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Search for new antimicrobial agents: from old pharmaceuticals to new synthetic compounds

Célio Dinarte Sousa Neves

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FACULDADE DE FARMÁCIA

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Search for new antimicrobial agents: from old pharmaceuticals to new synthetic compounds

Dissertation presented to the Faculdade de Farmácia da Universidade do Porto, to obtain the degree of Master in Pharmaceutical Chemistry

Work developed under the scientific supervision of
Professor Eugénia Pinto and Professor Honorina Cidade

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Abstract

The increase in the number of species with pathogenic activity and increased resistance to the most common antibiotics in clinical practice culminated in a growing interest in the discovery of new therapeutic agents.

This work is based on the exploration of two routes used to obtain new drugs: the repurposing of previously established drugs, and the study of new synthetic compounds. Nature is an immeasurable source of new products, resulting from natural optimization over millions of years, and a source of inspiration for the synthesis of so many others. Flavones, chalcones and xanthones have already been shown to have potential antimicrobial activity. Thus, these families of natural compounds stand out as promising sources for the development of new antimicrobial drugs.

This work presents the antimicrobial susceptibility testing, by broth microdilution, of 7 commercially available compounds, with a known therapeutic application, and of 34 new synthetic compounds, including flavones, chalcones and xanthones. From this evaluation 7 compounds with antimicrobial activity were identified. Moreover, HPLC analysis was performed in order to evaluate the effect on ergosterol biosynthesis of three chalcones with promising antifungal activity.

Keywords:

Infections; Drug repurposing; Flavones; Chalcones; Xanthones; Broth microdilution; Ergosterol; HPLC.

Resumo

O aumento do número de espécies com potencial patogénico e de resistência aos antimicrobianos mais comuns na prática clínica, culminaram num crescente interesse na descoberta de novos agentes terapêuticos.

Este trabalho tem por base a exploração de duas vias utilizadas na obtenção de novos fármacos: a reutilização de fármacos previamente estabelecidos e o estudo de novos compostos sintéticos. A natureza é uma fonte imensurável de novos produtos, resultante da otimização natural ao longo de milhões de anos, e uma fonte de inspiração para a obtenção sintética de análogos. As flavonas, calconas e xantonas já mostraram possuir potencial atividade antimicrobiana. Assim sendo, estas famílias de compostos naturais destacam-se como fontes promissoras para o desenvolvimento de novos fármacos com esta aplicação terapêutica.

Neste trabalho apresenta-se a avaliação antimicrobiana, através do método de microdiluição em caldo, de 7 compostos disponíveis comercialmente, e com conhecida aplicação terapêutica, e de 34 novos compostos sintéticos, incluindo flavonas, calconas e xantonas. Desta avaliação foram identificados 7 compostos com atividade antimicrobiana. Para além do trabalho descrito, foi utilizado o HPLC com o intuito de avaliar o efeito na biossíntese do ergosterol de três das calconas com atividade antifúngica mais promissora.

Palavras-chaves:

Infeções; Reutilização de fármacos; Flavonas; Calconas; Xantonas; Microdiluição em caldo; Ergosterol; HPLC.

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List of abbreviations

ADMET	Absorption, distribution, metabolism, excretion and toxicity
ATCC	American Type Culture Collection
CFU	Colony-forming Unit
CLSI	Clinical & Laboratory Standards Institute
CNS	Central Nervous System
DMSO	Dimethylsulfoxide
EMA	European Union Agency
ERG11	Lanosterol 14-α-demethylase
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight
FDA	Food and Drug Administration
HIV	Human Immunodeficiency Virus
HPLC	High-Performance Liquid Chromatography
HTS	High-Throughput Screening
IFI	Invasive Fungal Infection
MHA	Mueller-Hinton Agar
MHB2	Mueller-Hinton Broth 2
MIC	Minimum Inhibitory Concentration
MLC	Minimum Lethal Concentration
MOPS	3-(N-Morpholino)-propanesulfonic Acid
MYC	Mycobiotic Agar
NIR	Near-infrared
ROS	Reactive Oxygen Species
SAR	Structure-activity Relationship
SDA	Sabouraud Dextrose Agar
SOSA	Selective Optimization of Side Activities
TOR	Target of Rapamycin
UV	Ultraviolet
WHO	The World Health Organization

1. Introduction

Millions of symbiotic microorganisms coexist in the human body, constituting the human microbiota, where they play an important role in the clinical condition of the human being. They intervene in obtaining nutrients and in the processing of xenobiotics, providing to the human being unique and specific biochemical enzymes and pathways. They also provide to the host protection against pathogens, through competitive exclusion and production of antimicrobial substances.[1]

Under certain circumstances, the human microbiota can be harmful to us. The abnormal presence of the microbiota, outside its natural location, coupled with an imbalance between the body's natural defences and the pathogenic properties of microorganisms, trigger the manifestation of infection.[2]

Infections are caused not only by invasion or colonization of the body by external pathogenic microorganisms, but also by microorganisms normally present in the body, whose immunological conditions of the host allow the manifestation of its pathogenic properties. These diseases differ from most other pathologies because of its rapid mutability. Infectious diseases remain the second leading cause of death worldwide. Bacterial infections are responsible for the deaths of about 17 million people worldwide, but many others die of fungal, viral and parasitic diseases.[3]

The first concept of chemotherapy was dated from the work of Paul Ehrlich (1854-1915) in Germany. Ehrlich recognized the selective toxicity of chemical agents against infectious organisms by hypothesizing that substances that bind selectively only to the pathogen, and not to host cells, appear as effective drugs without producing side effects. To this ideal chemotherapeutic agent was assigned the term, metaphorical, "magic bullet".

