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# Characterisation of the antifungal effects of a plant-based compound, CIN-102, on the main septal filamentous fungi involved in human pathology



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## ABSTRACT

**Objectives:** Today, the increase of invasive fungal infections and the emergence of resistant strains are observed in medical practice. New antifungals are expected, and the plant world offers a panel of potentially active molecules. CIN-102 is a mixture of seven different compounds of plant origin developed from the formulation of cinnamon essential oil.

**Methods:** The in vitro activity of CIN-102 was characterised against *Aspergillus* spp., *Fusarium* spp. and *Scedosporium* spp. by studying the minimum inhibitory concentration (MIC), inoculum effect, germination inhibition, fungal growth, post-antifungal effect (PAFE) and synergy.

**Results:** MICs determined for the three genera followed a unimodal distribution and their mean values ranged from 62–250  $\mu\text{g}/\text{mL}$ . CIN-102 demonstrated an inoculum effect similar to voriconazole and amphotericin B, 100% inhibition of spore germination and a PAFE.

**Conclusion:** CIN-102 has significant activity against filamentous fungi involved in human pathologies and should be further explored as a potential new treatment. Other studies regarding its mechanisms of action as well as animal investigations are awaited.

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## 1. Introduction

The incidence of invasive fungal infections (IFIs) has been increasing over the past 20 years. Approximately 3600 people develop an IFI each year in France [1]. Moreover, the incidence of invasive aspergillosis is estimated at 1.4 cases per 100 000 people per year and has been gradually increasing between 2001 and 2010 [1]. It is important to note the emergence of infections caused by strains resistant to triazoles, which can be associated with therapeutic failures. Other less common fungal infections that are diffi-

cult to cure have increased in the last two decades in developed countries, such as fusariosis and scedosporiosis, occurring mainly during deep immunosuppression [2]. At the same time, only a few new antifungal drugs have been marketed in the last decade. At present, the antifungal arsenal available for the management of IFIs consists of ten drugs divided into four major classes. In addition, these drugs are often expensive and usually present significant toxicity and drug interactions. These limitations, combined with the emergence of resistance, justify the search for new therapeutic strategies [3,4].

The resources of the plant world in active substances used for therapeutic purposes are truly significant, although still underexploited. Essential oils extracted from plants (leaves, flowers, roots or bark) have different pharmacological properties, in particular

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**Table 1**  
Clinical and reference strains studied for the genera *Aspergillus*, *Scedosporium* and *Fusarium*

Genus/species	Reference strains	Clinical strains	Total strains
<i>Scedosporium</i> spp.			
<i>S. apiospermum</i>	4	3	7
<i>Lomentospora prolificans</i>	3	2	5
<i>S. aurantiacum</i>	2	0	2
<i>S. dehoogii</i>	1	1	2
<i>S. boydii</i>	1	0	1
<i>S. minutisporum</i>	2	0	2
<i>Aspergillus</i> spp.			
<i>A. fumigatus</i>	5	13	18
<i>A. flavus</i>	2	4	6
<i>A. niger</i>	1	3	4
<i>A. nidulans</i>	1	1	2
<i>A. versicolor</i>	1	1	2
<i>A. terreus</i>	2	1	3
<i>A. ochraceus</i>	1	0	1
<i>A. calidoustus</i>	1	0	1
<i>A. glaucus</i>	0	1	1
<i>A. clavatus</i>	1	0	1
<i>Fusarium</i> spp.			
<i>F. solani</i>	9	1	10
<i>F. duoseptatum</i>	1	0	1
<i>F. moniliforme</i>	1	0	1
<i>F. proliferatum</i>	1	0	1
<i>F. chlamydosporum</i>	1	0	1
<i>F. incarnatum</i>	1	0	1
<i>F. dimerum</i>	1	0	1
<i>F. verticillioides</i>	1	1	2
<i>F. keratoplasticum</i>	0	1	1

anti-infectious and antioxidant effects. Cinnamon essential oil is being studied for its analgesic, antitubercular, antioxidant, anti-hyperglycaemic and antifungal activities. It has an inhibitory effect on fungal and yeast growth of several species, including *Aspergillus* spp. and *Candida* spp. [5]. The main constituent of cinnamon essential oil is cinnamaldehyde, which can also be synthesised [6]. Recent studies demonstrated promising data regarding the effects of cinnamaldehyde on the main fungal genera involved in human pathology and on toxigenic fungi [6–9]. Cinnamaldehyde is an active inhibitor of yeasts and filamentous fungi growth. It acts by inhibiting ATPase activity and biosynthesis of cell walls and through the alteration of membrane structure and integrity [6]. Consequently, cinnamaldehyde constitutes a potential new class of antifungal agents.

In the present study, we characterised the antifungal activities of a mixture, named CIN-102, of seven different chemical compounds, comprising three cinnamaldehyde derivatives, that was developed from the formulation of cinnamon essential oil available in the Pharmacopoeia, and from which the genotoxic compounds have been removed (safrole, eugenol and coumarin).

## 2. Materials and methods

### 2.1. Fungal isolates

A total of 77 strains of filamentous fungi were used for the determination of minimum inhibitory concentrations (MICs). These strains comprised fungi belonging to the *Aspergillus* (39 strains), *Fusarium* (19 strains) and *Scedosporium* (19 strains) genera. They belong to the main species complexes (Table 1) and have different origins, including 44 reference strains and 33 clinical strains (Supplementary Tables S1–S3). Clinical strains were obtained from patients at the University Hospital (CHRU) of Nancy, France, and were identified in the laboratory.

**Table 2**  
Sequence of specific primers used to identify *Aspergillus*, *Scedosporium* and *Fusarium* strains

Genus	Primer name	Primer sequence (5'→3')
<i>Aspergillus</i> spp.	CMD5	CCG AGT ACA AGG ARG CCT TC
	CMD6	CCG ATR GAG GTC ATR ACG TGG
	Btub1F	AATGGTGCCGCTTTCTGG
	Btub2R	AGTTGTCGGGACGGAATAG
<i>Scedosporium</i> spp.	NL1	GCAT ATCA ATAA GCGGAGG AAAAG
	V3-12	GGTCC GTGTT TCA AGACG
	Bt2a	GGT AAC CAA ATC GGT GCT GCT TTC
	Bt2b	ACC CTC AGT GTA GTG ACC CTT GGC
<i>Fusarium</i> spp.	EF1	ATG GGT AAG GAA GAC AAG AC
	EF2	GGA AGT ACC AGT GAT CAT GTT

### 2.2. Clinical strain sequencing

Each clinical strain was identified by microscopy for its genus and by sequencing for its species complex. The  $\beta$ -tubulin (Btub1F/Btub2R) and calmodulin (CMD5/CMD6) genes were sequenced for *Aspergillus* strains [10],  $\beta$ -tubulin (Bt2a/Bt2b) and D1/D2 (NL1/V3-12) for *Scedosporium* strains [11], and elongation factor (EF1/EF2) for *Fusarium* strains [12] (Table 2). Following DNA extraction, PCR was performed in an iCycler iQ5™ (Bio-Rad) using a TaqPurple Kit (Ozyme) with primers specific for each genus. Sequencing according to the Sanger method was carried out by GenoScreen (France) using primers used to perform PCR. Sequence analyses were interpreted using BioEdit software [13] and were compared with the Mycosm.jgi.doe.gov and *Aspergillus*genome.org databases for species complex identification.

