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EPIGALLOCATECHIN-3-GALLATE ANTIMYCOTIC AND AZOLE RESISTANT MODULATOR POTENCIAL AGAINST RESISTANT FUNGI

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Master in Clinical and Laboratory Technologies

Lisbon, 2021

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Abstract

Systemic-opportunistic fungal infections caused by triazole-resistant fungi, including *Aspergillus fumigatus*, are an emergent cause of human disease worldwide, as *Aspergillus* spp. are one of the most prevalent fungi in occupational environments. The antifungal drugs resistance makes it imperative to assess new compounds with antimicrobial and/or synergistic properties, to be utilized as adjuvants in combination antifungal therapy. Epigallocatechin-3-gallate (EGCG), the main green tea constituent, has demonstrated anti-infective properties in several studies. The aim was to assess the antifungal potential of EGCG against triazole-resistant *Aspergillus fumigatus* isolates collected in different occupational settings, and the potential synergistic effects between ECGC and commonly used antifungal drugs – itraconazole (ITR), voriconazole (VOR) and posaconazole (POS). Triazole-resistant *Aspergillus fumigatus* isolates (N=3) were grown in Sabouraud dextrose 4% agar. Inoculum suspensions (OD=0.060) were prepared with EGCG at final concentrations of 250µg/mL, 100µg/mL, 50µg/mL, 25µg/mL and with no EGCG. Each suspension was spread onto RPMI-1640 medium and Sabouraud dextrose 4% agar supplemented with 4µg/mL ITR, 2µg/mL VOR, 0.5µg/mL POS or with no supplementation, and incubated at 34-37ºC for 7 days (adapted from the EUCAST E.Def 10.1 method). One isolate grew on RPMI-1640 supplemented with 2µg/mL VOR alone and with 25 and 50 µg/mL of EGCG, with no visible growth with EGCG concentrations above 100µg/mL, implying chemosensitization of the isolate. These results suggest that EGCG may be used as a possible adjuvant for combination antifungal therapy against triazole-resistant *Aspergillus fumigatus*. However further studies with greater sample sizes should take place before the translation of these results into clinically relevant strategies.

Keywords: Epigallocatechin-3-gallate; *Aspergillus fumigatus*; Antifungal resistance; Synergism

Resumo

Vários fungos resistentes aos triazóis, incluindo *Aspergillus fumigatus* são uma causa emergente de infeções sistémicas oportunistas, já que os *Aspergillus* spp. são dos fungos mais prevalentes em ambientes ocupacionais. O aumento da resistência aos antifúngicos torna imprescindível a avaliação de novos compostos com propriedades antifúngicas e/ou sinérgicas, para serem utilizados como adjuvantes na terapia fúngica combinada. A epigalocatequina-3-galato (EGCG), o principal constituinte do chá verde, demonstrou propriedades anti-infeciosas em vários estudos. Objetivou-se avaliar o potencial antifúngico da EGCG contra isolados de *Aspergillus fumigatus* resistentes a triazóis, colhidos em diferentes ambientes ocupacionais, e os potenciais efeitos sinérgicos entre a EGCG e os antifúngicos comumente usados – itraconazole (ITR), voriconazole (VOR) e posaconazole (POS). Os isolados de *Aspergillus fumigatus* resistentes a triazóis (N=3) foram cultivados em Sabouraud e preparadas suspensões fúngicas (OD=0.060) com EGCG em concentrações finais de 250µg/mL, 100µg/mL, 50µg/mL, 25µg/mL e sem EGCG. Cada suspensão foi inoculada em RPMI-1640 e Sabouraud suplementados com 4µg/mL ITR, 2µg/mL VOR, 0.5µg/mL POS ou sem antifúngico e incubada a 34-37ºC durante 7 dias (adaptado do método EUCAST E.Def 10.1). Um isolado cresceu em RPMI-1640 suplementado apenas com 2µg/mL VOR e na presença de 25 e 50 µg/mL de EGCG, mas não apresentou crescimento em concentrações de EGCG superiores a 100µg/mL, indicando sensibilização do isolado. Estes resultados sugerem que a EGCG poderá ser usada como potencial adjuvante na terapia antifúngica combinada contra *Aspergillus fumigatus* resistentes a triazóis. Não obstante, mais estudos com amostras maiores devem ser realizados antes da translação destes resultados para abordagens terapêuticas.

Palavras-chave: Epigalocatequina-3-galato; *Aspergillus fumigatus*; Resistência antifúngica; Sinergismo

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List of Abbreviations and Acronyms

5FC	5-fluorocytosine
ABC	ATP-Binding Cassette
ABPA	Allergic Bronchopulmonary Aspergillosis
AMB	Amphotericin B
ATP	Adenosine Triphosphate
bp	Base Pair
CLSI	Clinical and Laboratory Standards Institute
CPA	Chronic Pulmonary Aspergillosis
EBV	Epstein-Barr Virus
EGCG	Epigallocatechin-3-gallate
EFSA	European Food Safety Authority
EUCAST	European Antimicrobial Susceptibility Testing Committee
FLC	Fluconazole
FRPD	Filtering Respiratory Protection Devices
GMS	Grocott Methenamine Silver
H&E	Hematoxylin and Eosin
IA	Invasive Aspergillosis
IFI's	Invasive Fungal Infections
ITR	Itraconazole
KET	Ketoconazole
LDM	Lanosterol 14-α-demethylase
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight
MBIC	Minimum Biofilm Inhibitory Concentration
MCZ	Miconazole
MFS	Major Facilitator Superfamily
MIC	Minimum Inhibitory Concentration
MOPS	3-(N-morpholino) Propane-Sulfonic Acid
MPG	Mechanic Protection Gloves
MS	Mass Spectrometry
MST	Mean Survival Times
ncRNA	non-coding RNA
PAS	Periodic Acid-Schiff
PCR	Polymerase Chain Reaction
POS	Posaconazole

PTM	Post-translational Modification
RNAi	RNA interference
ROS	Reactive Oxygen Species
SAB	Sabouraud dextrose 4% agar
SCARE	Surveillance Collaboration on *Aspergillus* Resistance in Europe
SMIC	Sessile MIC
TR	Tandem Repeat
VOR	Voriconazole

1. Introduction

Antifungal resistance is a growing threat. It develops when antifungal drugs are used properly for long periods of azole treatment and prophylaxis or improperly to treat sick people in clinical settings (e.g., when dosages are too low, or when treatment courses are not long enough), and may even arise from the exposure of fungi to fungicides in agricultural practice (1,2). Systemic, opportunistic and invasive fungal infections (IFI's) caused by azole-resistant fungi, including *Aspergillus fumigatus*, are emerging as an important cause of human disease with associated high morbidity and mortality rates worldwide. Infections primarily occur in immunocompromised patients, such as those undergoing chemotherapy in health facilities. However, infections of otherwise healthy people are on the rise (3). This can be explained by the fact that the genus *Aspergillus* is one of the most prevalent regarding fungi in several highly contaminated occupational environments (4). Since fungi adapt to survive in warmer temperatures (5), global warming may further increase the prevalence of fungal disease in humans.

In this context, increasing resistance to the limited arsenal of antifungal drugs is a serious public health concern, becoming essential the development of new approaches and strategies to tackle antifungal resistance. This includes the assessment of new compounds with antimicrobial properties, to be utilized as adjuvants in antifungal pharmacologic therapy (6–8). Combination therapy with such compounds has become popular in clinical practice as a potential strategy to fight resistant fungal isolates (9). Epigallocatechin-3-gallate (EGCG), the main constituent of green tea, has demonstrated anti-infective properties in several *in vitro* studies against bacteria, virus and fungi (10). While there has been much research done on *Candida* spp., information regarding EGCG antimycotic activity against *Aspergillus fumigatus* is scarce. Moreover, as far as I know, there is no data available focused on triazole-resistant *Aspergillus fumigatus* strains and the potential synergistic interactions between EGCG and commonly used triazole antifungal drugs.

With this in mind, I aimed to assess the *in vitro* antifungal potential of EGCG against triazole-resistant *Aspergillus fumigatus* isolates collected during exposure assessment campaigns performed in the last ten years in Portugal and abroad in different work environments, including waste sorting plants, sawmills, dwellings, bakeries, swine farms and taxis, by air sampling and passive sampling using masks,

filters, electrostatic dust collector (EDC), among others. I also aimed to assess potential synergistic effects between increasing concentrations of EGCG and commonly used triazole antifungal drugs, namely itraconazole (ITR), voriconazole (VOR) and posaconazole (POS). To accomplish these goals a modification of the European Antimicrobial Susceptibility Testing Committee (EUCAST) method "Screening procedure E.Def 10.1 for the detection of azole resistance in *Aspergillus fumigatus* isolates using four-well azole-containing agar plates" (11) was used.

First of all, an overview of state-of-the-art topics was conducted in Chapter 2. "Literature Review", giving a clear picture of the current knowledge about the *Aspergillus fumigatus*, its azole-resistance mechanisms, the antifungal drugs used against it, and the health-promoting effects of EGCG and its potential effects against azole-resistant *A. fumigatus* and other fungi. In Chapter 3. "Methodology" aside from research design and sampling methods, I discussed the adaptations made to the EUCAST method used to do the present research. The results are presented and discussed in Chapter 4. Finally, the conclusions of the study are drawn in the last chapter.

2. Literature Review

In this chapter I provide an overview of the current knowledge about three main topics. I start by exploring the kingdom fungi, narrowing it down to *Aspergillus fumigatus* and its laboratory diagnosis. Then I synthesize the main antifungal agents used against this microorganism, also identifying relevant theories that may explain the emergence of triazole-resistance among *A. fumigatus*. Finally, EGCG's origin, health benefits against *A. fumigatus* and other fungi, but also disadvantages to its medical and dietary use are scrutinized.

2.1. Fungi Classification, Diagnostic and Occupational Burden

Fungi are eukaryotic organisms that contain a well-defined nucleus, mitochondria, Golgi bodies, and endoplasmic reticulum, and are classified in their own separate kingdom – Kingdom Fungi. They are distinguished from other eukaryotes by a rigid cell wall composed of chitin and glucan and a cell membrane in which cholesterol is substituted for ergosterol as the major sterol component (12). Taxonomic classification of fungi does not always meet consensus among experts. Until now it was heavily based on morphology and mode of spore production, but the advances in genome sequencing methodologies have resulted in a revolution in fungal taxonomy based on a phylogenetic approach to species recognition that relies on comparative analysis of variable nucleic acid characters to define a fungal species.

Fungi can exist either in a unicellular form (yeast), which can replicate asexually by budding or fission and form pseudohyphae or in a multicellular filamentous form (mold), which can replicate asexually and sexually. Molds are organisms consisting of tubular structures called hyphae (coenocytic or septate) that together produce the mycelium. When growing on agar, the hyphae may be termed vegetative or aerial, depending if it grows on or above the surface of the culture medium, respectively. The latter may produce structures known as conidia (asexual reproductive elements), either by blastic (budding) or a thallic process, in which hyphal segments fragment into individual cells or arthroconidia. These conidia can become airborne and disseminate the fungus. Some fungi of medical importance have a mold form in the environment and a spherical form in the body at 37°C – dimorphic fungi (12).

Fungi reproduce by the formation of spores that may be sexual (meiosis) or asexual (mitosis). The form that produces sexual spores is termed the teleomorph (most

often in the human body), and the form producing asexual spores is termed the anamorph (most often in culture). Asexual spores consist of two types: sporangiospores (produced in a containing structure or sporangia) and conidia (borne naked on specialized structures) as seen in *Aspergillus* spp. Sexual reproduction leads to the development of ascospores, produced in a specialized saclike structure – the ascus (12).

2.1.1. *Aspergillus* spp.