Since penicillin was discovered and introduced as a powerful antibacterial agent, antimicrobials have become critical in the fight against infectious diseases caused by bacteria and other microbes. As microorganisms emerge, new treatment and prevention strategies are also presented.[4] The increase of pathogenic microorganisms resistant to conventional antibiotics has resulted in an increase in the economic costs associated with the application of the treatments, as well as in a worrying inefficacy in their activity. Consequently, it triggered a growing interest and investment in the discovery of new therapeutic agents.[5] However, despite all the effort and progress made in discovering new compounds, infections continue to be a major cause of death in the world.

The progress of modern medicine, despite all the benefits, has triggered a substantial increase in the incidence of fungal infections, making them an important cause of morbidity and mortality. In developed countries, these infections occur mainly due to increased use of immunosuppressive therapies, such as immunomodulatory agents used

in the treatment of cancer, and organ transplantation. Moreover, clinical conditions that lead to immunocompromised hosts, such as the presence of Human Immunodeficiency Virus (HIV), are an important factor in the manifestation of these infections. Within this opportunistic window during which individuals are immunocompromised, they are susceptible to invasion by certain fungi.[6] Late diagnosis, the spread of infections to places where, for pharmacokinetic reasons, current treatments are not effective and increased resistance to medications, are just some of the causes, responsible for the high mortality resulting from the incidence of mycoses.[7]

The World Health Organization (WHO) does not present a program to combat and control fungal infections, and most public health agencies do poor or none mycological surveillance.[6] The financing of medical mycology is quite underrepresented when compared to other infectious diseases. Therefore, it is crucial not only to improve the mycological surveillance, but also to develop new antifungal agents to circumvent the resistance to the currently available and to be effective against new species involved in human infections.

1.1. Fungi kingdom

Fungi are currently organized in a separate kingdom of plants, animals and bacteria, the *Fungi* Kingdom (Eumycota).[8]

According to the morphology, fungi can be organized into distinct groups: yeasts, filamentous fungi and dimorphic fungi. Yeasts are usually unicellular, non-filamentous, typically spherical or oval. They produce rounded, pasty, or mucoid colonies on agar, resulting from the agglomeration of individual organisms.[9] Filamentous fungi (multicellular) present a set of filamentous tubular structures made up of connected cells, the hyphae. The hyphae may be divided by septa, conferring the designation of septate hyphae, or without septa, called aseptic or cenocytic hyphae. When the environmental conditions are favourable, the hyphae grow giving rise to a filamentous mass visible to the naked eye, the mycelium.[9] Moreover, dimorphic fungi present the two forms of growth during their life cycle. This third group is notoriously associated with pathogenic fungi, and their transition process, between groups, is closely dependent on external and internal conditions, such as the temperature or CO_2 concentration.[8] Taking advantage of their dimorphism, they assume the form of growth that is more suitable for their development.

The taxonomic organization and identification of fungi was classically based on macroscopic and microscopic morphology and biochemical parameters. In last decade, methodologies based on gene sequencing, in addition to the phenotypic characters, brought a more accurate identification to species level.[10] In addition, mass spectroscopy techniques

such as the matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) has emerged as an important tool for yeasts and filamentous fungi identification.[11-12] However, the methods based in DNA and proteomics have to be supported by the morphologic characteristics, to confirm the identity.

Fungi can be found in a wide range of different environments. Despite being primary terrestrial organisms, some may be found in freshwater or marine environment, playing an important recycler role in several organic cycles, and representing a positive force in the cycle of all organic matter.[13] These organisms have an important role in the pharmaceutical industry, being used in the production of organic acids, drugs (such as ergometrine and cortisone); antibiotics (such as penicillin and griseofulvin) and immunosuppressive substances (such as cyclosporine).[14] It is recognized that among the approximately 100,000 species of fungi described, not all have exclusively advantages, fewer than 400 are medically important, and less than 50 species cause more than 90% of the fungal infections of humans and other animals.[15,16]

1.2. Fungal infections

Human fungal infections can be classified into four groups according to the anatomical location: superficial, cutaneous, subcutaneous and systemic mycoses. Superficial mycoses are limited to the outermost part of the skin and hair due to changes in the normal conditions of secretions or flora. Cutaneous mycoses are caused by fungi that infect only the superficial and keratinized structures of the body (skin, hair and nails). Subcutaneous mycoses are initiated when objects contaminated with fungi, which normally reside in soil or vegetation, cause a traumatic injury. After entering the subcutaneous tissue, these invading organisms can proceed with the infection. Systemic mycoses are fungal infections that, theoretically, can involve any part of the body, such as the bloodstream, central nervous system (CNS), or organs.[15, 17]

Two types of microorganisms, the primary and opportunistic pathogens, cause fungal infections, most of which are opportunistic infections. In the case of opportunistic pathogens, when the host is healthy, no manifestations of disturbances are detected in his presence. However, when the clinical condition of the host is compromised, manifestations of infection occur. In contrast, primary pathogens are naturally able to establish an infection in the healthy population. It may be noted that many previously non-pathogenic species are now recognized as opportunistic pathogens.[18]

Most people throughout their lives contract superficial fungal infections, which are usually easy to cure, but millions of individuals around the world contract invasive fungal

infections (IFIs), whose diagnosis and treatment are more difficult.[19] In general, the term IFI is used only to characterize systemic, generalized, deep-seated, visceral and severe, life-threatening fungal infections, in contrast to superficial, local, benign, self-limiting fungal diseases. IFIs are often caused by the yeasts *Candida* and *Cryptococcus* and by filamentous fungi such as *Aspergillus*, *Fusarium* or *Mucor* species. In addition, these infections can be caused by some dimorphic fungi such as *Coccidioides*, *Blastomyces* and *Histoplasma* species.[6]

