</i>	<i>A. niger</i>	<i>A. oryzae</i>	<i>A. nidulans</i>
<i>A. fumigatus</i>	77.5%	76.6%	78%	73.9%

Nowadays, aspergillosis is treated with antifungal drugs, which usually consist of azoles, ergosterol interfering agents (Amphotericin B<sup>®</sup>) and echinocandins (Caspofungin<sup>®</sup>)<sup>2</sup>. However, these drugs suffer from numerous drawbacks, which limits their efficacy and practicability. For instance, azoles have been shown to interact with numerous other drugs. In addition, recent studies have noted an increase in the prevalence of azole resistance in *A. fumigatus*. Ergosterol interfering agents have serious side effects such as renal toxicity leading to renal insufficiency, and may cause allergic reactions (e.g., skin rashes, bruising, and troubled breathing). Finally, echinocandins are typically delivered through parenteral injections, which limit their use outside of the hospital. Therefore, there is a need for alternative treatments to aspergillosis that are less toxic to patients, more convenient to deliver, and more effective<sup>2,5,19</sup>.

Our goal was to identify new candidate drugs against aspergillosis. Previous studies have investigated the antifungal properties of certain essential oils (EOs), which are aromatic extracts usually obtained through the steam distillation of plants<sup>3,12–16,20</sup>. Therefore, we decided to test five EOs against *A. oryzae*: *Mentha x piperita*, *Melaleuca alternifolia*, *Thymus zygis*, *Origanum compactum*, *Cinnamomum cassia*. Most of these EOs have known activity against *Aspergillus* strains. For instance, *Mentha x piperita* appeared effective against *A. flavus* and *A. parasiticus*<sup>12</sup>. *Melaleuca alternifolia* showed antifungal activity against *A. niger*<sup>13</sup>. *Thymus zygis* and *Origanum compactum* revealed antifungal properties against both *A. flavus* and *A. niger*<sup>14</sup>. *Origanum compactum* contains a phenolic compound called carvacrol which could explain its inhibiting activity on some *Aspergillus* strains<sup>14,15</sup>. Finally, *Cinnamomum cassia* also showed a high antifungal activity against *A. niger*, probably because it contains cinnamaldehyde, an antifungal molecule<sup>16</sup>.

In this paper, we investigate the antifungal activity of these five EOs at various concentrations against *A. oryzae*. We further evaluate both the fungistatic (ability to prevent fungal growth) and fungicidal (ability to kill fungi) activity of two EO that showed promising antifungal activity at low concentrations. Overall, *Cinnamomum cassia* appears to be the best candidate, as it exhibits both fungistatic and fungicidal activities against *A. oryzae*.

## Methods

### Fungal Strain and Preculture

*Aspergillus oryzae* var. *oryzae* CBS 816.2 was purchased from the CBS-KNAW Fungal Biodiversity Centre (Netherlands).

Prior to the experiments, we prepared fungal precultures as follows: we seeded *A. oryzae* into a Czapek Agar medium (0.01 g/L ferrous sulfate, 0.5 g/L magnesium sulfate, 0.5 g/L potassium chloride, 1 g/L potassium phosphate, 3 g/L sodium nitrate, 30 g/L D-sucrose, 12 g/L agar) containing 1 M of KCl. The preparation was incubated at 30 °C for 6 days. We then collected the fungi with 0.05% Tween-20<sup>®</sup> (ref. P1379, Sigma Aldrich, Burlington MA, USA). Finally, the fungal concentration was adjusted to 10<sup>5</sup> conidia/mL.

dia/mL.

### Essential Oil Safety

All essential oils used in this study have been graded as “GRAS” (Generally Recognized As Safe) by the Flavor Extract Manufacturers Association. In addition, we manipulated essential oils following safe laboratory practices and all manufacturer’s recommendations. Nevertheless, all EOs, even when graded as GRAS and commercially available, may bear toxic effects at high doses, which includes sensitization, dermatitis, as well as digestive and neurotoxic effects<sup>21</sup>.