### 2.3. Culture and storage

Strains were subcultured on Sabouraud agar medium (Sigma-Aldrich). After 48–72 h of incubation at 31°C, colonies were picked from the medium using sterile swabs and were suspended in 0.9% NaCl or RPMI-MOPS (US Biological) medium. Strains were stored

and preserved in a mixture containing 1 mL of glycerol and 1 mL of physiological saline at  $-20^{\circ}\text{C}$ .

## 2.4. Minimum inhibitory concentration (MIC) determination

### 2.4.1. Liquid conditions

The Clinical and Laboratory Standards Institute (CLSI) M38-A2 reference protocol was followed for fungal MIC determination [14]. Two antifungal agents included in the protocol, namely amphotericin B (AMB) and voriconazole (VRC) (Sigma-Aldrich) (dilution range 0.03–16  $\mu\text{g}/\text{mL}$ ), were used in addition to CIN-102 (Septeos). After culturing, conidia were suspended in 0.9% NaCl and were then filtered, adjusted to  $5 \times 10^4$  conidia/mL using a spectrometer at 530 nm, and diluted to 1:50 to obtain the working suspension.

An inoculum of 100  $\mu\text{L}$  was then distributed in 96-well plates containing the antifungal dilutions. These plates were incubated at  $37^{\circ}\text{C}$  for 48 h. All strains were tested in duplicate. The MIC was determined as the lowest drug concentration resulting in complete growth inhibition after 24–48 h of incubation. For each 96-well plate, a control strain was tested in parallel as indicated in the CLSI protocol. A plastic film was put on every plate to limit evaporation and contamination. The density of the inoculum was checked by seeding 10  $\mu\text{L}$  of the working suspension diluted to 1:10 on Sabouraud agar medium after 1 day of incubation at  $31^{\circ}\text{C}$ . One to ten colonies must be counted to validate the test. After a preliminary test, the dilution range chosen for CIN-102 ranged from 1.9–1000  $\mu\text{g}/\text{mL}$ .

### 2.4.2. Solid conditions

Solid MICs were evaluated on a solid Sabouraud medium. CIN-102 was added to each Petri dish just before solidification of the medium to obtain final concentrations of 15.6, 31.25, 62.5, 125 and 250  $\mu\text{g}/\text{mL}$ . A drop of each inoculum was placed on the four quarters of the Petri dish for the concentrations tested. Following incubation at  $31^{\circ}\text{C}$  for 72 h, the MIC was determined as the lowest concentration at which the strain did not grow on the Petri dish.

## 2.5. Inoculum effect

The methodology was the same as for MIC determination in liquid medium, using an inoculum dilution gradient ranging from  $10^4$  to  $10^8$  spores/mL. A control without antifungal was carried out to verify the absence of inhibition effect due to a high quantity of spores. We considered that there was an effect when a difference in MIC greater than two dilutions was observed [6].

## 2.6. Germination assay

A conidia germination assay was carried out using the protocol of Gameda et al. [15]. Spores were collected by adding 5 mL of sterile water mixed with 0.1% Tween 80 to the Petri dish after peeling off with a rake three times. The recovered suspension was centrifuged for 5 min at 2000 rpm and conidia were counted on Kova cells and diluted at  $10^6$  spores/mL. Different antifungal solutions (AMB, VRC and CIN-102) were prepared in liquid Sabouraud medium and were used at 0.5  $\times$ , 1  $\times$  and 4  $\times$  MIC of the tested strains. Then, 1 mL of the spore suspension was added to each tube and incubated at  $31^{\circ}\text{C}$  with shaking for 48 h. A tube without antifungal served as a control. Germinated spores were counted by microscopy with Kova cells. The percentage inhibition was determined according to the following formula:

$$\% \text{ inhibition of germination} = (\text{SC} - \text{ST}) / \text{SC} \times 100$$

where SC is the percentage of spores germinated in the control tube and ST is the percentage of spores germinated in the test tube.

## 2.7. Growth curve with antifungals

The methodology was the same as for MIC determination in liquid medium. Following preparation, the plate was incubated in a plate reader (SpectraMax iD3; Molecular Devices) for 48 h at  $37^{\circ}\text{C}$  and measurement of the optical density at 405 nm ( $\text{OD}_{405}$ ) was carried out every 10 min. Each strain was seeded in duplicate and the experiment was performed three times. The calculated delay was the time difference between the start of growth for the test and control strain.

## 2.8. Synergy assay

The synergistic effect of CIN-102 combined with VRC or AMB was determined using the protocol of Cardoso et al. [16] with modification of a few elements. In a 96-well plate containing a series of dilutions of CIN-102, VRC or AMB, 0.5  $\times$  MIC of the antifungal agent tested was added to each well, except in column 11 and 12, which served as positive and negative controls. For each antifungal tested, a control line was carried out (same preparation as for the determination of MIC). The plates were then inoculated with  $10^4$  conidia/mL and were incubated at  $37^{\circ}\text{C}$  for 24–72 h. The experiment was conducted in triplicate. The result was visually determined when 100% of fungal growth was inhibited. The fractional inhibitory concentration (FIC) for each substance and the fractional inhibitory concentration index (FICI) for their combinations with another antifungal (VRC, AMB or CIN-102), were calculated as follows: FIC = concentration that inhibits 100% of growth in a combination/concentration that inhibits 100% of growth when used alone; and FICI = sum of the FICs of each drug tested. FICI values of  $\leq 0.5$  and  $> 4.0$  indicate synergistic and antagonistic interactions, respectively [17].