The fungi belonging to the phylum Ascomycota (Ascomycetes) produce both sexual and asexual spores, and account for most of the medically important fungi. This phylum is divided in four classes: Pneumocystidomycetes; Saccharomycetes; Sordariomycetes; and Eurotiomycetes. The latter comprises several taxonomic orders, including the Eurotiales, which contain the teleomorphs of the anamorphic mold genera *Aspergillus* spp. (12). The genus *Aspergillus* contains about 400 species divided into 8 sub-genera (*Aspergillus*, *Fumigati*, *Circumdati*, *Candidi*, *Terrei*, *Nidulantes*, *Warcupi*, and *Ornati*), which in turn are subdivided into several sections or species complexes (13), but less than 20 are known to cause disease in humans (14). *Aspergillus* species are saprotrophic fungi that obtain their nutrition by decomposing organic matter, such as dead plants or animals, and are widely distributed throughout the environment (15,16).

2.1.2. *Aspergillus fumigatus*

Aspergillus fumigatus, the most common *Aspergillus* species, is a major opportunistic fungal pathogen and often causes disseminated infections in immunocompromised hosts, with a relatively high mortality. These includes aspergillosis – a broad spectrum of diseases comprising allergies, invasive aspergillosis (IA), pulmonary aspergilloma/Chronic Pulmonary Aspergillosis (CPA), and allergic bronchopulmonary aspergillosis (ABPA) (17) (see Table 2.1.). Less commonly non-*fumigatus Aspergillus* species such as *Aspergillus flavus*, *A. niger*, *A. terreus*, *A. clavatus* and *A. nidulans* can cause these diseases too (16). All the mentioned species actually belong to a species complex that contain morphologically indistinguishable cryptic species, some of which may exhibit important antifungal resistance profiles and pathogenic features (12), according to molecular taxonomic studies. *Aspergillus* section *Fumigati* (*A. fumigatus* complex) contains at least 25 different species, with 7 anamorphs other than *A. fumigatus* itself (18). It is permissible to refer to a fungus only by its asexual designation if that is the form usually obtained in culture so, from hereon, it'll be referred to as *Aspergillus fumigatus*.

Table 2.1. Clinical syndromes associated with *Aspergillus* spp. species

Type of Aspergillosis		**General Description and Clinical Manifestation**	**Ref.**
Allergies	Allergic Rhinitis	Nasal obstructions, pleuritis, rhinorrhea, and sneezing	
	Allergic Sinusitis	Occurs when *Aspergillus* spp. hyphae causes inflammation in the sinuses and symptoms of sinus infection (drainage, stuffiness, headache), but doesn't cause infection nor tissue invasion.	(14,19)
	ABPA	Occurs due to hypersensitive bronchial lumen reaction to spore and hyphal surface antigens, which leads to persistent inflammation in the lungs and allergy symptoms (coughing, wheezing), but doesn't cause an infection.	(14,19)
	Hypersensitivity Pneumonitis (extrinsic allergic alveolitis)	Occurs when repeated conidia inhalation leads to irreversible lung damage, stimulating antigen-induced lymphocytes and precipitating antibodies.	(14)
Mycoses	Cutaneous (skin) Aspergillosis	Occurs when *Aspergillus* spp. enters the body through a break in the skin (*e.g.*, after surgery or burn wound) of immunocompromised patients and causes infection, or if IA spreads to the skin from somewhere else in the body (*e.g.*, lungs).	(19)
	Mycetoma	Occurs when localized cutaneous and subcutaneous tissues fungal infection leads to locally invasive tumor-like abscesses that rupture resulting in ulcers, swelling, and distortion of the infected part of the body.	(14)
	Pulmonary Aspergilloma/CPA	Occurs when long-term (≥3 months) *Aspergillus* spp. infection forms one or more balls of hyphae plus host cells, tissue debris, *etc.* in cavities (aspergillomas) within the lungs, but doesn't invade surrounding lung tissue.	(14,19)
	IA	IA usually affects people who have weakened immune systems, such as those who have had an organ or a stem cell transplant. It starts from the primary focus of infection, which is usually the lower respiratory tract, resulting from inhaled spores, though less commonly invasion can be via the sinuses or skin through catheter insertion sites etc. From the primary focus, *Aspergillus* spp. invades blood vessels and spreads to other organs, particularly the brain.	(14,19)

(continuation)

Mycotoxins	Gliotoxin	Inhibits macrophage phagocytosis, as well as T-cell activation and proliferation, contributing to *A. fumigatus* virulence in IA patients.	(14,20)
	Aflatoxins (B1, B2, G1, G2)	Produced by *A. flavus*, *A. parasiticus*, and *A. nominus*. May manifest as acute (hepatitis) or chronic disease (hepatic cancer), being the most active natural carcinogenic substances known.	(14)

A. fumigatus is a ubiquitous pathogen due to a number of reasons: (i) its ability to survive and grow in a wide range of environmental conditions, (ii) its effective dispersal in the air, (iii) its physical characteristics that allow conidia to reach the distal airways, and (iv) its swift adaptability to the host environment (21). Although *A. fumigatus* optimally grows at 37ºC and a pH 3.7 to 7.6, it can be isolated in the wild wherever decaying vegetation and soil temperatures range between 12ºC and 65ºC (21). This ability to survive and grow in a wide range of environmental conditions is, in part, due to the presence of successful defense systems such as the production of potent secondary metabolites (gliotoxin) and efflux pumps (ABC transporters), and the presence of melanin in their cell wall, which protects the fungus from ultraviolet irradiation (21).

Under adverse conditions, *A fumigatus* produces conidia (spores) on specialized hyphal structures called conidiophores, by asexual reproduction (budding from a mother cell) (12,16). Ascospores, the propagules produced in the sexual cycle, are produced in a structure called ascus under very specific non-physiologic circumstances. The ascospores germinate after heating at 70ºC for 30 min, a condition never found on the human body, and are therefore unlikely to initiate aspergillosis (12,16,21). *A. fumigatus* conidia are dispersed more efficiently in the air than those of most other molds because of the small size of their spores (2-4μm) and their hydrophobic characteristics conferred by the surface rodlet layer encoded by the *rodA* gene (14,21). Once inhaled, airborne conidia does not typically colonize de human respiratory tract and are readily cleared of the immunocompetent host by cilia on airway epithelial cells and resident alveolar macrophages (16). Although fairly uncommon in healthy hosts, *A. fumigatus* is an opportunistic pathogen that generally cause infection when defects in the host immune system allow conidia to penetrate, colonize, and reproduce in the host (12). Immunodeficiency caused by chemotherapy or immunosuppressive drugs after hematopoietic stem cell transplantation; to prevent graft rejection after solid organ transplantation; or to treat autoimmune diseases contribute to an increased incidence of *Aspergillus*-related disease. This individuals are unable to clear the lungs from inhaled

conidia that readily germinates into short hyphae known as germ tubes, within 4-6h at body temperature, since 37ºC is the optimum temperature for both germination and growth (16,21). In such scenarios, the germinated conidia form a colony composed of septate multinucleated cells (hyphae) embedded in an extracellular matrix (ECM) to form a biofilm. The cells efficiently sense and respond to the altered host environment, adapting their physiology by utilizing a coordinated gene expression program that enables adaptation to cation (iron, zinc and calcium) limitation, nitrogen and glucose deprivation, hypoxic and alkaline stresses, and other unfavorable conditions (16,21). Left untreated, hyphae secrete proteases and may invade pulmonary epithelium and vasculature, leading to thrombosis and infarction of tissue and to hematogenous dissemination to other target organs, particularly the brain (12).

According to some authors, in patients with high-risk conditions, including stem cell and organ transplant recipients, IA (even without azole-resistance) mortality exceeds 50% (22). An understanding of the occupational hazard posed by *Aspergillus* spp. and an early IA diagnosis can help reduce this burden.

2.1.3. Laboratory Diagnosis of Invasive Aspergillosis

Regardless of where the exposure to the conidia takes place, once a person is infected, a confirmed diagnosis of IA is only possible with laboratory testing. However, getting good answers from the laboratory is dependent upon the quality of the clinical sample, specimen selection and collection timing. When selecting specimens for culture and microscopic examination, attention should be given to the information obtained from clinical examination and radiographic studies. An adequate amount of clinical material must be collected aseptically or after proper cleaning and decontamination of the site to be sampled, preferably before antifungal therapy is given (12,23).

Direct microscopy examination is considered the most rapid and cost-effective means for the detection of hyphal structures directly in clinical specimens. It serves to guide the laboratory in selecting the most appropriate means to culture the specimen and is helpful in determining the significance of culture results. A number of different stains and microscopic techniques may be used to detect and characterize *Aspergillus fumigatus* directly in clinical samples, but the ones used most often include Gram stain, Hematoxylin and Eosin (H&E), Periodic Acid-Schiff (PAS) and Grocott Methenamine Silver (GMS) (12).

Nonetheless, the most sensitive and usually conclusive means of establishing the diagnosis of a fungal infection is to isolate in culture the fungus from a patient sample.

When suspected of IA, respiratory samples (sputum, bronchoalveolar lavage, etc) should be cultured. General purpose media commonly used for fungal culture is Sabouraud dextrose 4% agar (SAB), but selective culture media can be used directly on specimens to select the growth of *Aspergillus* species. After incubation, and if colonies sporulate, they should be inspected carefully for presumptive identification to at least genus level according to their macroscopic and microscopic morphology (see Table 2.2.) using lactophenol cotton blue (24). The detection of invasive fungal infection by blood culture in special bottles within an automated system is an important measure in diagnosing IA. Although contamination of blood cultures with a fungus may take place, for the most part, blood cultures positive for fungi within 5 to 7 days are significant (12).

Table 2.2. Characteristic features of *A. fumigatus* in clinical specimens and cultures (12)

Microscopic Morphologic Features in Clinical Specimens	**Morphologic Features in Culture**	
	Macroscopic	**Microscopic**
Septate, dichotomously branched hyphae of uniform width (3-6 μm)	Blue-green to gray	Conidiophores with enlarged vesicles covered with phialides Hyphae are hyaline and septate

Until recently, only a positive microbiology culture could truly define a fungal infection. However, this could take days, if not weeks. With improved diagnostics now available, rapid, sensitive, and specific confirmation of the cause of a fungal infection is possible by immunologic, biochemical (serological), molecular (polymerase chain reaction [PCR]), and/or proteomic methods of microorganism detection and identification. Antigen or antibody tests for *Aspergillus* spp. may be useful in diagnosing infection and allergy but are most important in managing the patient's response to therapy. Commercially available antigen tests are directed against specific *Aspergillus*-synthesized and released carbohydrates into body fluids, such as cell wall components (galactomannan, 1,3-β-glucans or chitin) or metabolites (D-mannitol) (25). Several test formats are used for the detection of antibodies in patient's serum including immunodiffusion enzyme-linked immunosorbent assay or, less commonly, hemagglutination and radioimmunoassay (26).

The molecular revolution in biology have allowed the for the description of new cryptic species among the *Aspergillus* genera, which are almost impossible to differentiate by classic morphological tools and some are more resistant to antifungal drugs (13). Alastruey-iziquierdo and colleagues (2012) (13) recommend performing the identification of such cryptic species by β-tubulin sequencing or matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) (13), which uses species-specific patterns of peptides and protein masses to identify microorganisms (12,24). As these techniques cannot detect strains with secondary resistance, they also advise performing antifungal susceptibility testing of the isolates routinely to assess their antifungal susceptibility profile *in vitro*, for better management of antifungal therapy (13).

A simple, fast and sensitive diagnosis of IA is key to an early antifungal treatment course. Therefore, innovative diagnostic methods may very well contribute to an increase in the survival of patients who suffer from this burden.

2.1.4. *Aspergillus fumigatus* Occupational Burden

The presence of fungal species in occupational settings has attracted increasing attention, as it represents an occupational burden for workers who are exposed to the conidia, increasing risk of infection, especially in those immunocompromised hosts. Owing to the *A. fumigatus* ability to survive and grow in a wide range of environmental conditions, is it globally distributed and there have been previous reports of *A. fumigatus* isolated from the air and surfaces of several occupational environments in Portugal and abroad. In their study, Viegas et al (2017) (4) reported the highest prevalence of *Aspergillus* spp. were found in wastewater (69,3%) and waste (34,8%) treatment plants and poultry feed industry (6,3%), even though it was also prevalent in cork industry (0,9%), slaughter houses (1,6%) and swine farms (7,4%) (4). Other studies also found wild and resistant *A. fumigatus* in bakeries (27,28), dwellings, veterinary clinics, dairy farms (28), taxis (28,29), industrial sawmills from Norway (30), and captive birds (zoos, pets and poultry) from Australia (31); apart, of course, from clinical biological samples (sputum, bronchial secretions, bronchoalveolar lavage, pulmonary tissue, etc) and hospitals indoor air and surfaces (32,33).