Invasive and superficial mycoses have become, and continue to be, a major public health problem, with considerable morbidity and mortality values. For example, in the early 2000s for people living with HIV, it was estimated that there were more than 1 million cases per year of disseminated cryptococcosis, and about 600,000 of these cases resulted in death.[20]

1.3. Antifungal agents

An ideal antifungal agent should be active and highly selective against specific targets present in fungi, fungicidal rather than fungistatic due to its mechanism of action, exhibit suitable ADMET properties and appropriate for formulation by both oral and intravenous route.[21]

Humans and fungi are similar, eukaryotes cells. In antifungal therapy, numerous potential targets can be identified. However, the compounds should be optimized for substantial selectivity, in order to block or destroy the eukaryotic machinery of fungi, causing limited or no damage to the cellular functions of the host.[22] In fungi, we can highlight the exclusive constituents of its cell wall (chitin, β-(1,3)-glucan and mannoprotein) and cellular membrane (ergosterol). The molecular differentials confer the selectivity to the different chemotherapeutic agents.

The antifungal drugs currently available include polyenes (amphotericin B and nystatin), azoles (such as fluconazole, ketoconazole and itraconazole), pyrimidines (flucytosine), echinocandins (such as caspofungin and micafungin) and allylamines (terbinafine and naftifine).[23]

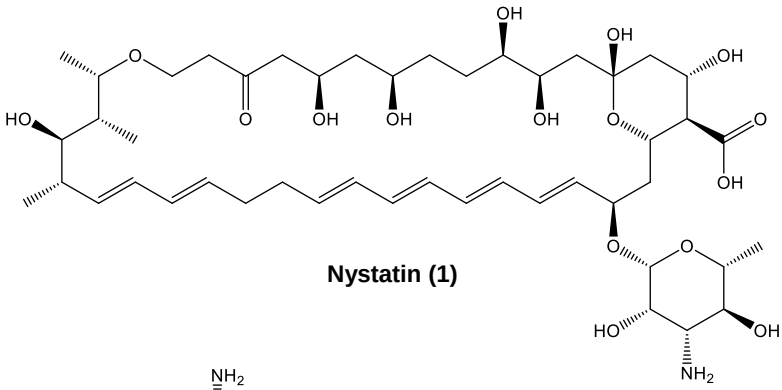
Knowledge of the intrinsic resistance of a pathogen of concern is important in clinical practice to avoid inappropriate and ineffective therapies. Antifungal resistance can be primary (intrinsic) or secondary (acquired). Intrinsic resistance is the innate ability of a microbial species to resist to the activity of an antifungal agent, thanks to its structural or functional characteristics that allow the presence of tolerance to a drug or class of drugs. Acquired resistance consists on the achievement of the ability to resist the activity of an

antifungal agent to which it has been susceptible. This last form of resistance results from genetic changes in genes involved in physiological processes and cellular structures. The overexpression of efflux pumps in fungal cells, with consequent reduction of drug concentrations in the interior is an example of a mechanism involved in the acquired resistance.[21] Resistant strains are increasing in number for some antifungal agent classes, particularly for the azoles and the echinocandins.[24]

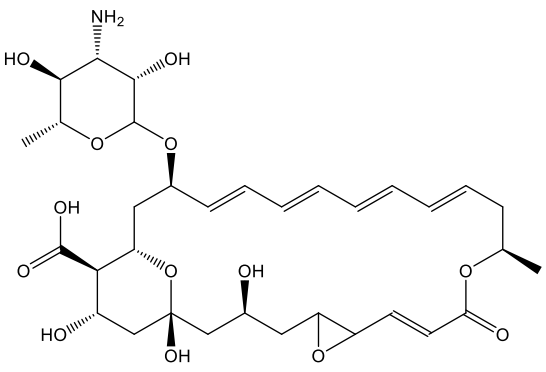
Polyenes

The polyenes, discovered in late 1950s, are fungicidal and exhibit a broad spectrum of antifungal activity against yeast and filamentous fungi, superior to other clinically available agents.[25] They are widely used for the systemic treatment of *Candida*, *Cryptococcus*, *Aspergillus* infections and many other IFIs,[6] but have also been reported as having other clinical applications, such as antibacterial, antiparasitic, immunosuppressive and antitumor.[26]

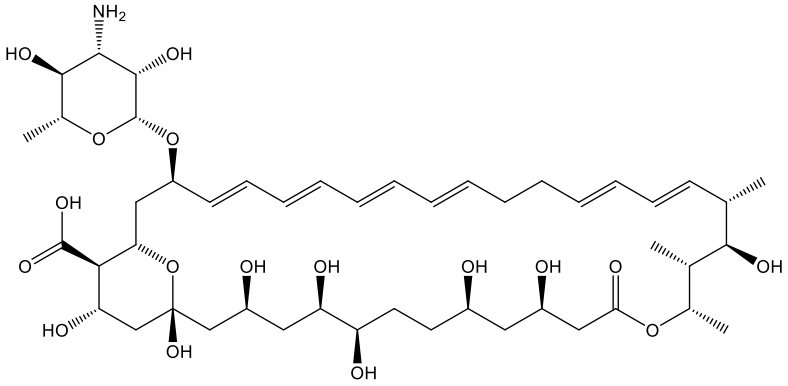
The antifungal agents nystatin (**1**), natamycin (**2**), and amphotericin B (**3**) (Figure 1) are the three polyenes in clinical use and represent a fraction of the over 200 polyene macrolide products.[27-28] They are mainly produced by the Gram-positive bacteria of the genus *Streptomyces, S. nodosus.*[26]



Nystatin (1)



Natamycin (2)



Amphotericin B (3)

Fig. 1: Structure of nystatin (**1**), natamycin (**2**) and amphotericin B (**3**).