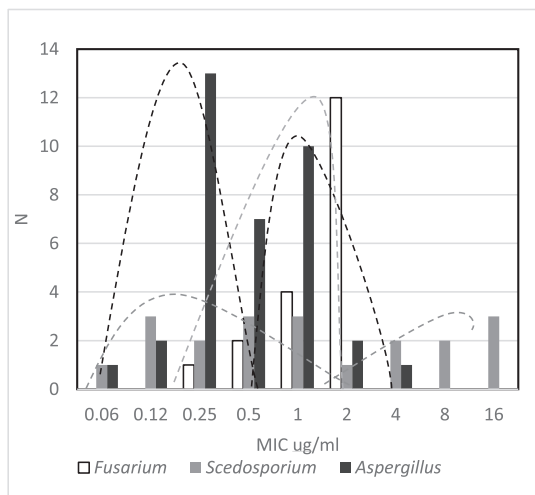
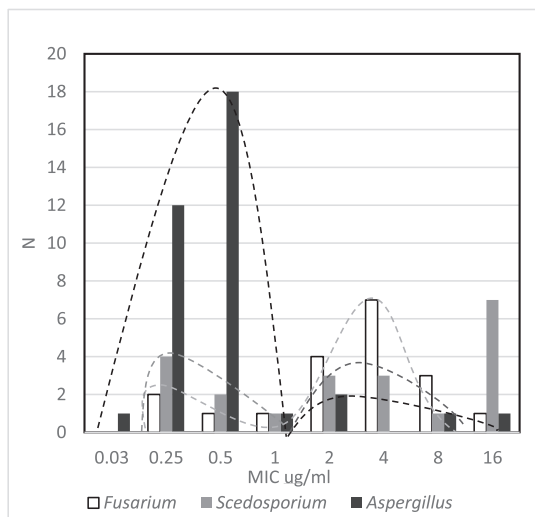
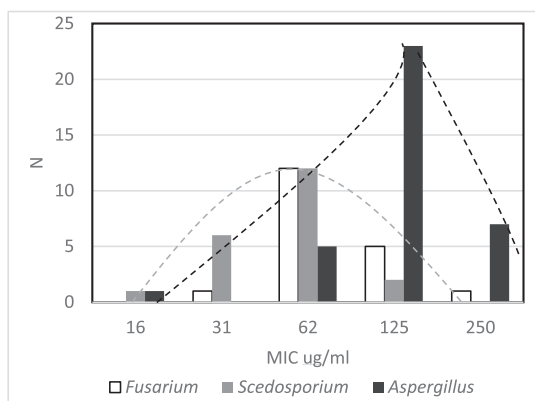
## 2.9. Post-antifungal effect (PAFE)

The PAFE, i.e. the antifungal activity on the growth of fungus after removing the antifungal from the culture medium, was determined by following the protocol of Vitale et al. [18] with a few modifications. The inoculum was prepared in RPMI at a concentration of  $4 \times 10^5$  conidia/mL and was incubated for 24 h at  $37^{\circ}\text{C}$  with shaking. After adding the antifungal for 4 h, three washes were carried out, comprising a centrifugation for 15 min at 3500 rpm and re-suspension of the pellet in 10 mL of NaCl. At the end of the third wash, the pellet was re-suspended in 10 mL of RPMI. Then, 30  $\mu\text{L}$  from the control and test tubes was seeded to perform a colony count determination for inocula verification after 24–48 h of incubation at  $37^{\circ}\text{C}$ . Briefly, 200  $\mu\text{L}$  from each tube was distributed in a 96-well plate and growth was measured at  $\text{OD}_{405}$  over 48 h using a SpectraMax iD3 plate reader. The experiment was conducted in triplicate. The PAFE was calculated by a graphical approach using the formula:  $\text{PAFE} = T - C$ , where  $T$  is the time of the first significant increase in  $\text{OD}_{405}$  of the exposed conidia or spores after removal of the drug and  $C$  is the time of the first significant increase in  $\text{OD}_{405}$  of the control.

## 3. Results

### 3.1. Minimum inhibitory concentration determination and spectrum of activity

Determination of MICs in liquid medium revealed a CIN-102 median MIC of 125  $\mu\text{g}/\text{mL}$  (range 16–250  $\mu\text{g}/\text{mL}$ ) for *Aspergillus* spp. The median MIC was 62  $\mu\text{g}/\text{mL}$  for *Fusarium* spp. (range 31–250  $\mu\text{g}/\text{mL}$ ) and 62  $\mu\text{g}/\text{mL}$  for *Scedosporium* spp. (range 16–125  $\mu\text{g}/\text{mL}$ ). For AMB, the median MICs were, respectively, 0.25  $\mu\text{g}/\text{mL}$



**Fig. 1.** Distribution (A) CIN-102, (B) voriconazole and (C) amphotericin B minimum inhibitory concentrations (MICs) for *Aspergillus* spp., *Fusarium* spp. and *Scedosporium* spp. The dotted curves make it possible to visualise the distribution of the strains.

(range 0.06–4 µg/mL) for *Aspergillus* spp., 2 µg/mL (range 0.25–4 µg/mL) for *Fusarium* spp. and 2 µg/mL (range 0.06–16 µg/mL) for *Scedosporium* spp. Finally, for VRC these data were 0.5 µg/mL (range 0.03–8 µg/mL) for *Aspergillus* spp. and 4 µg/mL (range 0.25–16 µg/mL) for *Fusarium* spp. and *Scedosporium* spp. (Fig. 1). A unimodal distribution of CIN-102 MIC values was observed for all tested strains. In contrast, for VRC and AMB, two populations stand out, one sensitive and one with a resistant phenotype (Fig. 1). In addition, the MIC range for the three genera was narrower for CIN-

**Table 3**  
Minimum inhibitory concentration (MIC) differences for CIN-102 between solid and liquid conditions for *Fusarium* spp.

	MIC difference <sup>a</sup>			
	0	+1	-1	+2 or -2
% of strains	61	11	27	0

<sup>a</sup> '+1' difference means that the MIC in liquid medium is one higher concentration than in solid medium.

**Table 4**  
Average minimum inhibitory concentrations (MICs) for susceptible and resistant strains to CIN-102, voriconazole (VRC) and amphotericin B (AMB)

Strain	MIC (µg/mL)		
	CIN-102	VRC	AMB
<i>Aspergillus fumigatus</i> G54E	250	0.25	0.25
<i>A. fumigatus</i> TR46	250	16	0.5
<i>A. fumigatus</i> TR34	250	8	0.5
<i>Aspergillus calidoustus</i>	62	16	16
<i>Lomentospora prolificans</i>	62	16	8
<i>Fusarium</i> sp.	62	4	2
<i>Scedosporium</i> sp.	62	0.5	4
<i>Scedosporium boydii</i>	62	0.12	2
Non-resistant <i>A. fumigatus</i>	125	0.5	0.25

102 than for VRC and AMB: 3 and 5 dilution gaps for CIN-102, 6 and 7 dilution gaps for VRC, and 4 and 9 dilution gaps for AMB (Fig. 1). No significant difference was visible between the clinical strains and the reference strains (MIC difference <2 dilutions). No difference greater than two dilutions was demonstrated for MICs between the liquid and solid condition (Table 3).