There are several air and surface sampling methods for fungal assessment. The most used direct air sampling method is the Millipore Air Tester using a pre-determined flow rate (L/min), and impacted directly onto culture media plates, according to the manufacturer's instructions (4,33). Sampling of settled dust into a sterilized bag (27,33),

from filtering respiratory protection devices (FRPD), mechanic protection gloves (MPG) or electrostatic dust collector (EDC) comprise the passive sampling air methods (33). The latter collection device is increasingly being used because it is electrostatic, inexpensive, easy to obtain, and effective at collecting dust (27). As of surface sampling techniques, most researchers apply the methods described in the ISO 18593, which specifies horizontal methods for sampling techniques using contact plates, stick swabs, sponges and cloths on surfaces in the food chain environment (4,33,34).

2.2. Triazole Antifungal Agents

Antifungal therapy has undergone a tremendous transformation in recent years. Since the mid 1950s, with the domain of the amphotericin B (AMB) and 5-fluorocytosine (flucytosine, 5FC), which are toxic and difficult to use, several new, systemically active agents and new formulations of older agents with proven antifungal activity have been discovered (12). Currently, four antifungal drug classes are used by clinicians for systemic treatment of IFI's: (i) polyenes (AMB), (ii) azoles, (iii) echinocandins and the (iv) pyrimidine analogue 5FC (35), each of them with particular targets and mechanisms of action. The azole class of antifungals is divided in terms of structure into imidazoles (two nitrogens in the azole ring) and triazoles (three nitrogens in the azole ring) (see Table 2.3.). As *A. fumigatus* is intrinsically resistant to the commonly used triazole fluconazole (FLC) (36), specific first-line antifungal therapy of IA usually involves the administration of first-generation triazole – ITR – and/or one or both the second-generation ones – VOR and POS – which have improved pharmacological profiles. Triazoles present broad-spectrum activity but suffer from erratic absorption and have many drug interactions. The most common serious side effect, hepatotoxicity, occurs often with VOR in up to 31% of the cases (12,35).

Table 2.3. Triazoles Characteristics (12,35)

Triazole	Route	Chemical Structure
ITR	Oral	
VOR	Oral IV	
POS	Oral IV	

The mechanism of action of triazoles is based on the inhibition of the ergosterol biosynthetic pathway by targeting the microsomal cytochrome P450 (CYP450) monooxygenase-dependent enzyme – lanosterol 14-α-demethylase (LDM) (see Figure 2.1.). LDM is encoded by the *cyp51A* gene in filamentous fungi (35,37) and is essential for converting lanosterol to ergosterol, a key component of the fungal cell membrane. The demethylation of fungal lanosterol is a two-step process involving the reduced form of nicotinamide dinucleotide phosphate and oxygen. As nitrogen from the triazole ring binds to the LDM heme iron, oxidation of the lanosterol methyl group is prevented, inhibiting the ergosterol production. The accumulation of toxic sterol intermediates, in combination with ergosterol depletion, results in the disruption of the cell membrane, hence the fungistatic effect (35).

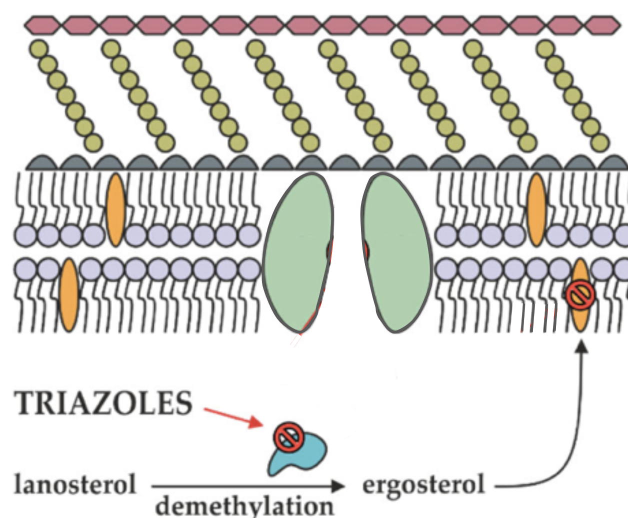

Figure 2.1. Triazoles mechanism of action (35)

2.3. Triazole-Resistant *Aspergillus fumigatus*

Triazole antifungals have greatly improved survival, however, the emergence of triazole-resistant *A. fumigatus* infections has been growing worldwide and is associated with increased treatment failure and mortality. A prospective multicenter international surveillance study of azole resistance in *A. fumigatus*, conducted by Surveillance Collaboration on *Aspergillus* Resistance in Europe (SCARE) network (38), reported a prevalence of azole resistance among patients with *A. fumigatus* species complex isolates ranging from 0.0%–26.1% among the centers, settling an overall prevalence of 3,2% of the total number of strains analyzed. Of the 195 cases with IA, azole-resistance was documented in 5,1%, with a case-fatality rate for this cohort of 70%. Yet another five-year single-center prospective survey of *Aspergillus* species isolated from clinical specimens in Japan (39) showed a 3% prevalence of azole-resistant isolates, among the *A. fumigatus*.

Azole resistance is mainly caused by two mechanisms: (i) mutations of the target protein and the (ii) overexpression of efflux pumps. Several single-point mutations in the *cyp51A* gene, that lead to amino acid substitutions (single or multiple amino acid changes) at codons G54, G138, G448, P216, F219 and M220 have been described (2,40). These mutations may directly block the entry or modify the LDM heme binding site of the drug, thereby reducing affinity of drug-enzyme interaction (17). However, the most common pan-azole-resistance mutations are combinations of tandem repeat (TR) sequences of different sizes inserted into the promoter region of *cyp51A* associated, or not, with point mutations in the gene (2), causing significant overexpression of the

cyp51A referring with azole resistance (17). It could be either amino acid changes of tyrosine to phenylalanine at codon 121 and threonine to alanine at codon 289, coupled with a TR of a 46-base pair (bp) sequence in the promoter region (TR_{46}/Y121F/T289A) (41,42); a 34-bp-long TR in the promoter region associated with a leucine-to-histidine substitution in codon 98 (TR_{34}/L98H) (43), which is associated with an 8-fold increase in the gene expression (36); or even a single 53-bp long TR in the promoter region (TR_{53}) with no mutations in the *cyp51A* gene (40,42).

Several reports show a predominance of the TR_{34}/L98H mechanism. The previously mentioned SCARE study (38) reported a 48,9% prevalence. This mechanism was present in 30 of 32 (94%) ITR resistant isolates of Snelders et al (2008) study (43). Another report by van der Linden et al (2013) (42) stated that 47 of 63 patients (74.6%) harbored the TR_{34}/L98H resistance mechanism and only 13 patients (20.6%) the TR_{46}/Y121F/ T289A (42). In Portugal, the first report of environmental isolates bearing the TR_{34}/L98H mutation, isolated from occupational environments, was published in 2021 (33).

Overexpression of efflux pumps account for the majority of the non-*cyp51*-mediated azole resistance. Many important efflux pumps belong to adenosine triphosphate (ATP)-binding cassette (ABC) transporters (AtrF and AtrI) or members of major facilitator superfamily (MFS) – MdrA (16,17). ABC transporters are composed of two transmembrane and two cytoplasmic nucleotide-binding domains, and use ATP to pump azoles and/or toxic metabolites out of the fungal cell (2), and MFS proteins cross the cell membrane by proton gradients (17). Increased expression of transporters is also associated with phenotypic resistance in *A. fumigatus*, since there is a decreased amount of intracellular drug available to bind to its target.

Two hypotheses have been proposed to explain the origin of triazole resistance. It could be due to (i) improper use of triazole drugs to treat sick people in clinical settings (*e.g.*, when dosages are too low, or when treatment courses are not long enough), or even when antifungal drugs are used properly for long periods of azole treatment and prophylaxis – "non-fungicide-driven" resistance mechanisms (1,2). It could have also arisen (ii) from the extensive use of triazole fungicides in agriculture to prevent and treat fungal diseases in crops – fungicide-driven resistance mechanisms (1). The use of triazole fungicides in agriculture is associated with TR-mediated resistance mechanisms (2). Astoundingly, the use of certain azole fungicides, which were shown to induce the TR_{46}/Y121F/T289A resistance mechanism (41), was approved in Europe in the 1990s, and only 10 and 20 years later the first clinical isolate harboring the TR_{34}/L98H (43) and

TR_{46}/Y121F/T289A (42) mutations were described, respectively. This environmental stress led to natural selection of triazole-resistant *A. fumigatus* in the environment which is able to infect the susceptible population (43). Several reported cases of triazole-resistant IA developing in triazole-naïve human patients (2) support the environmental origin. This fungicide-driven mechanism is thought to be widespread worldwide (44).

To distinguish whether microorganisms are resistant to antifungals or not, the EUCAST established limit values expressed as minimum inhibitory concentrations (MIC's), indicating the minimum concentration of the drug that inhibits the growth of fungi. According to the most recent EUCAST (45) definition, a microorganism can be categorized as "S" (susceptible under standard dosing regimen), "I" (susceptible to increased exposure by adjusting the dosing regimen or its concentration) or "R" (resistant even when there is increased exposure) (45). Several *in vitro* studies have reported that triazole-resistant *A. fumigatus* harboring any of the above-mentioned resistance mechanisms, show elevated EUCAST MIC values against all triazoles (see Table 2.4.). Importantly, TR-mediated mechanisms confer resistance to all mold-active medical triazoles without incurring a fitness cost or survival disadvantage to the fungus (22).

Table 2.4. *In vitro* susceptibility profile of azole-resistant *A. fumigatus* isolates to triazoles

MIC Breakpoints (µg/mL)			**Mutation**	**Ref.**
ITR	**VOR**	**POS**		
1	1	0,125	*wild type*	(46)
8	8	1	TR_{46}/Y121F/T289A	(41)
4	8	1	G448	
2	>16	0,5	TR_{46}/Y121F/T289A	(42)
8	4	1	TR34/L98H	(33)
>8	>8	>1	TR_{46}/Y121F/T289A	(47)

Values determined by the EUCAST standardized broth microdilution method are shaded

As mentioned earlier, IA mortality exceeds 50%, even in patients infected with azole-sensitive *A. fumigatus* (22). Triazole antifungals have greatly improved survival, however, triazole-resistant *A. fumigatus* infections are increasingly reported worldwide and are associated with increased treatment failure and mortality rates reaching an outstanding 88% for patients with triazole-resistant IA (48). The poor outcome for this patients, along with the common serious side effects, namely hepatotoxicity, that occurs most often with VOR (in 31% cases) (35), and the increasing resistance to the limited

arsenal of antifungal drugs make the assessment of new compounds with antimicrobial properties, an imperative matter to tackle the antifungal resistance crisis.

2.4. Epigallocatechin-3-gallate

EGCG is the most abundant (~60%) and active green tea catechin (49). Tea is one of the oldest and most popular beverages consumed across the world. All teas come from the leaves of the same plant (*Camellia sinensis*), that is mainly grown in sub-tropical regions such as Japan, China and Taiwan (50). There are four main types of tea produced, determined by how the tea leaves are processed, specifically by drying and fermentation methods – green, white, Oolong, and black tea. Green tea is produced when the mature leaves are picked up, quickly dried and applied heat to prevent any fermentation and preservation of polyphenols (49,51). White tea is processed the least and uses very young leaves and leaf buds. Oolong tea is produced by partially fermenting the leaves, and black tea by fully fermenting them (52). The chemical composition of green tea includes an extensive range of compounds (50), but the ones who account for the aroma and health benefits of green tea are the polyphenols (53). The most pertinent polyphenols are the flavonoids and in turn catechins, that comprise 80-90% of the flavonoids and around 30-40% of the water-soluble solids in green tea (52,54). These polyphenols are present in much higher concentrations in green tea than black, white or oolong tea (50,53), mainly because of the way it is processed after harvesting. Among the varieties and brands of green tea infusions and green tea extracts consumed, there is some variation in catechin content due to a number of factors such as growth conditions, processing techniques and brewing temperature and length (52).