Chemically the polyenes are hydrophobic molecules with a polyhydroxylic lactone ring of 20–40 carbon atoms with 4–7 conjugated double bonds.[28] This hydrophobicity is the structural feature most likely to contribute to the mechanism of action, since it provides hydrophobic interactions with the membrane sterols. These compounds also present a polar side chain, comprising a large number of hydroxyl groups and the amino sugar aminosamine, which gives them amphipathic nature and chemical instability.[27]

Through a close association with ergosterol, they form a ring of 8-10 molecules of polyene, culminating in the formation of aqueous pores in the fungi cellular membrane, which allow small essential molecules to escape from the cytoplasm (Figure 2). In addition, these drugs can have alternative mechanisms. For example, amphotericin B (**3**) directly binds to ergosterol and leads to electron transfer in the cell membrane, thus generation of reactive oxygen species (ROS) and creating oxidative stress.[29]

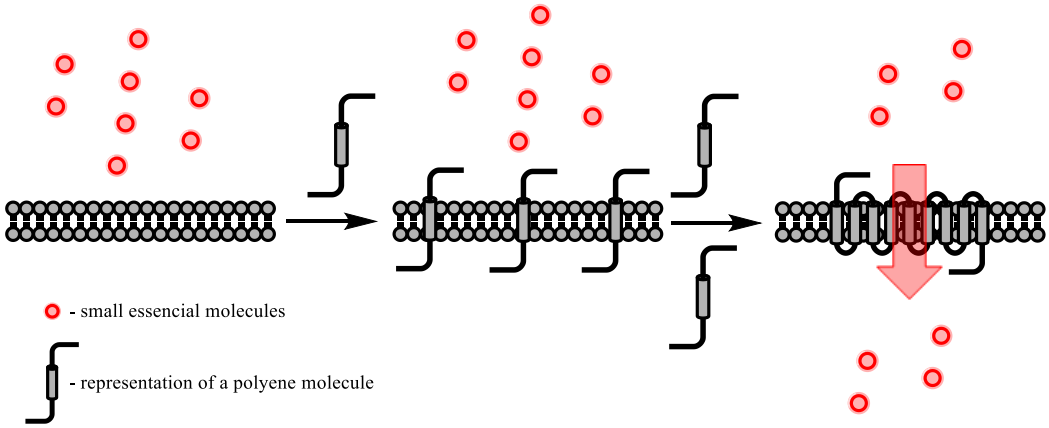
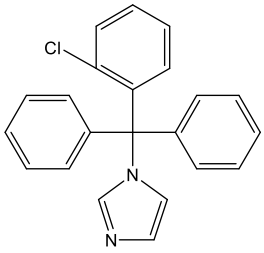


Fig. 2: Formation of ion channels by polyenes.

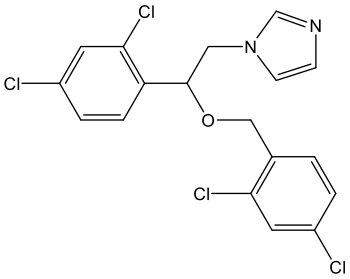
The affinity for cholesterol, the sterol present in human cells, confers them toxicity, thus limiting their use. For this reason, they are often used only in the initial phase of clinical treatment. Despite the reported toxicity, among the systemic antifungal agents currently in clinical, amphotericin B (**3**) remains the most potent fungicide and has the broadest antifungal spectrum of activity.[22] The development of novel formulations (liposomes, lipid complexes and colloidal dispersions) appear as a novel approach to limit toxicity and to obtain more efficient forms of administration.[27]

Azoles

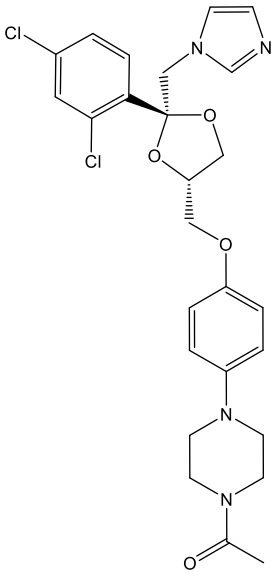
Azoles, first introduced in 1960s as derivatives of N-substituted imidazole, are the most widely used class of antifungal agents.[28] They are a large family of synthetic organic compounds, possessing a broad spectrum of activity with mainly fungistatic effect. They are classified into two main groups, imidazoles and triazoles, depending on the presence in the azole ring of two, or three nitrogen atoms, respectively (Figure 3).[30]



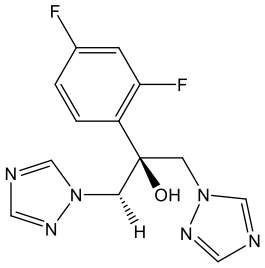
Clotrimazole (4)



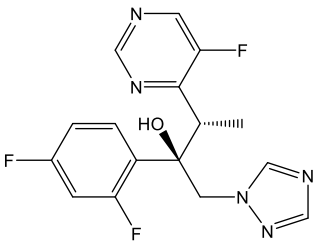
Miconazole (5)