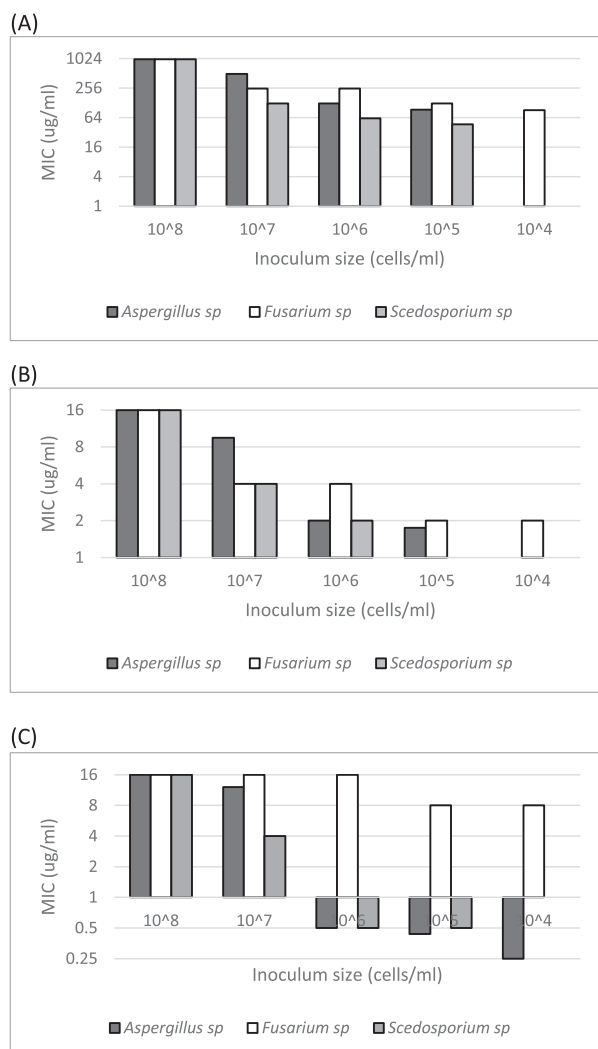
CIN-102 had comparable in vitro effects against fungi with natural or acquired resistance to antifungals used in clinical practice and those described as sensitive. For example, for *Lomentospora prolificans*, naturally resistant to VRC (MIC > 16 µg/mL) and AMB (MIC = 8 µg/mL), and *Scedosporium boydii*, which is sensitive to VRC (MIC = 0.12 µg/mL), both have an identical MIC of 62 µg/mL for CIN-102. There was almost no difference between *Aspergillus fumigatus* having acquired resistance to VRC, *Aspergillus calidoustus* (naturally resistant to VRC and AMB) and the other *Aspergillus* spp. strains considered sensitive to CIN-102 (Table 4).

### 3.2. Inoculum effect

The inoculum effect is an in vitro phenomenon described as an increase in the MIC of an antibiotic when the quantity of inoculated micro-organisms is increased. An inoculum effect was observed for all strains of *Aspergillus* spp., *Fusarium* spp. and *Scedosporium* spp. with the three substances tested (VRC, AMB and CIN-102) and it appeared with an inoculum greater than 10<sup>5</sup> and 10<sup>6</sup>. We noted that for VRC the inoculum effect was more visible for *Fusarium* strains, where the average calculated MIC (4 µg/mL) was never reached (Fig. 2).

### 3.3. Inhibition of spore germination

Inhibition of spore germination for the three genera and the three antifungals was dose-dependent. In general, CIN-102 was the product with the most effectiveness against the three genera at 1 × and 4 × MIC. A minimum of 50% inhibition was achieved for the three genera with CIN-102 and AMB at 4 × MIC, and at the CIN-102 MIC for the genus *Aspergillus*. VRC reached the threshold of 50% for *Aspergillus* and *Scedosporium* genera, respectively, at 1 × and 4 × MIC. We also observed that CIN-102 was the only



**Fig. 2.** Determination of the inoculum effect for *Aspergillus* spp., *Fusarium* spp. and *Scedosporium* spp. with (A) CIN-102, (B) amphotericin B and (C) voriconazole. MIC, minimum inhibitory concentration.

product able to approach 100% inhibition of germination for *Aspergillus* spp. and *Scedosporium* spp. In our study, CIN-102 was the only antifungal to have an effect against *Scedosporium* spp. at 0.5 × MIC (Fig. 3).

At very high doses of AMB (64 × MIC), total inhibition of spore germination was never reached. No difference was observed between susceptible and resistant strains. Morphological changes in the spores in contact with CIN-102 were the same as those observed with AMB. Spores appeared smaller, rounder and with a shrunken appearance in comparison with control spores.

### 3.4. Growth kinetics in the presence of antifungals

For the two tested antifungal agents (CIN-102 and AMB), growth delay was dose-dependent. The higher the concentration of antifungal agent, the longer the delay in the onset of fungal growth. Furthermore, we no longer saw growth at 2 × and/or 4 × MIC with AMB and CIN-102 even after 48 h for the majority of strains. Only *Fusarium dimerum* began to grow for ~11 h at 4 × MIC with CIN-102. At 0.25 × and 0.45 × MIC, the observed delay was between 0–3 h for CIN-102 and 0–10 h for AMB for all tested strains. For a concentration equivalent to the MIC, the de-

lay was between 3–23 h for CIN-102 and between 0–33 h for AMB (Fig. 4).

Generally speaking, the optical density decreased for all strains when the antifungal was added, which supposes a reduction in the number of cells in the medium. We note that for AMB the OD<sub>450</sub> is reduced by >50% compared with the control with a concentration equal to the MIC for all strains (except *Aspergillus flavus*) and for *Scedosporium apiospermum* at a concentration of 0.5 × MIC. For CIN-102, the OD<sub>450</sub> was reduced by >50% for *A. fumigatus* TR46 and TR34 and *L. prolificans*. It also decreased by >50% at 0.5 × MIC for *L. prolificans* and 0.25 × MIC for *L. prolificans* and *S. apiospermum* (Table 5).