### Antifungal Screening

We first screened the five EOs for their antifungal abilities. We prepared a stock Sabouraud medium (10 g/L peptone, 20 g/L glucose) containing 0.05% Tween-20<sup>®</sup>. For each EO, we then prepared an initial dilution consisting of a weighted amount of the EO added to the stock medium. Further dilutions were obtained through serial dilutions. Below are the resulting EO concentrations, as well as the concentration for the controls *Ocimum basilicum* (an EO with no known antifungal properties) and Amphotericin B<sup>®</sup>:

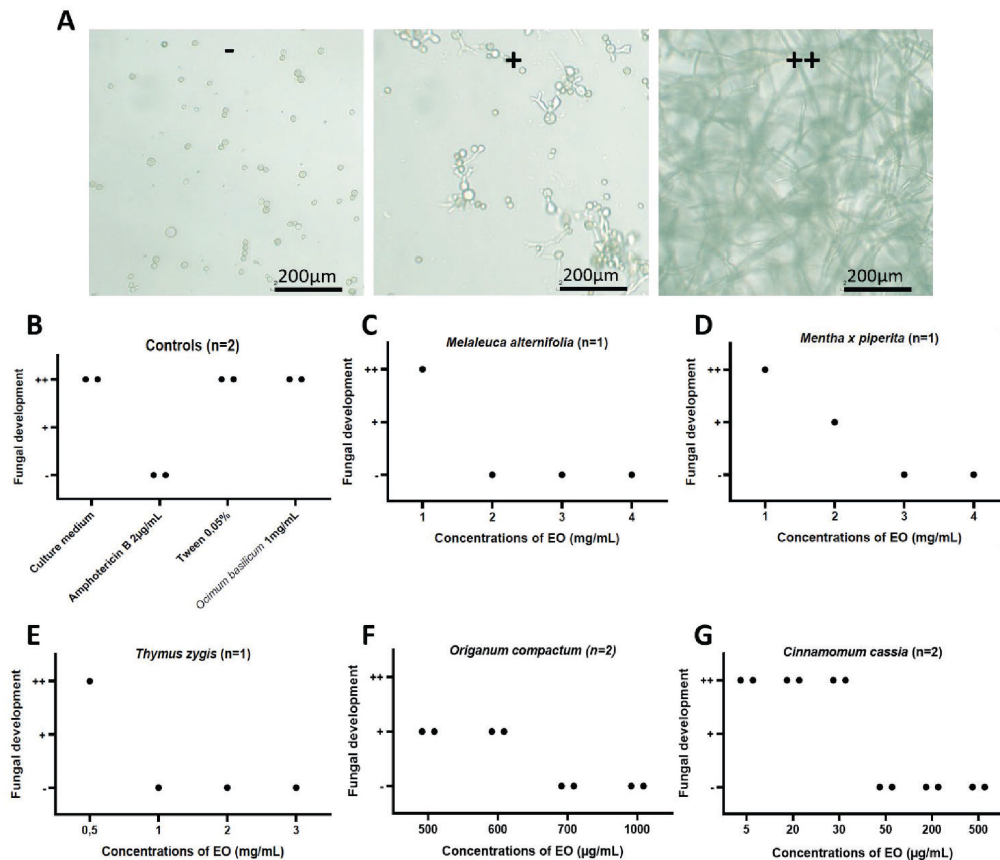
- *Melaleuca alternifolia* (EAN 5420008503917, Pranarôm, Ghislenghien, Belgium): 1 mg/mL; 2 mg/mL; 3 mg/mL; 4 mg/mL.
- *Mentha x piperita* (EAN 3401560104783, PurEssentiel, Bruxelles, Belgium): 1 mg/mL; 2 mg/mL; 3 mg/mL; 4 mg/mL.
- *Thymus zygis* (EAN 3401599455122, Pranarôm, Ghislenghien, Belgium): 0.5 mg/mL; 1 mg/mL; 2 mg/mL; 3 mg/mL.
- *Origanum compactum* (EAN 3401599454002, PurEssentiel, Bruxelles, Belgium): 500 µg/mL; 600 µg/mL; 700 µg/mL; 1000 µg/mL.
- *Cinnamomum cassia* (EAN 5420008506826, Pranarôm, Ghislenghien, Belgium): 5 µm/mL; 20 µm/mL; 30 µm/mL; 50 µm/mL; 200 µm/mL; 500 µm/mL.
- *Ocimum basilicum* (EAN 3701056802378, PurEssentiel, Bruxelles, Belgium): 1 mg/mL.
- Amphotericin B<sup>®</sup> (ref. Y0000005, Sigma Aldrich, Burlington MA, USA): 2 µm/mL.