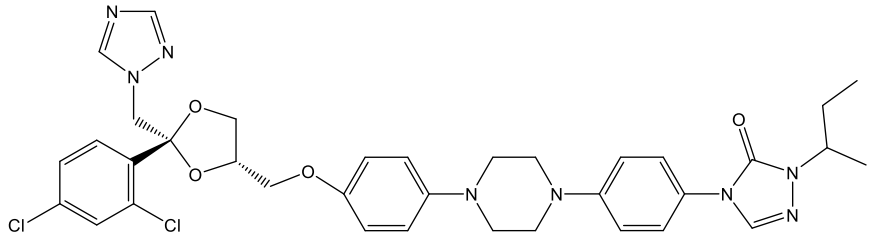
Ketoconazole (6)



Fluconazole (7)



Voriconazole (8)



Itraconazole (9)

Fig. 3: Structures of clotrimazole (**4**), miconazole (**5**), ketoconazole (**6**), fluconazole (**7**), voriconazole (**8**), itraconazole (**9**).

These antifungal agents prevent the synthesis of ergosterol by inhibiting the activity of lanosterol 14-α-demethylase (ERG11), a key enzyme in ergosterol synthesis, that is not present on the host cell membrane[22], responsible for the oxidative removal of the 14-α-methyl group from lanosterol (Figure 4). As a result, the levels of ergosterol are reduced with the concomitant accumulation of the intermediate precursors of ergosterol biosynthesis.[31] As ergosterol is the predominant sterol in the cell membrane of fungi, while mammalian membranes possess cholesterol, these drugs are quite selective for fungi.[6]

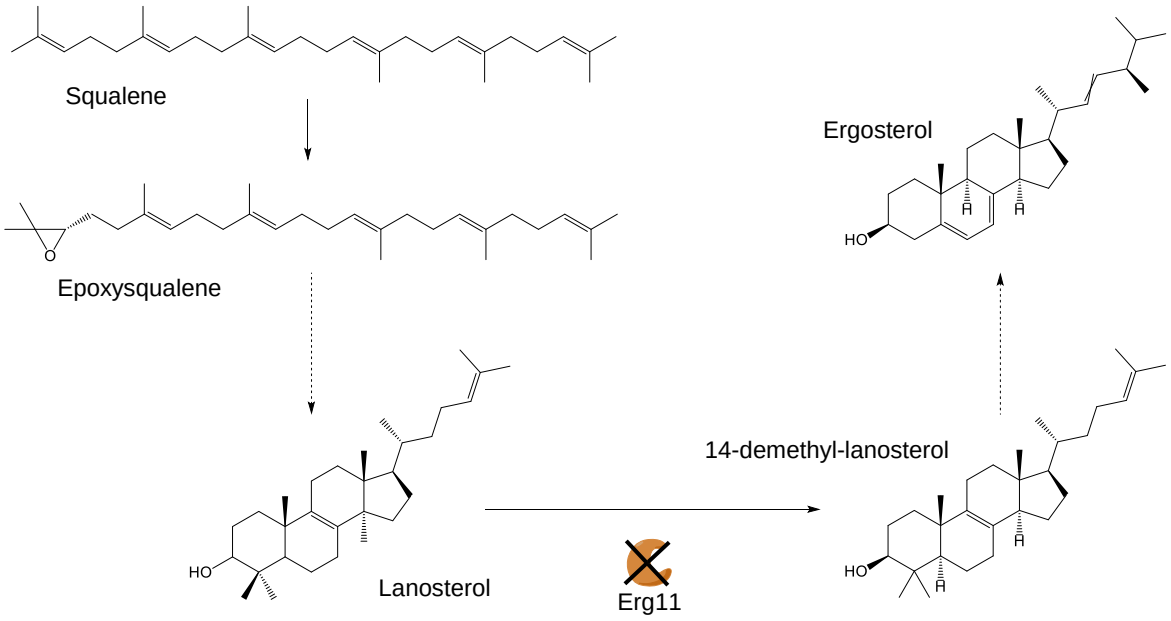
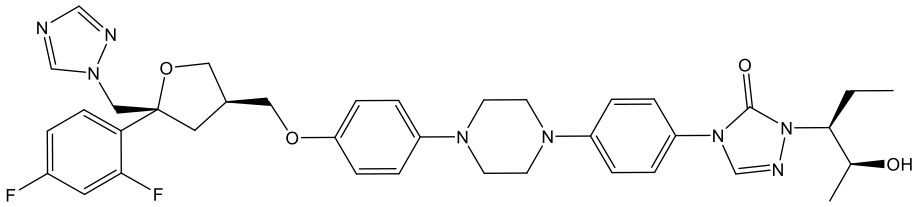


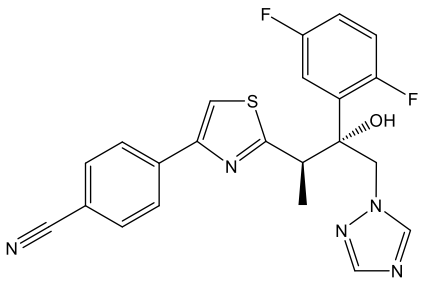
Fig. 4: Simplified scheme of ergosterol biosynthesis.