### 3.5. Synergy assay

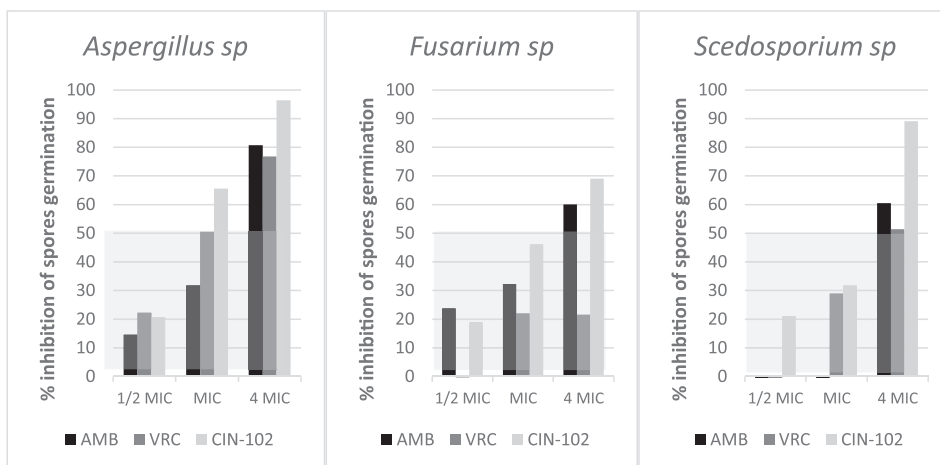
No synergistic effect could be demonstrated, either by combining 0.5 × MIC of VRC or AMB with CIN-102 or cinnamaldehyde or vice versa. All FICI values were between 1.062 and 2, i.e. no interaction. It should be noted, however, that no antagonistic effect was demonstrated (Table 6).

### 3.6. Post-antifungal effect (PAFE)

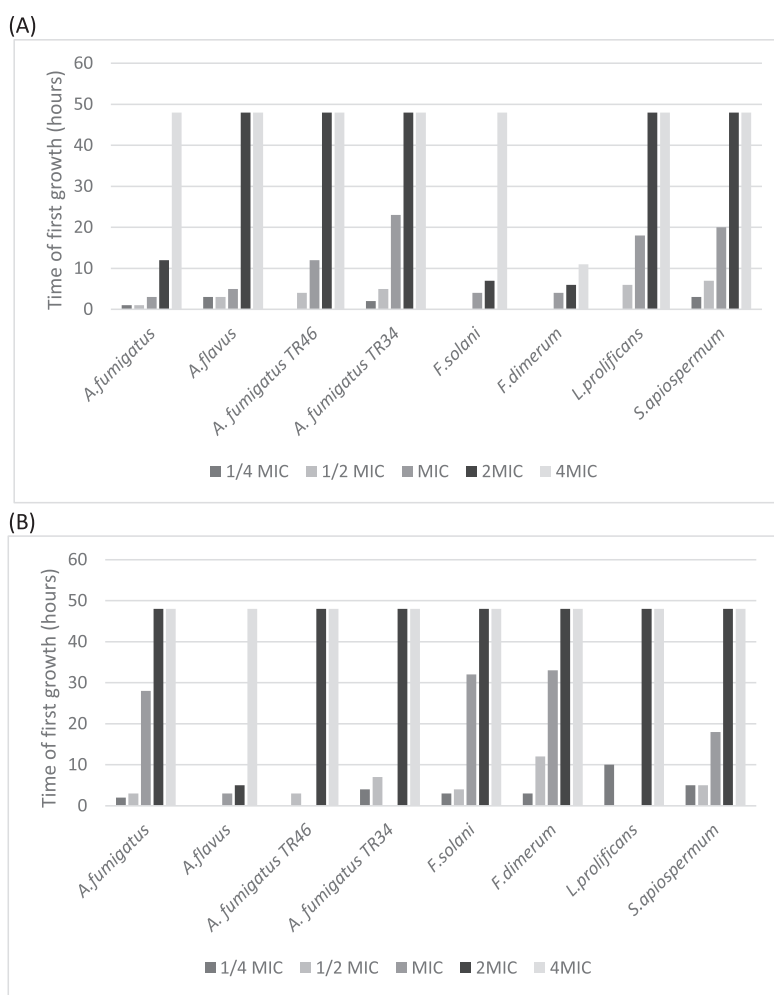
Three profiles of PAFEs emerged for CIN-102: a dose-dependent one, one with constant values whatever the dose of antifungal, and one without any PAFE (Fig. 5). The PAFE of *Fusarium* and *Aspergillus* strains was dose-dependent: 2 h at 1 × MIC and 11 h at 4 × MIC for *Fusarium solani* and 7 h at 1 × MIC and 23 h at 4 × MIC for *F. dimerum*; and 8 h at 1 × MIC and from 21–23 h at 4 × MIC for *A. flavus*, *A. fumigatus* TR34 and *A. fumigatus* TR46. For *Scedosporium* strains, the PAFE did not appear to be dose-dependent: it was 9 h whatever the initial dose of antifungal for *S. apiospermum*. For *L. prolificans*, no PAFE was observed (Table 7).

## 4. Discussion

The results of this study on filamentous fungi have shown that the activity spectrum of CIN-102 comprises the major genera involved in human pathology, namely *Aspergillus*, *Fusarium* and *Scedosporium*. The latter two have lower MICs than *Aspergillus*, which is generally the opposite for conventional drugs [19]. Although the MICs determined for CIN-102 are high compared with VRC or AMB, they are similar to those highlighted for cinnamaldehyde and other components derived from essential oils in the literature. A study by Homa et al. showed the effectiveness of cinnamon essential oil (*Cinnamomum verum*) against 18 *Fusarium* strains, with MICs between 31.25–500 µg/mL [20]. By comparison, itraconazole and fluconazole, two azole antifungals, when tested against several *Fusarium* strains showed MICs up to 128 µg/mL and 256 µg/mL, respectively [21]. The oral LD<sub>50</sub> (i.e. the dose required to kill one-half of the members of a tested population after a specified test duration) determined in rats for cinnamaldehyde (CNMA) is 2.2 g/kg (Fisher Scientific, safety data), 2 g/kg for VRC (Pfizer, safety data) and 5 g/kg (Fisher Scientific, safety data) for AMB. Thus, CNMA is not more toxic to humans than the drugs already used to treat IFIs. As CIN-102 has a composition close to CNMA, we can assume that its toxicity is similar. It is also important to note the unimodal distribution of CIN-102 MIC values. Indeed, this shows that no resistance against this antifungal has been identified in the tested strains. Strains usually resistant to antifungals have CIN-102 MICs comparable with sensitive strains. This is in favour of the hypothesis that existing antifungal resistance mechanisms of these strains have no impact on the efficacy of CIN-102. For *A. fumigatus*, the TR46 and TR34 CYP51A gene mutations confer azole resistance, in particular to VRC [22–24]. We therefore believe that CIN-102 does not act on the same target as VRC. This is in agreement with the



**Fig. 3.** Inhibition of spore germination of *Aspergillus* spp., *Fusarium* spp. and *Scedosporium* spp. for (A) CIN-102, (B) amphotericin B (AMB) and (C) voriconazole (VRC). MIC, minimum inhibitory concentration.