After we attained our desired EO dilutions, we added *A. oryzae* (final working concentration in each well: 10<sup>4</sup> conidia/mL). The mixtures of EO and fungus were then poured in a single well each of a 24-well plate (final volume: 1 ml per well). As additional controls, wells containing only the Sabouraud medium and the Sabouraud medium with 0.05% Tween-20<sup>®</sup> were also seeded with 10<sup>4</sup> conidia/mL to verify the viability of the *A. oryzae* strain and to ensure that any observed effects were not due to potential antifungal properties of the growth medium (final volume: 1 ml per well) (Table 2, rows 1 to 4).

The wells were then incubated for 48h at 30 °C. The results were observed with an inverted microscope (ref. AE31E, Motic).

### Fungicidal and Fungistatic Test

After the initial screening of the five EOs, we further investigated two EOs with promising profiles. We first prepared a stock solution of solid Sabouraud medium (10 g/L of peptone, 20 g/L glucose, 20 g/L agar). For each EO chosen for the fungicidal/fungistatic test, we then prepared an initial dilution by weighting the EO and adding it to the stock medium. Subsequent dilutions were obtained through serial dilutions. Below are the final EO concentrations, as well as concentration for the controls *Ocimum basilicum* and Amphotericin B<sup>®</sup>:



**Figure 1.** Discrimination criteria of *A. oryzae* growth after 48h incubation with EOs or control treatments. For all panels, the fungal development will be scored as follows: (-) no development, (+) start of germination, (++) network formation.

- *Origanum compactum*: 700 µg/mL, 800 µg/mL, 900 µg/mL.
- *Cinnamomum cassia*: 20 µg/mL, 30 µg/mL, 50 µg/mL.
- *Ocimum basilicum*: 1 mg/mL.
- Amphotericin B<sup>®</sup>: 2 µg/mL.

We poured the media into Petri dishes (diameter 55 mm, Sarstedt). As additional controls, Petri dishes containing only the solid Sabouraud medium and the Sabouraud medium with 0.05% Tween-20<sup>®</sup> were also prepared.

A 6 mm diameter cellulose disk (Merck) was then soaked in a preculture of 10<sup>4</sup> conidia/mL of *A. oryzae* and laid at the center of each Petri dish. The dishes were incubated for either 48h or 72h. After this first incubation, each diffusion disk was moved onto the center of a new Petri dish containing fresh Sabouraud solid medium and incubated for 48h.

We measured the diameter of the growth disk using a ruler at various times: end of first incubation, 24h after the start of the second incubation, and end of second incubation. All measurements of 6 mm or less were considered as an absence of fungal growth, as 6 mm was the diameter of the cellulose disk alone.