All antifungal azoles have the same mechanism of action. The differences among them are in avidity of enzyme binding, pharmacology, and side effects.[32] Although some side effects, such as nausea, vomiting and elevated liver enzymes, complicate the treatment of some patients, azoles may still be less toxic than other agents such as

amphotericin B (**3**).[33] More recently, other azole compounds with improved antifungal activity, safety, pharmacokinetics, and a broader spectrum of activity, such as posaconazole (**10**) and isavuconazole (**11**) (Figure 5) have been introduced in antifungal therapy or prophylaxis. Posaconazole (**10**) is structurally related to itraconazole (**9**) and possesses potent broad-spectrum activity against *Candida* species, *Aspergillus* species, and *Cryptococcus neoformans*. It was approved by the Food and Drug Administration (FDA) for use as prophylaxis against invasive *Aspergillus* and *Candida* infections in immunocompromised patients.[34] Isavuconazole (**11**) is a new triazole with a broad spectrum of activity against yeasts, molds, and dimorphic fungi. Its safety profile and pharmacokinetic characteristics make it a good choice for therapy, having been the latest FDA approval for the treatment of invasive aspergillosis and mucormycosis.[35]



Posaconazole (10)



Isavuconazole (11)

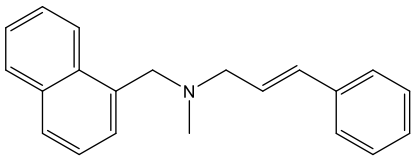
Fig. 5: Structures of posaconazole (**10**) and isavuconazole (**11**).

Allylamines

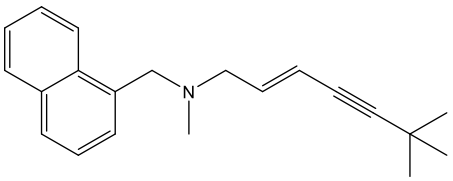
Allylamines are a group of synthetic antifungal agents which inhibit the activity of squalene epoxidase, a key enzyme in the early stages of ergosterol biosynthetic pathway, that catalyses the stereospecific epoxidation of squalene to 2,3-(*S*)-oxidosqualene in fungi.[36] The resulting ergosterol depletion and toxic squalene accumulation affect fungi cell membrane structure and function, eventually leading to growth inhibition and cell death.

Naftifine (**12**) and terbinafine (**13**) (Figure 6) are examples of this class of antifungals and are found to block the ergosterol biosynthesis of species such as *Saccharomyces cerevisiae*, *Candida albicans* and dermatophytes.[36] The first is considered a topical

antifungal, while the second, in addition to topical administration, may also be administered orally. Both antifungal agents can be administered topically for superficial skin infections, such as tinea cruris, tinea pedis, and other types of tinea corporis.[37] Allylamines appear as a preferential therapeutic possibility in relation to the azoles, due to the development of resistance to fluconazole (**7**), by *Candida* spp., and to itraconazole (**9**), by filamentous fungi species, as a result of increased use of azoles in therapy.[38-39]



Naftifine (12)

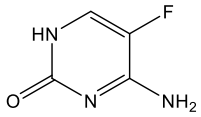


Terbinafine (13)

Fig. 6: Structures of naftifine (**12**) and terbinafine (**13**).

Pyrimidine analogues

5-Fluorocytosine or flucytosine (**14**) (Figure 7), an antimetabolite, was first synthesized in 1957 and its antifungal property discovered in 1964.[28] It is a fluorine analogue of cytosine, which inhibits nucleotide biosynthesis as it enters inside the fungal cells *via* cytosine permease. Following intracellular enzymatic modifications in the target cell, by a cytosine deaminase[22], that is not present in humans, flucytosine is transformed in 5-fluorouracil, 5-fluorodeoxyuric acid, and 5-fluoruridine, which exhibit antifungal activity. These compounds interfere in the synthesis of DNA and RNA, through enzymatic inhibition of the enzyme thymidylate synthetase and through incorporation into fungal RNA, instead of a normal nucleotide.[28, 40]



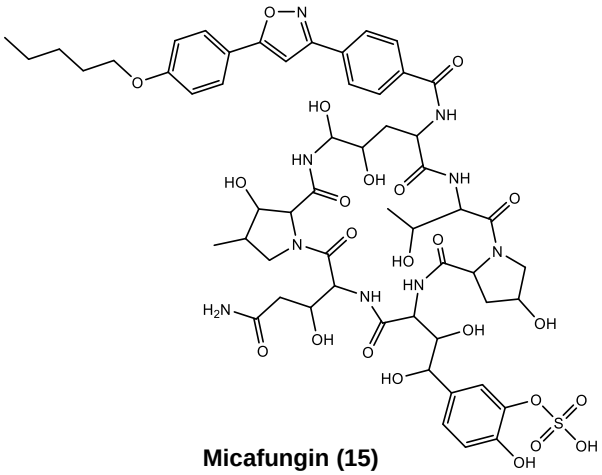
5-Fluorocytosine (14)

Fig. 7: Structure of 5-fluorocytosine (**14**).

Frequent development of mutational resistance by the target during therapy limits its application. For this reason, this drug is used as an adjunct to other drugs, such as fluconazole (**7**) or amphotericin B (**3**), instead of being used in monotherapie.[28, 40]

Echinocandins

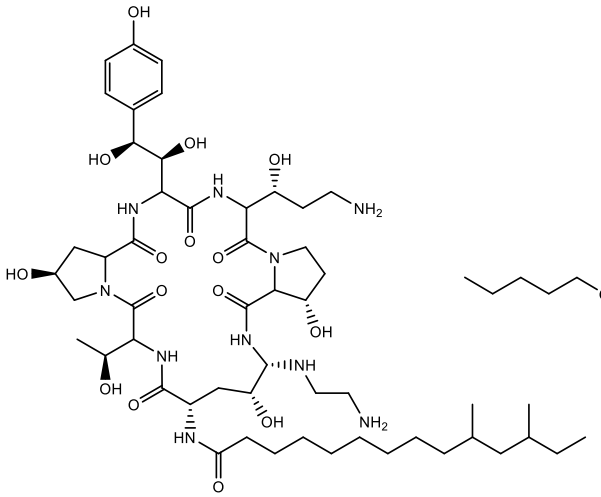
Echinocandins are the most recent antifungals available family. They are a class of semi-synthetic lipopeptides composed of cyclic hexapeptides, N-linked to a fatty acyl side chain, which have been synthetically modified from products resulting from the fermentation process, by several fungi. For example, micafungin (**15**) (Figure 8) is obtained through the cleavage of a hexapeptide in a natural process occurring in the *Coleophoma empedri* and subsequent synthetic addition of the fatty N-acyl side chain.[41]