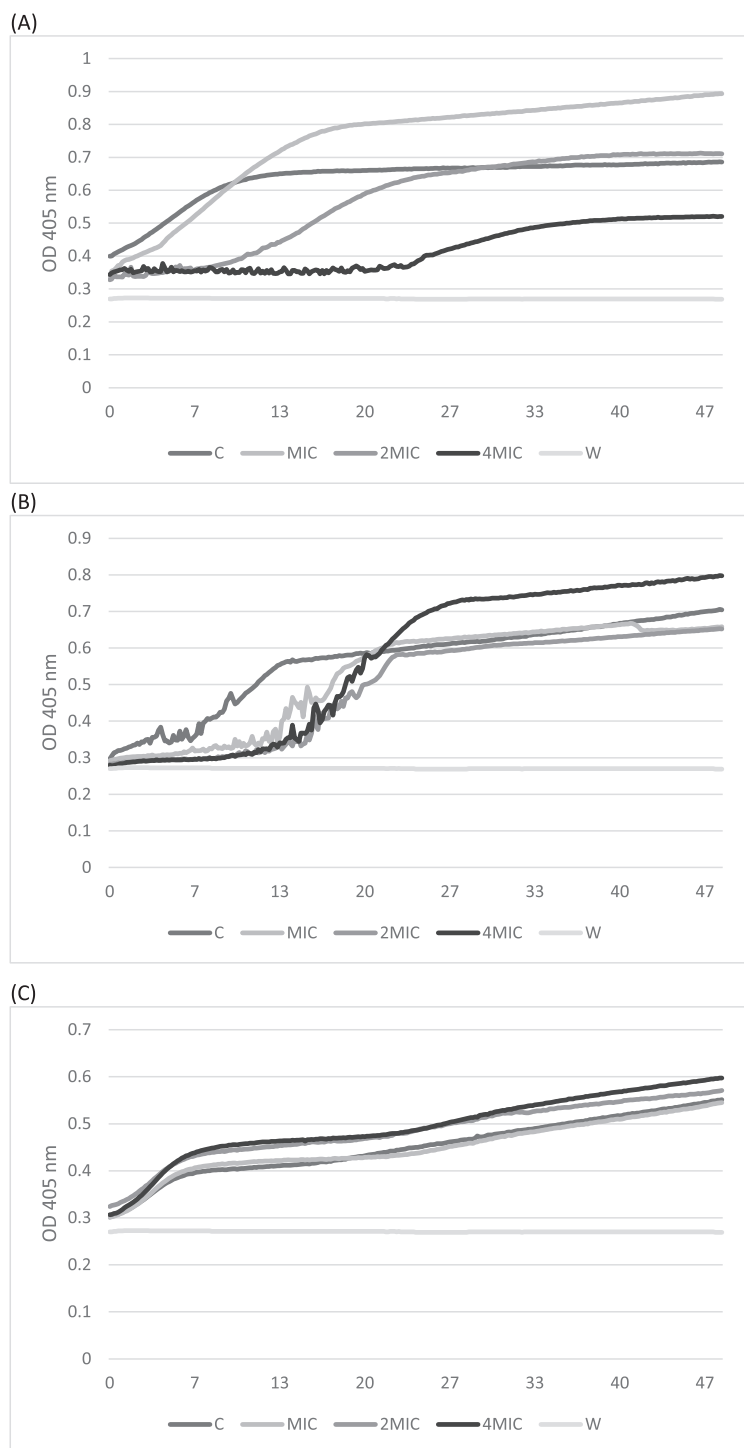


**Fig. 4.** Summary of the delays observed in the growth of strains in the presence of different concentrations of antifungal compared with the control for (A) CIN-102 and (B) amphotericin B. The maximum values were measured after 48 h of incubation. MIC, minimum inhibitory concentration.

possible mode of action of essential oils described in the literature, which are thought to act on the plasma membrane and cell wall [25]. The MICs obtained by the two methodologies, in liquid and solid medium, are comparable. This allows us to validate the model in liquid medium and to confirm the absence of bias due to the plastic film placed on the 96-well plate, which can limit the

passage of O<sub>2</sub>, reduce evaporation and could retain some CIN-102, which has hydrophobic characteristics.

During the CIN-102 study, we tested in parallel the MICs for CNMA on the same strains (data not shown). Although no significant difference existed between both MICs, CIN-102 appears to be a better candidate owing to lower toxicity compared with CNMA.



**Fig. 5.** Different profiles of post-antifungal effect: (A) dose-dependent (*Aspergillus* spp. and *Fusarium* spp.), (B) constant effect (*Scedosporium apiospermum*) and (C) no effect (*Lomentospora prolificans*). C0 serves as a positive control (no antifungal) and the blank (W) serves as a negative control. MIC, minimum inhibitory concentration.

Indeed, the compound CIN-102 corresponds to a mixture of different elements present in the essential oil of cinnamon, including CNMA, but genotoxic components have been removed [26–28]. We can therefore assume that CIN-102 is also effective but less toxic.