Among the biologically active compounds contained in *C. sinensis*, catechins stand out since they exhibit several health-promoting properties. Catechins contained in tea include epicatechin, epigallocatechin, epicatechin-3-gallate, and epigallocatechin-3-gallate (EGCG) (50). The latter is the most abundant (~60%) and active one (49). The basic structure of catechins involve a gallate moiety, esterified at the 3rd position of the C ring, a catechol group (3,4,5-tri- hydroxy groups) on the B ring, and hydroxyl groups at the 5th and 7th positions on the A ring (see Figure 2.2.) (54). The antioxidant activity potential of catechins depends mainly on the chemical structure, specifically on the number of hidroxyl groups and their distribution (50). The more hydroxyl groups, the more effective a free radical scavenger the catechin becomes. The structure of EGCG is an ortho-benzoyl benzopyran derivative, which has more hydroxyl bases than its

counterparts. Therefore, EGCG has the potential ability to scavenge free radicals because of the presence of the C ring gallate group (54).

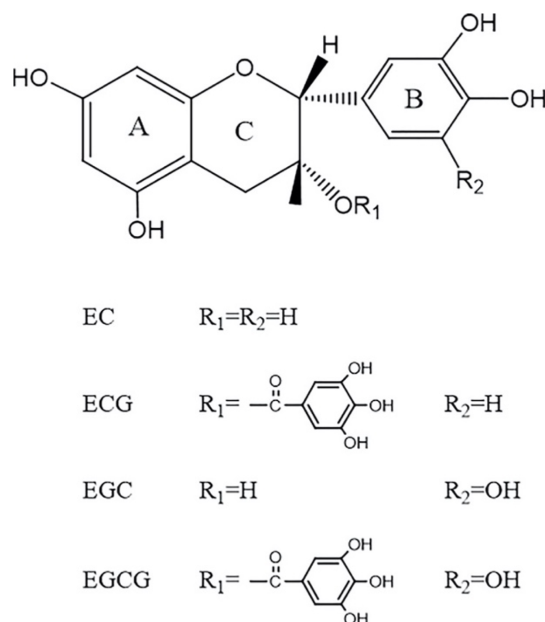

Figure 2.2. Chemical structure of the catechin components in green tea (54)

2.4.1. Health-Associated Effects of EGCG

Extensive research has shown that catechins – particularly EGCG – have a wide range of health-related benefits and therapeutic potential against various human diseases (see Table 2.5.).

Table 2.5. Health benefits of EGCG

Health Benefit	Mechanism of Action	Ref.
Antioxidant	The phenolic hydroxyl group provides hydrogen and undergoes oxidation reactions, scavenging reactive oxygen species (ROS)	(54,55)
Anti-neoplastic	Inducing apoptosis by inhibition of the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway	(55)
	Downregulation of PI3K/AKT, NF-κB, and Wnt signaling pathways, to inhibit cellular proliferation and inflammation and induce apoptosis	
	Protection against epithelial-mesenchymal transition (EMT) via an increase in the expression of E-cadherin, suppressing cell metastasis	
Anti-inflammatory	Suppression of NF-κB activation, downregulating expression of inflammatory cytokines and inflammation-related enzymes including TNF-α, IL-1β, COX-2, and MMP-9	(56)

(continuation)

Anti-obesity	Downregulation of genes involved in the synthesis of *de novo* fatty acids (*acc1, fas, scd1, c/ebpβ, pparγ,* and *srebp1*) and upregulation of genes associated with lipolysis (*hsl*) and lipid oxidization in adipose tissue of mice	(57)
Anti-diabetic	Stimulation of glucose uptake in response to insulin, by increasing expression of antioxidant enzymes (SOD, GPx) and phosphorylation of insulin receptor substrate-1 (IRS-1)	(58)
Hypotensive Effects	Attenuation of salt-sensitive hypertension via anti-oxidant, anti-inflammatory, and apoptosis-inducing effects on fibroblasts mediated by 67 kD laminin receptor	(59)
Fertility Improving	Improves sperm motility and viability and enhances oocyte and embryo quality, as well as fertilization and clinical pregnancy rates in females	(54)

2.4.2. Anti-infectious Effects of EGCG

Besides those broad health benefits, EGCG has also been shown to have specific anti-infectious properties and thus effective against a number of viruses, parasites, bacteria, fungi, and even prions. These topics are discussed next.

EGCG acts against virus by (i) inhibiting the virus from binding to and entering host cells; (ii) inhibiting viral RNA and DNA synthesis and viral gene transcription; (iii) destroying and functionally altering various viral molecules; and (iv) preventing infection with viruses in human and animal patients. Concerning prions, EGCG inhibits the β-sheet prions from changing the α-helical forms and could induce reversal of the β-sheet forms back to α-helical forms (52). Most studies showed antiviral properties within physiological concentrations of EGCG *in vitro* (10). Table 2.6. exemplifies some of the antiviral properties of EGCG against different viruses and prions, its mechanisms of action and MIC's necessary for either virustatic or virucidal activity.

Table 2.6. Antiviral properties of EGCG

Microorg.	**Mechanism of Action**	**MIC's**	**Effect**	**Ref.**
HIV Type-1	Inhibits virus entry and infection by binding to CD4 receptors.	MIC_{50}= 0,5-5,4 μg/mL MIC_{100}= 17-108 μg/mL	Virustatic	(60)
Epstein-Barr Virus (EBV)	Inhibits the lytic infection of EBV by decreasing the phosphorylation, and therefore suppressing, the activation of MEK/ERK1/2 and PI3-K/Akt signaling.	MIC_{50}=20 μM	Virustatic	(61)

(continuation)

Hepatitis B Virus	After treatment for 6 d, EGCG significantly inhibits the secretion of HBsAg and HBeAg by 53% and 44%, respectively.	MIC_{50}= 50 µg/mL	Virustatic	(62)
Yeast Prion Protein Sup35	EGCG binds to several regions within the unstructured N domain preventing the acquisition of β-sheet structure of select Sup35 prion strains.	*nd*	Virustatic	(63)

nd, not defined

Some interesting completed and ongoing clinical trials regarding the effects of EGCG against influenza virus have revealed that green tea catechins are able to prevent influenza infection. A randomized, double-blinded, placebo-controlled trial of 200 healthcare workers showed that the incidence of clinically defined influenza infection was significantly lower in a catechin capsules-given group (4.1%) compared with the placebo group (13.1%) (64). Another observational study found that green tea consumption is inversely associated with the incidence of influenza infection among schoolchildren in a tea plantation area of Japan (65). These results suggest that taking green tea and green tea catechins may be effective prophylaxis for influenza infection.

As with viruses, EGCG can also be used to difficult the parasite infection by (i) decreasing the number of parasites and its growth; (ii) fragmenting the parasite DNA or (iii) reducing their fatty acid synthesis (52). Studies with parasites include *Plasmodium falciparum*, *Babesia* spp., *Trypanosoma* spp., and *Leishmania braziliensis*. Several reports address the properties of EGCG against different parasites (see Table 2.7.).

Table 2.7. Antiparasitic properties of EGCG

Microorg.	Mechanism of Action	MIC's	Effect	Ref.
Babesia bovis & *B. bigemina*	Growth of *B. bovis* and *B. bigemina* was inhibited at 10 and 25 µM respectively and suppressed in the presence of 50 µM EGCG.	MIC_{50}(B. bovis)= 18 µM MIC_{50}(B. bigemina)= 25 µM	Parasitistatic	(66)
Trypanosoma brucei	EGCG exerts its effects on acetyl-CoA carboxylase (ACC) through activation of AMP-dependent protein kinase, which phosphorylates and inhibits *T. brucei* ACC and fatty acid synthesis.	EC_{50} of 37 µM and 55 µM for bloodstream form and procyclic form	Parasitistatic	(67)

(continuation)

Plasmodium falciparum	Green tea extract (25–100 μM EGCG) inhibits the growth of *P. falciparum* culture, by interfering with uptake of exogenous iron and depleting the intracellular labile iron pool in the red blood cells, leading to inhibition of their growth.	IC_{50}= 21.11 μM	Parasitistatic	(68)
Leishmania braziliensis (promastigote & intracellular amastigote life stages)	EGCG promotes ROS production, resulting in decreased mitochondrial membrane potential ($\Delta\Psi_m$) and reduced intracellular ATP concentrations.	Reduced promastigote viability and the infection index with IC_{50} of 278.8 μM and 3.4 μM	Parasiticidal	(69)

The kingdom bacteria is the main focus of EGCG studies concerning microorganisms. This may be due to the worldwide awareness raised by the world health organization about antibiotic resistance and/or the “ease-of-use” of these microorganisms in experimental *in vitro* studies, compared to other microorganisms like fungi. Reygaert et al (2018) (52), reviewed the possibilities for the use of green tea catechins in treating and preventing infectious diseases and realized that EGCG can successfully interfere with several bacterial processes due to its ability to bind to bacterial cell membranes (52). Such ability can lead to cell membrane damage, resulting in cell lysis; loss of function of transmembrane transporter proteins, like the pumps responsible for the efflux of toxins and antimicrobial agents; loss of bacterial ability to bind to host cells; and loss of the ability for quorum sensing and biofilm formation. Besides this capability, EGCG also affects basic bacterial functions such as fatty acid and folate biosynthesis pathways, by targeting different proteins; bacterial DNA gyrase and ATP synthase activities, by binding to the ATP binding site of the gyrase B subunit (70); and bacterial tyrosine phosphatase and cysteine proteases (52). Table 2.8. synthesizes some research papers reporting antibacterial properties of EGCG, as well as MIC’s against bacteria, that tend to be 10–100-fold higher than against virus (10).

Table 2.8. Antibacterial properties of EGCG

Microorg.	Mechanism of Action	MIC's	Effect	Ref.
Escherichia coli	Exposure of *E. coli* cells to EGCG induces an adaptive oxidative stress response resulting in an increase in intracellular ROS.	100-1000 μM	Bactericidal	(71)
Bacillus subtilis	EGCG inhibits the function of several membrane proteins in cell envelope, including the oligopeptide ABC transporter (Oppa), PTS system transporter, phosphate ABC transporter, and PBP5.	125 and 250 μg/mL	Bactericidal	(72)
Streptococcus mutans	EGCG inhibits biofilm formation (MIC_{50}=7,8 μg/mL) and reduces viability (MIC_{50}=156,25 μg/mL) of the preformed biofilm. EGCG at 15,6 μg/mL suppressed expression of *atpD*, *eno*, *ldh*, and *aguD* virulence factors.	MIC=31,25 μg/mL MBC=62,5 μg/mL	Bactericidal & Bacteriostatic	(73)
Fusobacterium nucleatum	EGCG damages the bacterial cell wall and interferes with the chelation of iron. It prevents biofilm formation at concentrations that did not interfere with bacterial growth (62,5 μg/mL) and decreases pre-formed biofilm viability (1000 μg/mL, time-dependent). It also decreases the adherence of *F. nucleatum* to epithelial cells and matrix proteins (31,25, 62,5 and 125 μg/mL, dose-dependent) and attenuated *F. nucleatum*-mediated hemolysis by 67% (125 μg/mL) and hydrogen sulfide production by 72.5% (2000 μg/mL).	MIC=500 μg/mL MBC=1000 μg/mL	Bactericidal & Bacteriostatic	(74)

For the purpose of this study, there's a need to emphasize the antifungal activity of EGCG. Most research papers mostly focus on the antifungal effects of this compound against *Candida* species. This may be due to the fact that candidiasis and candidaemia pose a much higher overall burden to public health than infections by other fungi such as *Aspergillus* spp. Actually, in a meta-analysis of population-based studies, the European incidence of candidaemia alone was approximately 79 cases per day, of which 29 patients might have fatal outcome at day 30 (75).