## Results

### Antifungal Screening

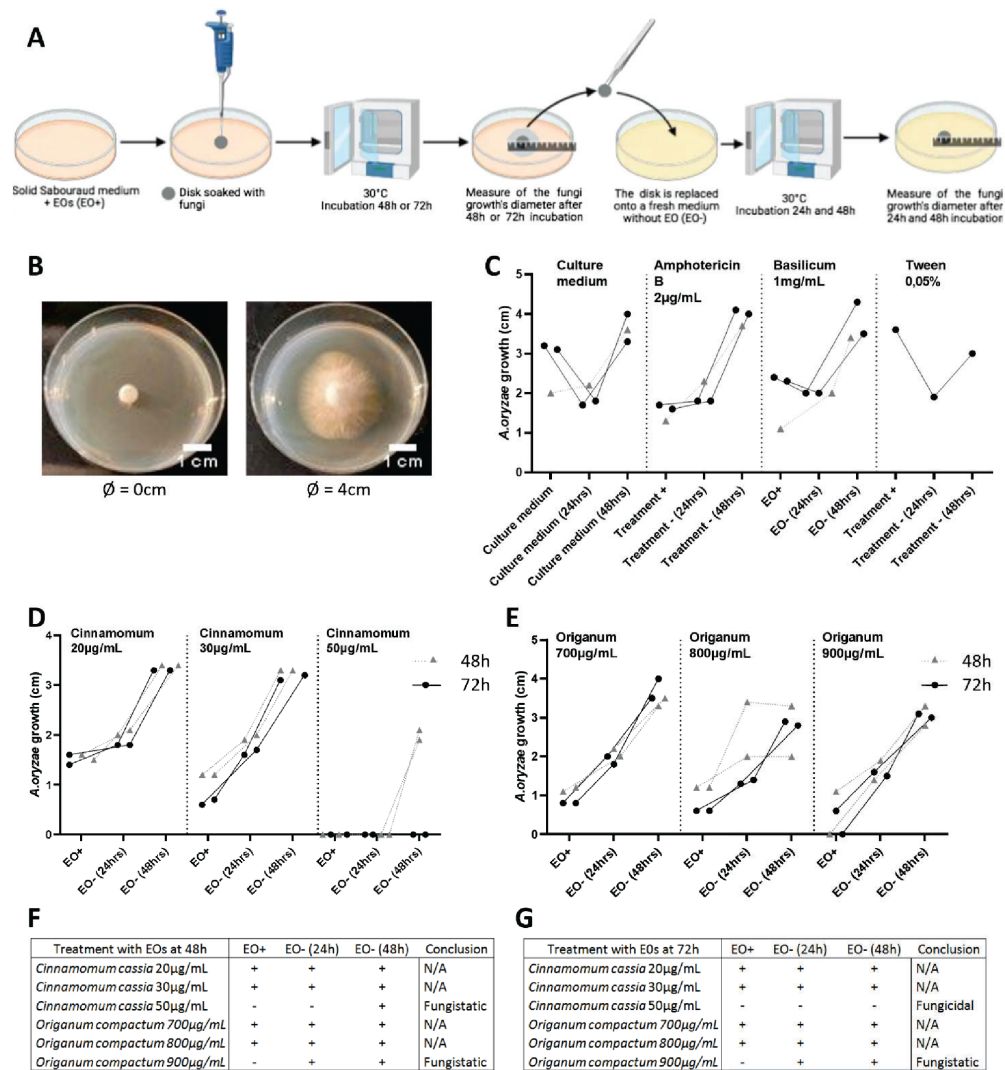
To screen which EOs had antifungal activity, we incubated *A. oryzae* spores in the presence of the EOs *Melaleuca alternifolia*, *Mentha x piperita*, *Thy-*

*mus zygis*, *Origanum compactum*, and *Cinnamomum cassia* at various concentrations for 48h (Figure 1, Table 2). *A. oryzae* germinates and forms a hyphal network in only 24h<sup>22</sup>. Therefore, for a given EO, if the growth medium was free of hyphae after 48h, we concluded that the EO was a potential inhibitor of *A. oryzae* development. On the contrary, if germination began or a network started to form after 48h, we concluded that the EO had no observable antifungal activity against our strain of interest (Figure 1). The results, including controls, are in Table 2.