CIN-102 appears to have a fairly significant inoculum effect; however, it does not differ from that observed for AMB or VRC. Inhibition of spore germination is stronger for CIN-102 than for conventional antifungals, and only CIN-102 was capable of inhibiting almost 100% of spore germination. The low inhibition rate demonstrated for *Fusarium* spp. with VRC is consistent with clinical data

in the literature [29]. The spore has a virulence, resistance and dormancy factor, and current antifungals are not very effective against this form (in particular for *Fusarium* spp. and *L. prolificans*). CIN-102 would therefore be interesting because it achieves a level of inhibition much higher than other products. Moreover, CIN-102 would also have an impact on the cell wall of fungi based on the visual impact observed on spores. We also demonstrated no synergistic effect between VRC and AMB with CIN-102 for the strains tested, but no antagonistic effect either. A PAFE is observed with CIN-102 for all strains, except *L. prolificans*. For *A. fumigatus*, the



**Table 5**

Percentage optical density in the medium with antifungal compared with the control without antifungal

Concentration	<i>Fusarium solani</i>		<i>Fusarium dimerum</i>		<i>Aspergillus fumigatus</i>		<i>Aspergillus flavus</i>		<i>A. fumigatus</i> TR34		<i>A. fumigatus</i> TR46		<i>Scedosporium apiospermum</i>		<i>Lomentospora prolificans</i>	
	CIN-102	AMB	CIN-102	AMB	CIN-102	AMB	CIN-102	AMB	CIN-102	AMB	CIN-102	AMB	CIN-102	AMB	CIN-102	AMB
MIC	66.67	26.09	82.35	10.00	80.00	–	71.43	100	–	–	15.00	–	70.00	25.00	30.77	–
0.5 × MIC	66.67	65.22	88.24	67.50	80.00	58.82	71.43	100	100	41.94	70.00	68.97	75.00	50.00	38.46	80.00
0.25 × MIC	73.33	82.61	94.12	75.00	80.00	100	100	100	100	70.97	85.00	75.86	50.00	100	46.15	80.00

AMB, amphotericin B; MIC, minimum inhibitory concentration.

**Table 6**

Fractional inhibitory concentration index (FICI) for the tested strains

Combination	FICI							
	<i>Fusarium solani</i>	<i>Fusarium dimerum</i>	<i>Lomentospora prolificans</i>	<i>Scedosporium apiospermum</i>	<i>Aspergillus fumigatus</i>	<i>Aspergillus flavus</i>	<i>A. fumigatus</i> TR34	
CIN-102/VRC	2	1.5	2	1.5	1.5	1.5	2	
CIN-102/AMB	1.5	2	1.25	1.5	1.5	2	1.062	
CNMA/VRC	2	2	2	2	2	2	2	
VRC/CNMA	1.5	2	2	2	2	2	2	

VRC, voriconazole; AMB, amphotericin B; CNMA, cinnamaldehyde.

**Table 7**

Post-antifungal effect (PAFE) determined for CIN-102 against different fungal strains with 4 h of contact before removal

Strain	PAFE (h) <sup>a</sup>		
	1 × MIC	2 × MIC	4 × MIC
<i>Fusarium solani</i>	2	11	11
<i>Fusarium dimerum</i>	7	NT	23
<i>Scedosporium apiospermum</i>	9	9	9
<i>Lomentospora prolificans</i>	0	0	0
<i>Aspergillus fumigatus</i>	8	8	21
<i>Aspergillus flavus</i>	8	8	23
<i>A. fumigatus</i> TR34	8	18	21
<i>A. fumigatus</i> TR46	8	17	23

MIC, minimum inhibitory concentration; NT, not tested.

<sup>a</sup> The PAFE time was calculated in relation to the control.

PAFE is between 9–10 h after 4 h of contact with AMB (4 × MIC) [18] and ~1 h after 2 h of exposure to posaconazole [30]. Since the PAFE is generally greater when the antifungal is fungicidal, and our results approach those of AMB, we can hypothesise that the mode of action of CIN-102 may rather be fungicidal, but it is necessary to confirm this with other experiments [31].

## 5. Conclusion

Although the amount of antifungal required to reach the MIC is greater than with current antifungals, we have shown CIN-102 activity in all three genera, an inoculum effect and a PAFE. In addition, the effect on spore germination is interesting, and no resistance was observed. CIN-102 may therefore be of interest in the treatment of IFIs induced by strains resistant to current antifungal agents. It now remains to discover its mechanism of action and to confirm these properties in vivo using animal models.

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## Competing interests

None declared.

## Ethical approval

Not required.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2021.03.017.