A study by Chen, et al (2015) (76) investigating the susceptibility of *Candida* and *A. fumigatus* species to different teas and EGCG isolated from green tea using the EUCAST microdilution method, showed potent *in vitro* antifungal activity of all teas and EGCG against *C. glabrata*, but none had effect on *A. fumigatus* at the concentrations

tested. The MIC_{50} of EGCG were 0.31 µg/mL against *C. glabrata* and 5 µg/mL against *C. albicans* and *C. parapsilosis*. At higher concentrations EGCG was fungicidal against *C. glabrata* (76). Hirasawa and Takada (2004) (7), in another study regarding *C. albicans*, noted it was sensitive to EGCG in a pH-dependent manner. EGCG was more active at slightly basic pHs. At pH 6.0 the MIC_{90} was 2000 µg/mL, while it ranged from 500–1000 µg/mL at pH 6,5 and as low as 15.6–250 µg/mL at pH 7.0 (7). The results from another investigation by Park, et al (2006) (77) evaluating the *in vitro* antifungal susceptibility of 21 clinical isolates of seven *Candida* species to EGCG and commonly used antifungal agents, using the Clinical and Laboratory Standards Institute (CLSI) protocol M27-A, were mainly in agreement with those obtained by Chen, et al (2015) (76) and Hirasawa and Takada (2004) (7). Among the tested species, *C. glabrata* exhibited the highest susceptibility to EGCG (MIC_{50}, 0.5–1 µg/mL and MIC_{90}, 1–2 µg/mL), while *C. albicans* (MIC_{50}, 4–8 µg/mL and MIC_{90}, >16 µg/mL) and *C. parapsilosis* (MIC_{50}, 1–4 µg/mL and MIC_{90}, 2–16 µg/mL) were also susceptible to EGCG, although less than *C. glabrata* (77). A few years after reporting inhibitory effect of EGCG on clinical isolates of *Candida* species, Park et al (2011) (78) investigated the *in vitro* antifungal activity of EGCG and antifungal agents against clinically isolated dermatophytes (*M. canis*, *T. mentagrophytes*, and *T. rubrum*), by the CLSI M38-A2 method. As expected, the dermatophytes isolates also revealed lower MIC values against FLC (MIC_{50}, 2-16 µg/mL, MIC_{90}, 4-32 µg/mL, and geometric mean (GM) MICs, 3.45-25.8 µg/mL) and 5FC (MIC_{50}, MIC_{90}, and GM MICs, >64 µg/mL) than EGCG (MIC_{50}, 2-4 µg/mL, MIC_{90}, 4-8 µg/mL, and GM MICs, 3.36-4 µg/mL), although they were less susceptible to AMB and ITR (78). More recently, Behbehani et al (2019) (79) examined the efficacy of EGCG and in combination with FLC/ketoconazole (KET) drugs against oral *Candida* isolates. The MIC ranges of EGCG alone, determined using the M27-A CLSI protocol, were between 3.91 to 15.63 mg/mL against all *Candida* isolates, higher than the MIC ranges of FCZ and KET (4 to 128 mg/mL and 4 to 32 mg/mL, respectively). Overall, the concentrations of EGCG for antifungal susceptibility in the published reports were slightly higher than those of tested antifungal agents (FLC, AMB, 5FC, ITR and KET), suggesting that EGCG may be effectively used as a possible agent or adjuvant for antifungal therapy in candidiasis and dermatophytosis.

Although proved that EGCG has antifungal potential against *Candida* spp. and dermatophytes, its mechanism of action remained unclear until 2006, when Navarro-Martínez et al (8), demonstrated that EGCG can indirectly disrupt ergosterol production, by inhibiting the *C. albicans* dihydrofolate reductase – a key enzyme in the biosynthesis

of purines, pyrimidines and several amino acids. The authors also determined the MIC values of EGCG for the strains tested at pH 7.4 by the CLSI broth dilution M27-A method, which varied from 1 to 32 µg/mL, falling within the range of previous reports (8). Later, Evensen and Braun (2009) (80) conducted experiments to determine the effects of tea polyphenols on *C. albicans* biofilm formation. Results showed that cultures treated with 1.0 mmol/L EGCG, displayed a 75% reduction of viable cells during biofilm formation, and that pre-established biofilms treated with EGCG were also reduced, by 80%. The authors demonstrated that *in vivo* proteasome activity was significantly decreased, contributing to cellular metabolic and structural disruptions, inhibiting of biofilm formation and maintenance by *C. albicans* (80).

2.4.3. Antifungal Synergism

Since EGCG alone is proved to have health beneficial properties, researchers have also been assessing the potential synergism of this compound in combination with other medically used drugs. Although research about drug synergism concerning EGCG have been primarily focused on a variety of drugs other than antifungals (81–84), antifungal effects of EGCG and other phytocompounds on *Candida* species alone and in combination with medically used antifungal agents have been attracting considerable attention, specifically on enhancing its susceptibility to those agents. Antifungal synergism is the combination of antifungal agents that have enhanced antifungal activity when used together compared with the activity of each agent alone (12).

Da Silva and colleagues (2014) (6) evaluated *in vitro* interactions of EGCG with FLC against FLC-resistant *Candida tropicalis*. Results show that EGCG alone had no antifungal activity within the concentration range tested, but when the FLC-resistant strains were exposed to various concentrations (0.25 to 128 µg/mL) of EGCG combined with various FLC concentrations (0.25 to 16 µg/mL), there was significant synergistic activity. All studied strains showed MIC_{50} values of 64 µg/mL to FLC, that came down to 16 µg/mL after cotreatment with 128 µg/mL of EGCG (6). In the Hirasawa and Takada (2004) (7) study, the authors also noted that various catechins present in green tea, including EGCG, have significant antifungal activities when combined not only with FLC but also AMB, indicating additive or synergistic effects. The research performed by broth dilution showed that combined treatment with 3.12–12.5 µg/mL EGCG plus AMB 0.5 µg/mL markedly decreased the growth of AMB-resistant *C. albicans*. Astonishingly, the combined use of 12.5 µg/mL EGCG and 10–50 µg/mL FLC inhibited the growth of FLC-resistant *C. albicans* by an impressive 98.5%– 99.7% (7).

Behbehani et al (2019) (79) research results were mainly in agreement with those obtained earlier in a sense that the combination of EGCG (MIC 3.91-15.63 µg/mL) with FLC or KET against the planktonic cells of all *Candida* isolates showed a remarkable decrease in the MIC values (MIC of FLC decreased from 4-64 to 1-8 µg/mL; MIC of KET decreased from 4-32 to 1-8 µg/mL) compared to each drug alone. Concerning *Candida* biofilm cells, the minimum biofilm inhibitory concentration (MBIC) ranges of EGCG against all tested isolates were between 62.5-125 µg/mL and in the range of 128-512 µg/mL (FLC) and 64 to 512 (KET) µg/mL, but when EGCG was combined with FLC or KET drugs, the MBIC values of drugs and EGCG decreased 3 to 5 fold indicating a synergistic effect in almost all the cases (79). Anand and Rai (2016) (85), who also evaluated synergistic effects of catechins isolated from green tea leaves with FLC and AMB against *C. albicans* and *C. glabrata*, showed synergistic activity with FLC and AMB against *Candida* species. The MIC_{90} values of FLC (64-128 µg/mL), AMB (1 µg/mL), green tea catechins (125-250 µg/mL) and EGCG (125-250 µg/mL) alone proved lower when the catechins and EGCG were used in combination of FLC or AMB. FLC MIC_{90} values ranged from 4-64 µg/mL, AMB's from 0.06-2 µg/mL, green tea catechins from 15.62-62.5 µg/mL, and EGCG's from 7.81-62.5 µg/mL. It is important to note that not all the purified catechins isolated from different green tea leaves had synergistic activity with FLC and AMB against *C. albicans*, but all have demonstrated synergistic inhibitory potential with AMB against *C. glabrata* (85).

As the results from *in vitro* experiments clearly suggested that EGCG and other phytocompounds could be an effective treatment or cotreatment for *Candida* spp. infections, Han (2007) took the leap from *in vitro* to *in vivo* experiments. He investigated synergic anticandidal effects of EGCG and its mechanism in a murine model of disseminated candidiasis caused by *C. albicans* and found that EGCG-given BALB/c mice inoculated with viable *C. albicans* yeast cells survived longer than the control mice group. EGCG treatment inhibited the hyphal formation, causing growth-inhibition of the *Candida* cells. In experiments determining synergic effects, mice given diluent (control), AMB (0.5 mg/kg of body weight), or EGCG (2 mg/kg) had mean survival times (MST) of approximately 10.9, 11.7, and 13.9 days, respectively. However, mice administered with a combination of AMB (0.5mg/kg) plus EGCG (2 mg/kg) had an MST value of up to 42.1 days, surviving an average of 30 days longer than the mice who received AMB alone. Actually, the MST from the combination-treated mice groups was much higher than MST from mice group that received four times the AMB dose. These results indicate that EGCG, which has *in vitro* and *in vivo* anticandidal activity by blockage of the hyphal

formation, presents synergistic effects against disseminated candidiasis when combined with AMB (81).

Confirmed the *in vitro* and *in vivo* anticandidal and synergistic effects of EGCG, it was time to translate all this knowledge to clinical trials. Recently, a randomized double-blinded controlled clinical trial was designed to evaluate and compare the antimicrobial efficacy of, among others, green tea mouth rinses against oral *Candida albicans* in children. Unfortunately, the efforts came unfruitful, as a statistically significant fall in colony count was not found (86).

2.4.4. Disadvantages of EGCG

Despite all the evidence pointing to the health benefits of EGCG, there are two major downsides to its possible medical and dietary use worth considering. First of all, EGCG alone suffers from poor *in vivo* bioavailability and stability (49). When consumed, EGCG is at higher concentration in the digestive system, but the issue of its bioavailability comes from the extreme pH conditions in the stomach and intestinal tract, as well as related digestive enzymes and biologically inactivating processes such as methylation (55,87). The main reason for low absorption rate of EGCG is because it is transported across intestinal epithelium predominantly by passive diffusion, and there is no specific receptors on the surface of small intestinal epithelial cells that have been found to carry EGCG into cells (87). Emerging technologies such as nanotechnology can, in part, provide solutions to encounter some of these limitations. For this reason, EGCG is mostly used either in combination with other components such as nanoparticles (88); encapsulated in edible coating with royal jelly proteins (89); or being modified as a prodrug (90) to enhance its stability and bioavailability. Cai et al (2018) (87) conducted a thorough review on the advances in bioavailability studies involving absorption and metabolic biotransformation of green tea catechins.

Secondly, there have been reports of hepatotoxicity linked to the consumption of large amounts of green tea extracts (91). After the report of 13 cases of hepatotoxicity, a green tea preparation (Exolise®) marketed for weight loss, was withdrawn in 2003. More recently SLIMQUICK® weight loss products, many of which contain green tea extract, have been associated with six cases of acute liver injury. Following concerns regarding their possible harmful effects on the liver, the European Food Safety Authority (EFSA) (91) has assessed the safety of green tea catechins from dietary sources. For green tea infusions brewed with hot water, and instant and ready-to-drink green tea beverages with similar catechin content, EFSA's experts concluded that there is

generally no indication of liver damage in the range of the mean daily intake of EGCG (90–300 mg/day), nor after high consumption of ≥ 5 cups per day (300–866 mg/day), and that the few cases of liver damage reported in humans are likely due to rare and unpredictable reactions. Concerning food supplements, EFSA's panel concluded – on the basis of human interventional clinical trials conducted with volunteers under medical supervision – that there was no indication of hepatotoxicity for doses below 800 mg/day taken for up to 12 months (91). According to these results, about seven cups of green tea correspond to the highest amount that could be drunk without expecting health consequences (92).