The control results are in rows 1 to 4. As expected, the control well containing the growth medium with no EO had fungus that formed networks

**Table 2. Screening of candidate EOs inhibiting *A. oryzae* growth.** The rows show the different conditions we tested (EOs and controls), while the columns represent the concentrations in mg/ml (n.a.: not applicable). For each condition, the fungal development is scored as follows: (-) no development, (+) start of germination, (++) network formation (see Figure 1). Conditions that were not tested appear as gray crossed out cells. The four first rows report the control conditions (culture medium alone; Amphotericin B; *Ocimum basilicum*; Tween-20), while the other rows show the results of our EO test screenings. To the right of each condition, n gives the number of replicates. For conditions with n greater than 1, the results of all replicates were always identical, hence we did not distinguish them in this table.

	(n.a.)	0.002	0.005	0.02	0.03	0.05	0.2	0.5	0.6	0.7	1	2	3	4
Culture medium (n=2)	++													
Amphotericin B (n=2)	-													
Tween 0.05% (n=2)	++													
<i>Ocimum basilicum</i> (n=2)											++			
<i>Melaleuca alternifolia</i> (n=1)											++	-	-	-
<i>Mentha x piperita</i> (n=1)											++	+	-	-
<i>Thymus zygis</i> (n=1)								++			-	-	-	-
<i>Origanum compactum</i> (n=2)								+	+	-	-	-	-	-
<i>Cinnamomum cassia</i> (n=2)		++	++	++	-	-	-	-	-	-	-	-	-	-



**Figure 2.** Fungicidal and fungistatic effects of EOs on *A. oryzae*. **(A)** Experimental approach. A cellulose disk saturated with *A. oryzae* is placed in a Petri dish containing solid Sabouraud medium with the EO of interest (EO+). The dish is incubated for 48 to 72h, after which fungal growth is measured. Then the disk is transferred in a new dish with EO-free medium (EO-). The fungal growth is measured at 24 and 48h of incubation. Fungicidal EOs would prevent growth in both EO+ and EO- conditions, while fungistatic EOs would prevent fungal growth in the EO+ condition only. **(B)** Exemplar growth disks. Left: no apparent growth. Right: 4 cm growth. We measured fungal growth as the diameter of the disk after incubation. All diameters of 6 mm or less were considered as an absence of fungal growth, as 6 mm was the diameter of the cellulose disk alone. **(C)** Control conditions (culture medium alone; Amphotericin B; *Ocimum basilicum*; Tween-20). For all graphs: all dots corresponding to the same cellulose disk are connected with a line. Grey triangles and dotted lines: the disks were left 48h in the EO+ medium. Black circles and solid lines: the EO+ incubation lasted 72h. **(D), (E)** Fungal growth in EO+ and EO- conditions. In **(D)**, the EO+ medium contained *Cinnamomum cassia*, while it contained *Origanum compactum* in **(E)**. **(F), (G)** Summary table of the measurements obtained from disks incubated for 48h **(F)** or 72h **(G)** in the EO+ condition. For all tables: (-) means an absence of fungal growth, while (+) represents an apparent growth.

inside the wells. This also confirmed the viability of the *A. oryzae* strain. The well containing Amphotericin B, a well-known antifungal drug<sup>23</sup>, prevented the development of *A. oryzae*. Finally, the well containing *Ocimum basilicum*, an EO with no known antifungal activity<sup>19</sup>, did not prevent fungal growth, as expected.

The results of screening the five EOs at various concentrations are in rows 5 to 9. Three EOs (*Melaleuca alternifolia*, *Mentha x piperita*, and *Thymus zygis*) showed antifungal activity only at concentrations higher than 1 mg/mL. Two, *Origanum compactum* and *Cinnamomum cassia*, showed antifungal activity at concentrations as low as 700 µg/mL and 50 µg/mL, respectively.