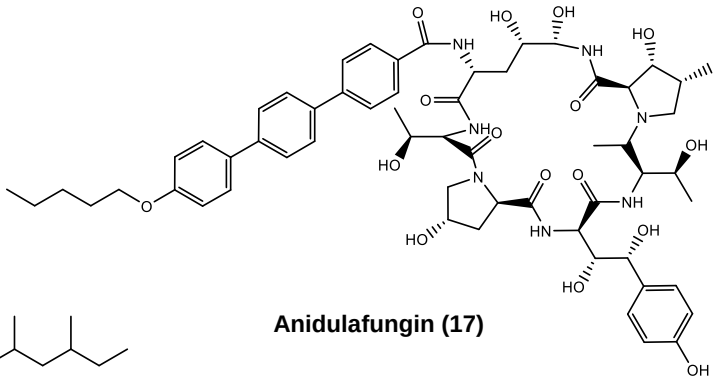
Micafungin (15)

Fig. 8: Structure of micafungin (**15**).

In addition to micafungin (**15**), other two echinocandins with similar activities are currently available, caspofungin (**16**) and anidulafungin (**17**) (Figure 9).



Caspofungin (16)



Anidulafungin (17)

Fig. 9: Structures of caspofungin (**16**) and anidulafungin (**17**).

The mechanism of action of echinocandins is related with the inhibition of the synthesis of β-(1,3)-glucans, important constituents of the fungal cell wall, through the inhibition of 1,3-β-D-glucansynthetase.[28] The absence of β-(1,3)-glucans causes morphological changes and osmotic instability, culminating in the death of the fungal organism. Since mammalian cells do not contain these constituents, this class of antifungal agents is only toxic to fungi, an important factor for their frequent use in a hospital environment.[22]

1.4. Development of new antifungal agents

Over time, microorganisms have developed survival mechanisms to counter threats to their development and growth. Their life cycles provide rapid expansion and it is this same accelerated growth, coupled with the many factors inherent in their survival, which trigger disturbances in man.

Biological systems allow understanding the interaction of the host cell with the microorganism, at the molecular level. At the present, the efforts are focused on identifying new fungal specific targets that are critical for fungal growth and have minimal similarity to targets among human proteins.

The development of resistance is only one of the factors that drive the constant search for new drugs. The new antifungals must respond to several parameters in order to complement or even replace existing therapies. They should present better fungicidal activity, rather than fungistatic activity, greater spectrum of activity against drug resistant fungi, better pharmacokinetic properties and pharmacodynamic parameters, and reduced toxicity through new and more selective mechanisms of action.[21] However, not everything is based on improving its clinical efficiency, the economic factor is also important. Since 2006, after the authorization of anidulafungin (**17**) by the FDA and the European Union Agency (EMA), that no new class of antifungals was approved.[40]

There is an urgent need for new antifungal agents, yet their development is characterized by a lack of investment resulting in limited development. The average costs involved in the development and introduction of a new drug on the market are estimated to be around $ 54 million in the 1970s, rising to about $ 800 million at the turn of the century. In addition to the cost increase, the time period for pharmaceutical companies to maximize their return on investment has decreased.[42] The probability of a compound in the preclinical development reaching the market is only 1 in 10,000 and the costs of developing new products continue to rise.[42]

The High-Throughput Screening (HTS) of large compound libraries is still the mainstay of hit generation in the pharmaceutical industry. However, this approach gives meagre results considering the investment in labour and machinery. In order to reduce the associated costs, smaller libraries, whose compounds present increased drug-likeness, are used.[43]

1.4.1. Repurposing

Some drugs have more than one therapeutic application, being the degree of potency for each pharmacological activity the factor that is decisive in the choice of the main therapeutic application. There are several examples of drugs or drug candidates that, after being introduced in the market for the treatment of a specific disease, or being in clinical trials, are explored to be used in other pathologies.

The process of adapting the drugs previously established for the treatment of new diseases, as a different approach to the expansion and development of new drugs, is defined as repurposing, also called repositioning, reprofiling and therapeutic switching.[44]

The "recycled drugs" may be included into the following categories:[44]

(i) drugs in clinical development;

(ii) drugs that failed to demonstrate the effectiveness of a particular indication in phase II or III clinical trials, but for which no safety concerns were identified;

(iii) projects of drugs that were discontinued for commercial reasons (budgetary issues for example, duplicate projects in the same area therapy or change in the overall strategy of the portfolio of a company);

(iv) marketed drugs for which patents are about to expire or already generic;

(v) drugs which were discovered, developed and launched in emerging markets, but were never released in the major markets like the United States of America.