Despite of the fact that, as stated before, most of the *in vitro* and *in vivo* data on the EGCG antifungal activity were assessed against *Candida* species and suggested its potential as additional or co-adjuvant therapeutic agent, further research is crucial to evaluate antifungal activity of EGCG against a number of different fungi such as *Aspergillus* spp, and other clinically relevant fungi. In this sense, this study fills a gap in the existing knowledge and pushes the boundary of scientific evidence. As far as I know, this is the first research focusing on the *in vitro* antifungal effects of EGCG both alone and in combination with different antifungals, namely ITR, VOR and POS, against triazole-resistant *Aspergillus fumigatus*. The ultimate goals when testing EGCG's anti-*Aspergillus* potential are (i) to enhance efficacy in the treatment of triazole-resistant IA infections, (ii) to broaden the spectrum of empiric antifungal therapy available, (iii) to lower the dosages of triazoles, thus preventing adverse effects and the emergence of triazole-resistant *A. fumigatus*, and/or (iv) to enhance the antifungal effects by achieving a synergistic killing effect.

3. Methodology

In this chapter it is described exactly how I collected and analyzed the data, as well as the overall approach designing this experiment.

3.1. Research Design and Location

This research took place in Lisbon School of Health Technology facilities, a part of Lisbon Polytechnic Institute, in collaboration with Health & Technology Research Center. It is classified as experimental and interventional (since the researcher play an active role by manipulating the EGCG and antifungal concentrations, to evaluate the effect of this intervention upon the presence or absence of fungal growth); with a factorial design (since more than one independent variable, each comprising at least two levels – absence or presence in different concentrations – are used to verify the combined action exerted by the independent variables on the dependent one) (93).

3.2. Variables

For data analyze and reproducibility purposes, the screening procedure was made in triplicate. The independent variables, the ones manipulated by the researcher, are the concentrations of EGCG, ITR, VOR and POS. The dependent variable, on which the effects of the independent variables are exerted, is the presence or absence of fungal growth on each plate. The former is classified as quantitative and the latter as qualitative of nominal scale, since the results were read visually by recording the presence or absence of growth on each plate (93).

3.3. *A. fumigatus* Isolates Characterization

The triazole-resistant *A. fumigatus* isolates (N=3) were previously studied and kindly provided by Doctor Carla Viegas, from H&TRC. These isolates came from a bank of *Aspergillus fumigatus* isolates collected in previous exposure assessment campaigns performed from January 2018 to February 2019 in different Portuguese indoor and occupational environments. Isolate number 299 was collected from the air of a dairy, while isolates number 873 and 1209 came from a waste sorting plant FRPD. *Aspergillus* species were identified at section level by macroscopic and microscopic morphology using a tease mount or Scotch tape mount and lactophenol cotton blue mount

procedures and using identification atlas (94). *A. fumigatus* isolates were confirmed by molecular methods (sequence analysis of the calmodulin or β-tubulin genes for *Aspergillus* spp.), and sequencing of the *cyp51A* gene and its promoter revealed pan-azole-resistance mutations in all three isolates (33). Table 3.1. synthesizes the growth pattern of these isolates as well as their MIC's and *cyp51A* mutation mechanism, obtained by Gonçalves et al (2021) (33). As positive control, an azole-sensitive *A. fumigatus* isolate was obtained from reference strains from the Mycology Laboratory of the National Institute of Health Doctor Ricardo Jorge (INSA).

Table 3.1. Growth pattern of the triazole-resistant *A. fumigatus* isolates, their MIC's and mutations found on the *cyp51A* gene (33)

Isolate	**Azole Screening Media**			**MIC's (µg/mL)**			***cyp51A* Mutation**
	VOR	POS	ITR	VOR	POS	ITR	
299	+	+	+	4	2	4	TR_{34}/L98H
873	+	+	+	2	1	4	
1209	-	+	+	4	1	8	

-: negative (no growth); +: positive (any type growth)

3.4. Screening of Azole Resistance and evaluation of EGCG effect in *Aspergillus fumigatus* Isolates

I set to answer our research question through the adaptation of the EUCAST method "Screening procedure E.Def 10.1 for the detection of azole resistance in *Aspergillus fumigatus* isolates using four-well azole-containing agar plates" (11). It was modified intending the phenotypic detection of *A. fumigatus* isolates resistant to ITR, VOR and POS, as well as the detection of synergism between EGCG and the triazoles.

3.4.1. Preparation of Stock Solutions

The ITR (Acros Organics, 452870050), VOR (Honeywell, 137234-62-9), POS (Sigma-Aldrich, 32103) and EGCG (Sigma-Aldrich; E4143) powders were weighed on an analytical balance (KERN & Sohn GmbH ABJ 220-4M; min=10mg; max=220g; readability=0,1mg).

Azole stock solutions were prepared in DMSO as follows:

1. For 500 mg/L solution with final volume of 10 mL, 5.1 mg of ITR (kept at 4ºC), 5 mg of VOR (kept at room temperature) and 5 mg of POS were weighted;
2. 10 mL of DMSO were added in a sterilized falcon and stirred until dissolved;
3. The azole stock solutions were kept at -80ºC (valid for 6 months without significant loss of activity).

EGCG stock solution was prepared in sterile water as follows:

1. For 5000 µg/mL solution with final volume of 1 mL, 5 mg (=0.005g) of EGCG (kept at 4ºC) were weighted;
2. 1 mL of sterile water was added in a sterilized Eppendorf and vortexed until dissolved;
3. The EGCG stock solution aliquots were kept at -20ºC and away from any light source since EGCG is light sensitive.

3.4.2. Media

For the purpose of this study it was used the Lonza BioWhittaker® (04-525F) selective media RPMI-1640 (with L-Glutamine and buffered with 3-(N-morpholino) propane-sulfonic acid [MOPS], at a final concentration of 0.165 mol/L, pH 7.0., but without sodium bicarbonate) supplemented with glucose to a final concentration of 2% (RPMI 2% G), as well as the VWR Chemicals BDH® (84663.0500) Sabouraud dextrose 4% agar, a general-purpose media for the cultivation and isolation of pathogenic and non-pathogenic fungi.

3.4.3. Preparation of Azole-Containing Plates

The azole-containing RPMI-1640 plates (nominal capacity of approximately 20 mL and 100 mm) supplemented with chloramphenicol (at a final concentration of 0.2 mg/mL) were prepared as follows:

1. Azole stock solution was defrosted in advance at room temperature;
2. D(+)-Glucose (Millipore, 1.08337.0250), Agar Bacteriologic (Frilabo, 61101) and Chloramphenicol (Fisher-Scientific, BP904-100) were dissolved in 900 mL of RPMI-1640 medium (see Table 3.2.);
3. Components were stirred until completely dissolved and then filled up to 990 mL with RPMI-1640 medium;

4. The solution was poured in 4 Erlenmeyer flasks (247.5 mL in each flask) and autoclaved at 121ºC for 15 minutes;
5. Agar was let to cool down until one was able to touch the flasks comfortably;
6. The different azole stock solutions were added to each flask (see Table 3.2.) and the remaining antifungal-free flask was used for the growth control plates, as follows:
   - Erlenmeyer 1: ITR (final concentration, 4 µg/mL)
   - Erlenmeyer 2: VOR (final concentration, 2 µg/mL)
   - Erlenmeyer 3: POS (final concentration, 0.5 µg/mL)
   - Erlenmeyer 4: growth control (antifungal-free plate);
7. The agar from each flask was poured (approximately 20 mL per plate) to the respective sterile plates and let to solidify;
8. The plates prepared were properly sealed and stored at 4ºC for up to 1 month from the date of preparation if there was no reduction in the height of agar (suggesting evaporation).

Table 3.2. Reagents to prepare 1 L of RPMI-1640 (~50 agar-containing plates)

Reagents	**Mass Weighted/Volume Pippeted**
RPMI-1640 (with L-Glutamine and 0,165 M MOPS Without Sodium Bicarbonate)[a]	10.4 g
D(+)-Glucose[b]	18.0 g
Agar Bacteriological[c]	20.0 g
Chloramphenicol[d]	0.180 g (final concentration: 0.2 mg/mL)
ITR[e] (stock solution)	1.980 mL in 247.5 mL of agar
POS[f] (stock solution)	0.248 mL in 247.5 mL of agar
VOR[g] (stock solution)	0.990 mL in 247.5 mL of agar

[a]Lonza BioWhittaker® (04-525F), [b]Millipore (R 1.08337.0250), [c]Frilabo (61101), [d]Fisher-Scientific (BP904-100), [e]Acros Organics (452870050), [f]Sigma-Aldrich (32103), [g]Honeywell (137234-62-9)

The azole-containing SAB plates (nominal capacity of approximately 20 mL and 100 mm) were prepared as follows:

1. Azole stock solution was defrosted in advance at room temperature;
2. 65g of SAB powder (VWR Chemicals BDH®, 84663.0500) (see Table 3.3.) were stirred until completely dissolved in 1 L of purified water and brought to boil with frequent stirring;
3. The solution was distributed in 4 Erlenmeyer flasks (250 mL in each flask) and sterilized by autoclaving at 121ºC for 15 minutes;
4. Agar was let to cool down until one was able to touch the flasks comfortably;
5. The different azole stock solutions were added to each flask (see table 3.3.) and the remaining antifungal-free flask was used for the growth control plates, as follows:
    - Erlenmeyer 1: ITR (final concentration, 4 μg/mL)
    - Erlenmeyer 2: VOR (final concentration, 2 μg/mL)
    - Erlenmeyer 3: POS (final concentration, 0.5 μg/mL)
    - Erlenmeyer 4: growth control (antifungal-free plate);
6. The agar from each flask was poured (approximately 20 mL per plate) to the respective sterile plates and let to solidify;
7. The plates prepared were properly sealed and stored at 4ºC for up to 1 month from the date of preparation if there was no reduction in the height of agar.

Table 3.3. Reagents to prepare 1 L of SAB (~50 agar-containing plates)

Reagents and Formula	Mass Weighted/Volume Pippeted
Sabouraud dextrose 4% agar (84663.0500)[a] D(+)-Glucose Meat peptone Casein peptone Agar	65 g
Purified Water	1000 mL
ITR[b] (stock solution)	2 mL in 250 mL of agar
POS[c] (stock solution)	0.250 mL in 250 mL of agar
VOR[d] (stock solution)	1 mL in 250 mL of agar

[a]VWR Chemicals BDH® (84663.0500), [b]Acros Organics (452870050), [c]Sigma-Aldrich (32103), [d]Honeywell (137234-62-9)

3.4.4. Preparation of the Inoculum Suspensions

Prior to the preparation of the inoculum suspensions, *A. fumigatus* isolates were cultured on SAB medium and incubated at 25ºC to be able to sporulate. Inoculum suspensions were then prepared from fresh, mature (3- to 5-day-old), non-contaminated cultures, as follows:

1. The conidia of up to five pure colonies of each isolate were carefully rubbed using a moist sterile cotton swab and subsequently suspended in 1000 µL of sterile water in a sterile Eppendorf and vortexed for 15 seconds with a vortex mixer at approximately 2000 rpm, ensuring homogeneity of the conidia suspension to avoid presence of clumps;
2. 500 µL of sterile water plus 500 µL of the previous suspension were pipetted to a spectrophotometer (UNICAM Spectronic Helios β) *cuvette*, before measuring the absorbance at 600 nm in duplicate;
3. The mean absorbance values of each suspension were calculated to a final optical density (OD) of 0.5 McFarland (OD_{600} = 0.060);
4. At last, final inoculum suspensions were made with a calculated volume of the remaining fungal suspension, EGCG at final concentrations of 25 µg/mL, 50 µg/mL, 100 µg/mL, 400 µg/mL and filled up to 1 mL with sterile water. For control purposes it was included an inoculum suspension without EGCG. Between experiments, the suspensions were kept frozen at -20ºC.

EGCG dosage was selected considering that 400 mg and 800 mg of EGCG intake, which are considered safe doses previously used in human clinical trials result in peak serum concentrations in the range of 100 to 400 ng/mL (95).

3.4.5. Inoculation and Incubation of Agar Plates

25 µL of each final inoculum suspension were pipetted onto the previously prepared agar-containing plates without touching the surface, within 30 minutes of the preparation of the inoculum suspension to maintain viability of the conidia. In the first two experiments the fungal suspensions were inoculated in RPMI-1640 medium and in the last one they were suspended in SAB medium. Although the EUCAST protocol recommends an incubation time of 48h, the agar plates were incubated without agitation at 34-37ºC in ambient air for 7 days as the inoculum suspension had diluted amounts of fungi compared to the original protocol.