## References

- [1] Bitar D, Lortholary O, Le Strat Y, Nicolau J, Coignard B, Tattévin P, et al. Population-based analysis of invasive fungal infections, France, 2001–2010. *Emerg Infect Dis* 2014;20:1149–55. doi:10.3201/eid2007.140087.
- [2] Blot M, Lanterrier F, Lortholary O. Epidemiology of visceral fungal infection in France and in the world [in French]. *Rev Prat* 2015;65:1318–21.
- [3] Rapp RP. Changing strategies for the management of invasive fungal infections. *Pharmacotherapy* 2004;24 4S–28S; quiz 29S–32S.
- [4] Robbins N, Caplan T, Cowen LE. Molecular evolution of antifungal drug resistance. *Ann Rev Microbiol* 2017;71:753–75. doi:10.1146/annurev-micro-030117-020345.
- [5] D'Agostino M, Tesse N, Fripiat JP, Machouart M, Debourgogne A. Essential oils and their natural active compounds presenting antifungal properties. *Molecules* 2019;24:3713. doi:10.3390/molecules24203713.
- [6] Shreaz S, Wani WA, Behbehani JM, Raja V, Irshad M, Karched M, et al. Cinnamaldehyde and its derivatives, a novel class of antifungal agents. *Fitoterapia* 2016;112:116–31. doi:10.1016/j.fitote.2016.05.016.
- [7] Liang D, Xing F, Selvaraj JN, Liu X, Wang L, Hua H, et al. Inhibitory effect of cinnamaldehyde, citral, and eugenol on aflatoxin biosynthetic gene expression and aflatoxin B1 biosynthesis in *Aspergillus flavus*. *J Food Sci* 2015;80:M2917–24. doi:10.1111/1750-3841.13144.
- [8] Morcia C, Tumino G, Ghizzoni R, Bara A, Salhi N, Terzi V. In vitro evaluation of sub-lethal concentrations of plant-derived antifungal compounds on *Fusaria* growth and mycotoxin production. *Molecules* 2017;22:1271. doi:10.3390/molecules22081271.
- [9] Khan MSA, Ahmad I. Antifungal activity of essential oils and their synergy with fluconazole against drug-resistant strains of *Aspergillus fumigatus* and *Trichophyton rubrum*. *Appl Microbiol Biotechnol* 2011;90:1083–94. doi:10.1007/s00253-011-3152-3.
- [10] Vidal-Acuña MR, Ruiz-Pérez de Pipaón M, Torres-Sánchez MJ, Aznar J. Identification of clinical isolates of *Aspergillus*, including cryptic species, by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). *Med Mycol* 2018;56:838–46. doi:10.1093/mmy/myx115.
- [11] Elizondo-Zertuche M, de J Treviño-Rangel R, Robledo-Leal E, Luna-Rodríguez CE, Martínez-Fierro ML, IP Rodríguez-Sánchez, et al. Molecular identification and in vitro antifungal susceptibility of *Scedosporium* complex isolates from high-human-activity sites in Mexico. *Mycologia* 2017;109:874–81. doi:10.1080/00275514.2017.1416260.
- [12] Sun S, Lui Q, Han L, Ma Q, He S, Li X, et al. Identification and characterization of *Fusarium proliferatum*, a new species of fungi that cause fungal keratitis. *Sci Rep* 2018;8:4859. doi:10.1038/s41598-018-23255-z.
- [13] Tippmann H-F. Analysis for free: comparing programs for sequence analysis. *Brief Bioinform* 2004;5:82–7. doi:10.1093/bib/5.1.82.
- [14] Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard—second edition. CLSI document M38-A2. Wayne, PA: Clinical and Laboratory Standards Institute (CLSI); 2008.
- [15] Gameda N, Woldeamanuel Y, Asrat D, Debella A. Effect of essential oils on *Aspergillus* spore germination, growth and mycotoxin production: a potential source of botanical food preservative. *Asian Pac J Trop Biomed* 2014;4(Suppl 1):S373–81. doi:10.12980/APJTB.4.2014C857.
- [16] Cardoso NNR, Alviano CS, Blank AF, Romanos MTV, Fonseca BB, Rozental S, et al. Synergism effect of the essential oil from *Ocimum basilicum* var. Maria Bonita and its major components with fluconazole and its influence on ergosterol biosynthesis. *Evid Based Complement Alternat Med* 2016;2016:5647182. doi:10.1155/2016/5647182.
- [17] de A Cordeiro R, Portela FV, Pereira LM, de Andrade AR, de Sousa JK, Aguiar AL, et al. Efflux pump inhibition controls growth and enhances antifungal susceptibility of *Fusarium solani* species complex. *Future Microbiol* 2020;15:9–20. doi:10.2217/fmb-2019-0186.
- [18] Vitale RG, Mouton JW, Afeltra J, Meis JFGM, Verweij PE. Method for measuring postantifungal effect in *Aspergillus* species. *Antimicrob Agents Chemother* 2002;46:1960–5.
- [19] Borman AM, Fraser M, Palmer MD, Szekely A, Houldsworth M, Patterson Z, et al. MIC distributions and evaluation of fungicidal activity for amphotericin B, itraconazole, voriconazole, posaconazole and caspofungin and 20 species of pathogenic filamentous fungi determined using the CLSI broth microdilution method. *J Fungi (Basel)* 2017;3:27. doi:10.3390/jof3020027.
- [20] Homa M, Fekete IP, Böszörményi A, Singh YRB, Selvam KP, Shobana CS, et al. Antifungal effect of essential oils against *Fusarium* keratitis isolates. *Planta Med* 2015;81:1277–84. doi:10.1055/s-0035-1546272.
- [21] Taj-Aldeen SJ. Reduced multidrug susceptibility profile is a common feature of opportunistic *Fusarium* species: *Fusarium* multi-drug resistant pattern. *J Fungi (Basel)* 2017;3:18. doi:10.3390/jof3020018.
- [22] Chen Y, Wang H, Lu Z, Li P, Zhang Q, Jia T, et al. Emergence of TR46/Y121F/T289A in an *Aspergillus fumigatus* isolate from a Chinese patient. *Antimicrob Agents Chemother* 2015;59:7148–50. doi:10.1128/AAC.00887-15.
- [23] Leonardelli F, Theill L, Nardin ME, Macedo D, Dudiuk C, Mendez E, et al. First itraconazole resistant *Aspergillus fumigatus* clinical isolate harbouring a G54E substitution in Cyp51A in South America. *Rev Iberoam Micol* 2017;34:46–8. doi:10.1016/j.riam.2016.05.005.
- [24] Chowdhary A, Kathuria S, Xu J, Sharma C, Sundar G, Singh PK, et al. Clonal expansion and emergence of environmental multiple-triazole-resistant *Aspergillus fumigatus* strains carrying the TR34/L98H mutations in the *cyp51A* gene in India. *PLoS One* 2012;7:e52871. doi:10.1371/journal.pone.0052871.
- [25] Bakkali F, Averbeck S, Averbeck D, Idaomar M. Biological effects of essential oils—a review. *Food Chem Toxicol* 2008;46:446–75. doi:10.1016/j.fct.2007.09.106.
- [26] Chiang S, Lee P, Lai M, Shen L, Chung W, Huang H, et al. Safrrole-2',3'-oxide induces cytotoxic and genotoxic effects in HepG2 cells and in mice. *Mutat Res* 2011;726:234–41. doi:10.1016/j.mrgentox.2011.09.014.

- [27] Maistro EL, de Souza Marques E, Fedato RP, Tolentino F, da Silva C de AC, Tsuboy MSF, et al. In vitro assessment of mutagenic and genotoxic effects of coumarin derivatives 6,7-dihydroxycoumarin and 4-methylscutletin. *J Toxicol Environ Health A* 2015;78:109–18. doi:[10.1080/15287394.2014.943865](https://doi.org/10.1080/15287394.2014.943865).
- [28] Maralhas A, Monteiro A, Martins C, Kranendonk M, Laires A, Rueff J, et al. Genotoxicity and endoreduplication inducing activity of the food flavouring eugenol. *Mutagenesis* 2006;21:199–204. doi:[10.1093/mutage/gel017](https://doi.org/10.1093/mutage/gel017).
- [29] Talas L, Szigeti ZM, Banfalvi G, Szeman-Nagy G. Effect of amphotericin B and voriconazole on the outgrowth of conidia of *Aspergillus fumigatus* followed by time-lapse microscopy. *AMB Express* 2019;9:43. doi:[10.1186/s13568-019-0769-6](https://doi.org/10.1186/s13568-019-0769-6).
- [30] Leung S, Poulakos MN, Machin J. Posaconazole: an update of its clinical use. *Pharmacy (Basel)* 2015;3:210–68. doi:[10.3390/pharmacy3040210](https://doi.org/10.3390/pharmacy3040210).
- [31] Manavathu EK, Ramesh MS, Baskaran I, Ganesan LT, Chandrasekar PH. A comparative study of the post-antifungal effect (PAFE) of amphotericin B, triazoles and echinocandins on *Aspergillus fumigatus* and *Candida albicans*. *J Antimicrob Chemother* 2004;53:386–9. doi:[10.1093/jac/dkh066](https://doi.org/10.1093/jac/dkh066).