Having identified *Origanum compactum* and *Cinnamomum cassia* as two promising EO candidates with antifungal activity at relatively low concentrations, we then decided to further investigate their antifungal properties.

### Fungistatic or Fungicidal Properties of Selected EOs

We performed fungicidal and fungistatic tests with *Cinnamomum cassia* and *Origanum compactum* against *A. oryzae* (Figure 2). Briefly, we placed a small cellulose disk saturated with a solution of *A. oryzae* spores in a Petri dish containing a layer of solid Sabouraud medium mixed with the EO of interest (EO+). The Petri dish was incubated for 48 to 72h, after which we measured the diameter of fungal growth. Then, we transferred the same paper disk in a new Petri dish with fresh, EO-free medium (EO-) and measured, once again, the fungal growth after 24 and 48h of incubation (Figure 2A, 2B). Fungicidal substances would prevent fungal growth in both EO+ and EO- conditions, while fungistatic compounds would prevent fungal growth in the EO+ condition only.

Prior to this, we conducted another series of control tests (Figure 2C). As before, we examined the viability of our fungus strain: *A. oryzae* success-

fully formed a growth disk both prior to and after transfer to a new Petri dish. In addition, we confirmed the fungistatic effect of Amphotericin B<sup>23</sup>, and the lack of antifungal activity from both *Ocimum basilicum*<sup>19</sup> and the culture medium (with and without Tween-20).

The results of the tests on *Cinnamomum cassia* and *Origanum compactum* are in Figure 2D-2G. Both EOs inhibited the growth of *A. oryzae* in the EO+ condition at the highest concentration tested (*Cinnamomum cassia*: 50 µg/mL; *Origanum compactum*: 900 µg/mL), which confirmed the antifungal activity previously observed. Furthermore, the disks transferred from the EO+ to the EO- condition after 48h of incubation displayed sizeable growth, indicative of a fungistatic effect from both *Cinnamomum cassia* and *Origanum compactum* (Figure 2D-2F).

However, after 72h of incubation in the EO+ condition, the two EOs showed different results. While we noted a fungistatic effect from *Origanum compactum*, the disk taken from *Cinnamomum cassia* EO+ medium developed no growth disk, indicating a fungicidal effect (Figure 2D, 2E, 2G).

To conclude, we confirmed that both *Cinnamomum cassia* and *Origanum compactum* have antifungal properties against *A. oryzae*. We also detected a fungistatic effect from *Origanum compactum*, and both a fungistatic and fungicidal effect from *Cinnamomum cassia* depending on the duration of the incubation in contact with the EO.

## Discussion

### *Cinnamomum cassia*, a Fungicidal EO Against *A. oryzae*

While all EOs exhibited some antifungal activity, *Origanum compactum* and *Cinnamomum cassia* were most effective, exhibiting antifungal activity at concentrations of 700 µg/mL and 50 µg/mL, respectively (Figure 1, Table 2). Both EOs exhibited fungistatic activity. Interestingly, when the fungus was in contact with the EO for 72h, *Cinnamomum cassia* also acted as a fungicidal agent.

The main antifungal ingredient of *Cinnamomum cassia* is cinnamaldehyde<sup>16-18</sup>. Cinnamaldehyde acts by impairing two essential biological processes: it disrupts ATPase activity and inhibits the synthesis of fungal cell walls<sup>17,18</sup>. Other varieties of cinnamon-based EOs exist, such as *Cinnamomum zeylanicum* (bark), which also contains cinnamaldehyde. A previous study found that *Cinnamomum cassia* was slightly better at inhibiting *A. niger* growth than *Cinnamomum zeylanicum*<sup>16</sup>. This is not surprising, considering the composition of these EOs: *Cinnamomum cassia* contains about 66% of cinnamaldehyde, while *Cinnamomum zeylanicum* contains 64%<sup>16</sup>. Therefore, one would expect *Cinnamomum cassia* to also show greater antifungal activity against *A. oryzae* than *Cinnamomum zeylanicum*. This hypothesis is open for testing.