3.4.6. Reading and Interpretation of Results

After incubation, the endpoint was read visually by recording the presence or absence of growth on each plate at 2, 4 and 7 days. Any growth on one or more azole-containing agar plates was considered. The presence or absence of fungal growth on the surface of the four plates yielded the following preliminary classification: azole-susceptible isolate: growth on the antifungal-free plate and absence of growth on the azole-containing plates at a given time; potentially an azole non-susceptible isolate: growth on the antifungal-free and on any of the azole-containing plates (11).

The gathered data was then organized in tables using Microsoft® Excel (v. 16.36).

3.5. Ethic

The research project was previously approved by the course council in its own model, and then sent to the Lisbon School of Health Technology ethics board, obtaining a positive feedback (CE-ESTeSL-Nº. 24-2020). The authors certify that there was no actual or potential conflict of interest in relation to this research. As this was an in vitro study, without the need of animal models or biological samples from patients, no special ethical authorization was required.

4. Results and Discussion

Tea is the most widely consumed beverage in the world and has been reported to have antimicrobial activities (10,96–98). Specifically, EGCG isolated from green tea has shown to have antifungal activity against a number of fungi (7,10). Thus, we aimed to find out if EGCG has *in vitro* antifungal potential against triazole-resistant *Aspergillus fumigatus* collected during exposure assessment campaigns, and if there are synergistic effects between EGCG and the clinically used triazoles.

As mentioned earlier, the screening procedure was made in triplicate. In the first two experiments the fungal suspensions were inoculated in RPMI-1640 medium and in the last one they were suspended in SAB medium. Next, I analyze and discuss one by one, the results obtained from each experiment.

4.1. *A. fumigatus* isolates have divergent growth patterns in azole-containing plates (RPMI-1640 medium) in co-exposure with EGCG at different concentrations

In Experiment I (see Table 4.1.) isolates 299 and 873 proved resistant to 2 µg/mL VOR and 4 µg/mL ITR since they grew in the presence of these drugs alone and with increasing concentrations of EGCG at all tested time points. Regarding POS-containing media both isolates initially (2d) showed no growth on 0.5 µg/mL POS but grew from 4d onward. This delay is possibly associated with the inherent dilution applied to the inoculum suspensions and goes according to the resistant phenotype of these isolates found in the Gonçalves et al (2021) (33) study. As such, the results presented so far point towards no effects of EGCG alone against these isolates nor synergism with any of the triazoles.

Isolate 1209 grew in all media when EGCG was present without any triazole drug, showing no effects of increasing concentrations of this compound against this isolate. After 2 days it proved resistant to 2 µg/mL VOR in the presence of 2 µg/mL VOR alone and with 25 and 50 µg/mL of EGCG but showed no growth when the EGCG concentrations raised above 100 µg/mL. This is the most interesting finding, in a way that shows a potential sensitization when this isolate was exposed to 2 µg/mL VOR in addition to 100 µg/mL (or higher) of EGCG. This finding comes to suggest that EGCG, not only have synergistic antifungal potential against resistant *C. albicans* (6–

8,12,79,85,99), but it might also have against triazole-resistant *A. fumigatus*. However, further studies should take place in order to prove strong relations between the use of EGCG and sensibilization of other-wise-resistant *A. fumigatus* isolates to triazoles.

This antifungal synergism relation – the combination of antifungal agents that have enhanced antifungal activity when used together compared with the activity of each agent alone (12) – between EGCG and antifungal drugs have been previously reported in other fungi such as *Candida* spp.. For example, Navarro-Martínez et al (2006) (8) reported antifungal synergism between EGCG and inhibitors of the *C. albicans* ergosterol biosynthesis pathway (ITR and KET), lowering the MIC values of these agents, that ranged from 0,01-16 µg/mL (ITR) and 0,01-4 µg/mL (KET) alone, to 0,003-0,01 µg/mL (ITR) and 0,002-0,25 (KET) in combination with EGCG (8). Ning et al (2015) (99) also observed synergism between the catechin and miconazole (MCZ), FLC and AMB against both planktonic and biofilm cells of *Candida* species. MICs against *Candida* planktonic cells ranged from 375-1500 µg/mL (EGCG), 0,25-1 µg/mL (MCZ), 0,5-4 µg/mL (FLC) and 0,063-0,25 µg/mL (AMB), while the lowest concentration of test agent which inhibited more than 80% metabolic activity of *Candida* biofilm cells (SMIC [sessile MIC]) showed obvious increases, ranging from 3000-24000 µg/mL (EGCG), 12,5-1600 µg/mL (MCZ), 156,25-6400 µg/mL (FLC), and 0,63-6,25 µg/mL (AMB), clearly showing that all tested *Candida* biofilm cells were highly (20–3200 times) resistant to EGCG and antimycotics compared with their planktonic counterparts. When EGCG was in combination with MCZ or AMB, synergistic effects were observed against all tested *Candida* planktonic cells (MICs of MCZ reduced from 0,25-1 to 0,031-0,25 µg/mL; MICs of AMB reduced from 0,063-0,25 to 0,016-0,063 µg/mL) and against most *Candida* biofilms (SMICs of MCZ reduced from 12,5-3200 to 0,20-800 µg/mL; SMICs of AMB reduced from 0,63-6,25 to 0,13-1,56 µg/mL). For the EGCG plus FLC combination, however, no synergism was observed against both planktonic and biofilm cells of *C. krusei* and *C. kefyr* (99).

However, none of that research showed synergism between EGCG and VOR specifically. As such, as far as I know this is the first report of an azole-resistant-*A. fumigatus* being potentially sensibilized to VOR in the presence of EGCG. This is particularly interesting, as the most common serious side effect, hepatotoxicity, occurs most often with VOR in up to 31% of the cases (12,35).

It is noteworthy that this isolate presented growth after an incubation period of 7d which might be explained by the acquisition of VOR-resistance after prolonged incubation time. Actually, a study by Ricardo et al (2014) (100) reported such a

phenomena in a *Candia* species. They exposed a susceptible *Candida krusei* isolate daily to 1 µg/mL VOR, and after 5 days of exposure to VOR, they achieved a higher MIC value than the original, which remained constant after 25 additional days of treatment with VOR and also after 30 consecutive days of incubation in VOR-free medium (100).

As to POS and ITR, isolate 1209 proved sensitive until day 7. The sensitive isolate didn't grow in any of the media, proving its phenotype.

Table 4.1. Experiment I: growth patterns of A. fumigatus isolates in different azole-containing plates (RPMI-1640 medium) and EGCG concentrations

Isolates		EGCG (µg/mL)	W/o Antifungal			2 µg/mL VOR			0.5 µg/mL POS			4 µg/mL ITR		
			2d	4d	7d	2d	4d	7d	2d	4d	7d	2d	4d	7d
Triazole-resistant Isolates	299	0	+	+	+	+	+	+	-	+	+	+	+	+
		25	+	+	+	+	+	+	-	+	+	+	+	+
		50	+	+	+	+	+	+	-	+	+	+	+	+
		100	+	+	+	+	+	+	-	+	+	+	+	+
		250	+	+	+	+	+	+	-	+	+	+	+	+
		400	+	+	+	+	+	+	-	+	+	+	+	+
	873	0	+	+	+	+	+	+	-	+	+	+	+	+
		25	+	+	+	+	+	+	-	+	+	+	+	+
		50	+	+	+	+	+	+	-	+	+	+	+	+
		100	+	+	+	+	+	+	-	+	+	+	+	+
		250	+	+	+	+	+	+	-	+	+	+	+	+
		400	+	+	+	+	+	+	-	+	+	+	+	+
	1209	0	+	+	+	+	+	+	-	-	-	-	-	-
		25	+	+	+	+	+	+	-	-	-	-	-	-
		50	+	+	+	+	+	+	-	-	-	-	-	-
		100	+	+	+	-	-	+	-	-	-	-	-	-
		250	+	+	+	-	-	+	-	-	-	-	-	-
		400	+	+	+	-	+	+	-	-	-	-	-	+
Triazole-sensitive Isolate		0	+	-	-	-	-	-	-	-	-	-	-	-
		25	-	-	-	-	-	-	-	-	-	-	-	-
		50	-	-	-	-	-	-	-	-	-	-	-	-
		100	-	-	-	-	-	-	-	-	-	-	-	-
		250	-	-	-	-	-	-	-	-	-	-	-	-
		400	-	-	-	-	-	-	-	-	-	-	-	-

-: negative (no growth); +: positive (any type growth)

The results from Experiment I triggers the will to know which phenomena is causing the sensibilization of the VOR-resistant isolate 1209. At this time, the reason for this is unknown. Further work is needed, and more isolates need to be tested to draw any conclusion from these observations. However, after some research, two plausible answers can be formulated. The VOR-resistant isolate 1209 may have been chemosensitized by EGCG at higher concentrations (≥ 100 µg/mL), leading to a vulnerability to VOR; or EGCG may play a role in epigenetic modulation on 1209 isolate phenotype, therefore contributing to the sensibilization to VOR when higher concentrations of EGCG are present.

Chemosensitization involves enhancing the effectiveness of antifungal agents by co-applying a second compound. The second compound does not necessarily need to have much antifungal potency alone, but debilitates the ability of the fungus to launch a protective response to the antifungal agent (101).

On the other hand, the fact that the three isolates presented differences in resistance patterns to VOR make us doubt the role genetics play in the resistance phenotype of *A. fumigatus*, as the *cyp51A* mutation found (TR_{34}/L98H) was common in all of them. Could different epigenetic mechanisms among the isolates, despite having the same triazole-resistance mutation, be affecting their phenotype? Epigenetic mechanisms refer to cellular changes mediated by factors other than DNA sequence modifications that momentarily affect the expression of target genes, providing an additional route via which microorganisms can rapidly respond to changing environments and stresses, including drug stress (102). Generally, the epigenetic mechanisms are divided in two main categories: RNA-based and chromatin-based mechanisms. RNA-based mechanisms include RNA interference (RNAi) and non-coding RNAs (ncRNAs). RNAi mechanism is mediated by small RNAs which selectively target complementary RNAs inducing degradation or inhibiting translation of target RNA and can also recruit heterochromatin proteins, inhibiting gene expression itself. ncRNAs are mainly long ncRNAs (> 200 bp), which comprise a high percentage of total RNA transcripts and also play a role in epigenetic gene regulation. Chromatin modifications can be chemical (post-translational modifications [PTMs] of histone proteins and DNA methylation) or structural (chromatin remodeling and DNA-DNA interactions). The N-terminal tail of histones in nucleosomes act as substrates for several PTMs, including methylation, acetylation, and phosphorylation. Histone modifications are able to alter gene expression levels by allowing or restricting binding of transcription factors or enhancers. The enzymes responsible for mediating these modifications are called histone acetyltransferases

(HATs), whereas the ones responsible for removing these modifications are the histone deacetylases (HDACs). Acetylated histones lead to an open chromatin conformation (euchromatin) promoting transcription, whereas deacetylated histones inhibit transcription since the chromatin is compact (heterochromatin). DNA methylation is a process by which DNA methyltransferases (DNMTs) add methyl groups to the cytosine bases of DNA molecule, originating the 5-methylcytosine base modification (102). Hypomethylation promotes the activation of genes that are normally silenced, increasing the transcriptional activity, while hypermethylation promotes the opposite (103). At last, chromatin structure is dependent upon nucleosome positioning in the genome, a critical factor required for transcription initiation. It requires ATP-dependent nucleosome remodelers or looping of DNA sequences to alter between compact (nontranscribed) or loosen (transcribed) states (102).