### *Cinnamomum cassia*, a Non-Toxic Potential Alternative to Current Aspergillosis Treatment

We showed that *Cinnamomum cassia* is an effective fungicidal agent against *A. oryzae* at concentrations as low as 50 µg/mL *in vitro*. Interestingly, *in vitro* studies suggest that *Cinnamomum cassia* is non mutagenic<sup>24</sup>. Furthermore, rats subjected to daily oral doses of this EO showed signs of potential toxicity only at very high doses (2 g/kg/day)<sup>24</sup>. Therefore, it may be possible to deliver a treatment containing *Cinnamomum cassia* orally. Another delivery route is directly to the lungs, using an inhaler – although this method is yet to be proven safe and should be tested *in vivo*. In addition, promising innovative approaches aimed at delivering volatile drugs, such as nanoemulsion, nanopreparations, and nanocarriers, are currently under investigation<sup>25-27</sup>. If proven effective, they may be suitable for *Cinnamo-*

*mum cassia*.

A recent study found that *Cinnamomum zeylanicum* and *Rosmarinus officinalis*, when combined, had a synergic effect against fungi developing on pears, meaning that the antifungal effect of the EO mix was greater than the sum of the antifungal activities measured separately<sup>28</sup>. As a future avenue, it would be interesting to test the potential synergic effect of *Cinnamomum cassia* and *Origanum compactum*.

## Conclusion

Aspergillosis is a nosocomial infection of the respiratory system caused by *Aspergillus*, a genus of fungi found pervasively in the environment both indoors and outdoors<sup>1,2</sup>. Conventional treatments consist of antifungal drugs, namely ergosterol interfering agents (Amphotericin B), echinocandins (Caspofungin), and azoles. However, these drugs have numerous drawbacks<sup>2,5,19</sup>. In addition, certain studies suggest that *Aspergillus* may still grow in the presence of these drugs<sup>2,19</sup>. Therefore, there is a need for alternative treatments against *Aspergillus*.

Several research teams have reported on the antifungal potential of essential oils against *Aspergillus* strains<sup>3,12-16,20</sup>. Therefore, we decided to characterize the antifungal activity of five essential oils against *Aspergillus oryzae*, a species known to cause specific forms of aspergillosis. These were *Melaleuca alternifolia*, *Mentha x piperita*, *Thymus zygis*, *Origanum compactum*, and *Cinnamomum cassia*. These EOs were already known for their antifungal activity against some members of the genus *Aspergillus*<sup>12-18</sup>. However, to our knowledge, no past study has assessed the effect of these EOs against *A. oryzae* specifically.

Two essential oils stood out from our initial screening: *Origanum compactum* and *Cinnamomum cassia*. Both EOs showed strong fungistatic activity against *Aspergillus oryzae*. In addition, when left in contact with the fungus for 72h, *Cinnamomum cassia* showed a clear fungicidal effect against *Aspergillus oryzae* at concentrations as low as 50 µg/ml. Therefore, *Cinnamomum cassia* may represent a valuable alternative to conventional treatments against aspergillosis. Its potential as a drug treatment should be assessed next through *in vivo* studies.

## Author Contribution

M.L., I.C., M.B., F.C., J.S.-B., and A.S.-P. designed the experiments. M.L., I.C., M.B., and F.C. performed the experiments, under the supervision of J.S.-B. and with precious advice from P.G. and F.Y. The first draft of the manuscript was written by M.L., I.C., M.B., and F.C. The manuscript was revised by J.G., J.S.-B., and A.S.-P. These experiments were conducted through the “Projets Fil Rouges” (Applied Transversal Projects), a student-led research program directed by F.Y. at Sup’Biotech.

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