After thorough research, it was found that the epigenetic modulator potential of EGCG specifically on *A. fumigatus*, let alone triazole-resistant *A. fumigatus* have not been defined yet and *in vitro* experiments are currently lacking. However, EGCG's epigenetic modulator potential have been proved on cancer physiopathology. Potenza et al (2020) (58) suggested that EGCG may carry out its health benefits not only due to its antioxidant nature, but also via receptor-mediated activation of multiple signaling pathways, and by acting as an epigenetic modulator of DNA methylation and chromatin remodeling, altering gene expression and modifying microRNA activities (58). Actually, several papers reported important anti-cancerous epigenetic properties of EGCG on certain types of human cancers, such as breast cancer (104), cervical cancer (105) or leukemia (106), among others. The DNA methylation mediated by specific DNMTs, is involved in the epigenetic silencing of multiple genes implicated in human breast carcinogenesis. Mirza et al (2013) (104) revealed that treatment with natural compounds, among which EGCG, resulted in a significant decrease in the transcript levels of all the DNMTs investigated, demonstrating that these compounds have the potential to reverse the epigenetic changes (104). In another study using human cervical cancer cell line HeLa, Khan et al (2015) (105) found that time-dependent EGCG-treated HeLa cells were found to have a significant reduction in the enzymatic activity of DNMT and HDAC, resulting in reactivation of known tumor-suppressor genes in HeLa cells due to marked changes in the methylation of the promoter regions of these genes (105). Borutinskaite et al (2017) (106) demonstrated EGCG might also be useful for acute promyelocytic leukemia (APL) treatment, due to its ability to inhibit APL cell proliferation and cause apoptosis through downregulation of epigenetic modifiers and hyperacetylation of

histones binding to the promoter regions of genes associated with cell cycle arrest and differentiation (106).

The results obtained for isolates 299 and 873 in Experiment II (see Table 4.2.) are exactly the same as for these isolates in experiment I. Concerning isolate 1209 and the triazole-sensitive isolate, the results were somewhat different from those obtained in experiment I. Isolate 1209 showed no effects of EGCG alone at tested concentrations (resistant to all). It was also resistant to 2 µg/mL VOR, although it showed no growth in the absence of EGCG at 2d. As to POS it remained sensitive throughout the incubation period. While for 4 µg/mL ITR, it proved sensitive although it showed some growth from day 4 onward when the concentration of EGCG was 400 µg/mL, perhaps meaning some tolerance to prolonged exposure to these compounds.

The triazole-sensitive isolate grew after 4 days in the media with no antifungal, confirming its phenotype. The fact that it didn’t grew in the presence of EGCG leads us to think EGCG exerted some kind of selective pressure in the azole-sensitive isolate. It interestingly demonstrates tolerance to 2 µg/mL VOR in the absence of EGCG after long exposure to it (7d incubation) and unsurprisingly did not grow in the POS and ITR-containing media, proving sensitive to those triazoles.

Table 4.2. Experiment II: growth patterns of A. fumigatus isolates in different azole-containing plates (RPMI-1640 medium) and EGCG concentrations

Isolates		EGCG (µg/mL)	W/o Antifungal			2 µg/mL VOR			0.5 µg/mL POS			4 µg/mL ITR		
			2d	4d	7d	2d	4d	7d	2d	4d	7d	2d	4d	7d
Triazole-resistant Isolates	299	0	+	+	+	+	+	+	-	+	+	+	+	+
		25	+	+	+	+	+	+	-	+	+	+	+	+
		50	+	+	+	+	+	+	-	+	+	+	+	+
		100	+	+	+	+	+	+	-	+	+	+	+	+
		250	+	+	+	+	+	+	-	+	+	+	+	+
		400	+	+	+	+	+	+	-	+	+	+	+	+
	873	0	+	+	+	+	+	+	-	+	+	+	+	+
		25	+	+	+	+	+	+	-	+	+	+	+	+
		50	+	+	+	+	+	+	-	+	+	+	+	+
		100	+	+	+	+	+	+	-	+	+	+	+	+
		250	+	+	+	+	+	+	-	+	+	+	+	+
		400	+	+	+	+	+	+	-	+	+	+	+	+
	1209	0	+	+	+	-	+	+	-	-	-	-	-	-
		25	+	+	+	+	+	+	-	-	-	-	-	-
		50	+	+	+	+	+	+	-	-	-	-	-	-
		100	+	+	+	+	+	+	-	-	-	-	-	-
		250	+	+	+	+	+	+	-	-	-	-	-	-
		400	+	+	+	+	+	+	-	-	-	-	+	+
Triazole-sensitive Isolate		0	-	+	+	-	-	+	-	-	-	-	-	-
		25	-	-	-	-	-	-	-	-	-	-	-	-
		50	-	-	-	-	-	-	-	-	-	-	-	-
		100	-	-	-	-	-	-	-	-	-	-	-	-
		250	-	-	-	-	-	-	-	-	-	-	-	-
		400	-	-	-	-	-	-	-	-	-	-	-	-

-: negative (no growth); +: positive (any type growth)

4.2. *A. fumigatus* isolates growth pattern in azole-containing plates (SAB medium) differ in co-exposure with EGCG at different concentrations when compared to RPMI-1640 medium

At last, the results form Experiment III (see Table 4.3.) were substantially different from those obtained from Experiments I and II. Isolates 299 and 873 demonstrated growth on all triazole and non-triazole-containing media.

Isolate 1209 showed no effect of exposure to EGCG, growing in all tested concentrations in media w/o triazoles. Initially, isolate 1209 didn't grew on VOR-containing media (except when in the presence of 400 µg/mL EGCG), but it proved resistant after 4d (for 0, 100 and 250 µg/mL EGCG) and only after 7d (for 25 and 50 µg/mL EGCG). Apparently, isolate 1209 is resistant to 2 µg/mL VOR when EGCG is at a final concentration of 400 µg/mL. It was resistant to 0.5 µg/mL POS in absence of EGCG, stopped growing when in mutual presence of 0.5 µg/mL POS and EGCG at any tested concentration. It was sensitive to 4 µg/mL ITR (except when in the presence of 400 µg/mL EGCG). At day seven it showed some kind of growth when the concentration of EGCG was either 0, 50 or 100 µg/mL, again suggesting tolerance after long incubation periods.

The triazole-sensitive isolate grew in the absence of EGCG or antifungal and was sensitive to the presence of any concentration of EGCG or triazoles.

Even though the growth of the triazole-resistant *A. fumigatus* isolates fits that from Gonçalves et al (2021) (33) (see Table 4.3.), attention should be given when analyzing the results from this experiment since the EUCAST screening procedure for the detection of azole resistance in *Aspergillus fumigatus* isolates indicates explicitly that fungal suspensions must be inoculated onto RPMI-1640 medium and not on the general-purpose SAB medium.

Table 4.3. Experiment III: growth patterns of A. fumigatus isolates in different azole-containing plates (SAB medium) and EGCG concentrations

Isolates		EGCG (µg/mL)	W/o Antifungal			2 µg/mL VOR			0.5 µg/mL POS			4 µg/mL ITR		
			2d	4d	7d	2d	4d	7d	2d	4d	7d	2d	4d	7d
Triazole-resistant Isolates	299	0	+	+	+	+	+	+	+	+	+	+	+	+
		25	+	+	+	+	+	+	+	+	+	+	+	+
		50	+	+	+	+	+	+	+	+	+	+	+	+
		100	+	+	+	+	+	+	+	+	+	+	+	+
		250	+	+	+	+	+	+	+	+	+	+	+	+
		400	+	+	+	+	+	+	+	+	+	+	+	+
	873	0	+	+	+	+	+	+	+	+	+	+	+	+
		25	+	+	+	+	+	+	+	+	+	+	+	+
		50	+	+	+	+	+	+	+	+	+	+	+	+
		100	+	+	+	+	+	+	+	+	+	+	+	+
		250	+	+	+	+	+	+	+	+	+	+	+	+
		400	+	+	+	+	+	+	+	+	+	+	+	+
	1209	0	+	+	+	-	-	+	+	+	+	-	-	+
		25	+	+	+	-	+	+	-	-	-	-	-	-
		50	+	+	+	-	+	+	-	-	-	-	-	+
		100	+	+	+	-	-	+	-	-	-	-	-	+
		250	+	+	+	-	-	+	-	-	-	-	-	-
		400	+	+	+	+	+	+	-	-	-	+	+	+
Triazole-sensitive Isolate		0	+	+	+	-	-	-	-	-	-	-	-	-
		25	-	-	-	-	+	-	-	-	-	-	-	-
		50	-	-	-	-	-	-	-	-	-	-	-	-
		100	-	-	-	-	-	-	-	-	-	-	-	-
		250	-	-	-	-	-	-	-	-	-	-	-	-
		400	-	-	-	-	-	-	-	-	-	-	-	-

-: negative (no growth); +: positive (any type growth)

4.3. Limitations

In the course of this study, we encounter some limiting factors. The major and most obvious one is the fact that laboratory-based *in vitro* experiments can't always accurately simulate real-life situations and don't necessarily translate to biological animal and human models, even though they are effective for testing causal relationships between variables and proving concepts. Another major limitation was the lack of knowledge and scientific evidence specifically about this topic, as there is some research directed to EGCG activity against several microorganisms, including fungi, but very little about the effects of triazoles and EGCG together against triazole-resistant *A. fumigatus*. The reduced number of isolates also posed a limitation, since it prevented us from applying statistical inference, thus reducing the certainty of the results presented. At last, other limiting factor common to studies of this kind was the limited budget. I couldn't forget to mention all the physical, psychological and logistic barriers imposed to master students who managed to finish their dissertations while fighting the SARS-CoV-2 pandemic.

4.4. Future Work

In order to overcome these limitations, I highly recommend further experiments be carried out with a more heterogeneous (perhaps including healthcare isolates) and greater sample size so the results are even more reliable and statistic conclusions can be drawn. Considering the previously described and established epigenetic modulator potential of EGCG against cancer (84,104–106), it would be extremely relevant to understand if there is any epigenetic mechanism leading to the different drug responses observed in the isolates, since all had the same triazole-resistant mutation. In a broader sense, it would be interesting to evaluate the epigenetic potential of these compound against fungi aiming the development of novel epigenetic-based antifungal therapy. As stated before, EGCG has in general a low bioavailability (87–89) hence, the translation of its antifungal synergistic *in vitro* effects into clinically relevant strategies is also a crucial aspect that need to be considered in future *in vivo* studies.

5. Conclusion

Invasive fungal infections caused by triazole-resistant fungi, including *Aspergillus fumigatus,* are becoming an important cause of human disease with associated high morbidity and mortality rates worldwide. Increasing resistance to the limited arsenal of antifungal drugs is a serious public health concern. As such, the development of new approaches and strategies to tackle the growing antifungal resistance crisis have become essential. This includes the assessment of new compounds with known antimicrobial properties, to be utilized as novel agents or adjuvants in antifungal pharmacologic therapy. In this regard, researchers mainly focused on the antifungal effects of EGCG against yeasts such as *Candida* spp. and molds such as dermatophytes, lacking scientific evidence towards *A. fumigatus*. In a way, this work came to fill this literature void. I tested and then discussed the *in vitro* antifungal effects of EGCG against triazole-resistant *Aspergillus fumigatus* isolates collected during exposure assessment campaigns performed in the last ten years in Portugal and abroad in different work environments, and its potential synergistic effects with clinically used triazoles, namely ITR, VOR and POS.

In fact, EGCG alone showed no effects on triazole-resistant *A. fumigatus* isolates, as it didn't have the ability to reverse the resistance phenotype of these strains. Regarding isolate 1209, it proved resistant to VOR in the presence of 2,0 µg/mL of VOR alone and with 25 and 50 µg/mL of EGCG but it showed no growth when the EGCG concentrations raised above 100 µg/mL, potentially showing it somehow sensitized the isolate to 2 µg/mL VOR. In summary, we demonstrated the potential synergistic effects of EGCG with 2 µg/mL VOR against triazole-resistant *A. fumigatus*. It comes as a way to lower the dosages of triazoles, thus preventing adverse effects and the emergence of triazole-resistant *A. fumigatus*, and to enhance the antifungal effects by achieving a synergistic killing effect.

There is still a long way to go, and future work is needed before EGCG can be routinely administered as an antifungal drug in immunocompromised patients. In the actual panorama however, we hope these exciting findings stimulate further research on EGCG and provide a scientific foundation that ultimately may translate into the use of these compound as a novel agent or adjuvant for antifungal therapy in IA.

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