With repurposing approach, it will possible identify safe and bioavailable drugs with diversified pharmacological applications. In contrast to HTS, using this approach, only a reduced number of drug molecules are screened for which bioavailability and toxicity studies have already been carried out and efficacy in humans has been confirmed. After this screening a hit can be generated and used as the starting point for a drug discovery program, aiming to transform the initial 'side activity' into the 'main activity', the so called selective optimization of side activities (SOSA) approach. With this approach there is a high probability of yielding safe, bioavailable, original and patentable analogues.[43]

As a prime example of this approach, can be referred the work developed by Blankenship et al.[45] In this work it was demonstrated that clinically used calcineurin and target of rapamycin (TOR) inhibitors have antifungal activity and synergize with fluconazole.

Another example is the use of sertraline in cryptococcal meningitis. Sertraline is a selective serotonin reuptake inhibitor that is mainly used to control depression. However, this antidepressant manifests fungicidal activity and synergetic effect with fluconazole, against *Cryptococcus*. One of its most valuable aspects as a potential anticryptococcal drug is its superior ability to accumulate in the CNS over other antifungal agents, which is particularly important in the treatment of cryptococcosis, since *Cryptococcus* preferentially proliferates in the brain.[46] Following a successful phase II exploratory study using sertraline as adjunctive therapy for cryptococcal meningitis[47], the researchers intend to determine the importance of adding this compound to a standard therapy for cryptococcal meningitis, through a phase III exploratory study.[22]

1.4.2. Natural sources and synthetic analogues

Through evolutionary pressure, Nature has guided the production of a wide range of compounds with a variety of biological purposes, such as antimicrobial activity. These secondary metabolites have played an important role in drug discovery. In the late 1920s, Alexander Fleming accidently discovered an antibiotic penicillin from the fungus *Penicillium notatum.* After, several analogues of penicillin or other antibiotics were discovered from natural sources or were synthesized. In addition to penicillins, many chemically diverse antimicrobial agents have been identified and obtained from natural sources, namely microorganisms and plants.[48] These natural compounds are often associated with defence and survival mechanisms. Most of the times, these natural products may present little or no significant activity, or they may have significant side effects. Therefore, these compounds most be afterward submitted to molecular modifications in order to potentiate its activity and improve selectivity and drug-like properties. Numerous examples of lead optimization programs have successfully optimized antimicrobial lead molecules from Nature.[49]

1.4.2.1. Flavonoids

The flavonoids are a group of natural products widely found in normal human diet.[50] They possess a fifteen-carbon skeleton consisting of two benzene rings (rings A and B) linked by a three carbon unity which may or may not form a third ring (ring C). They can be subdivided into several sub-classes as illustrated in Figure 10.

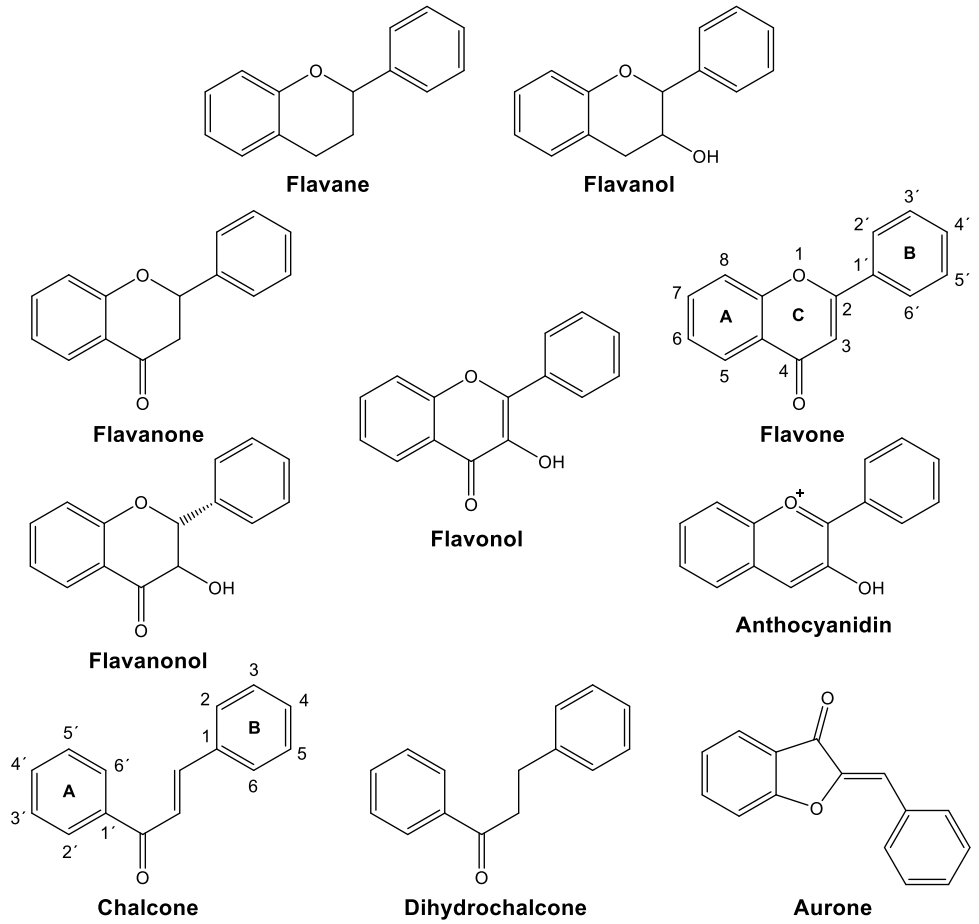


Fig. 10: Basic skeletons of several subclasses of flavonoids.

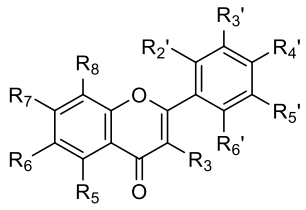
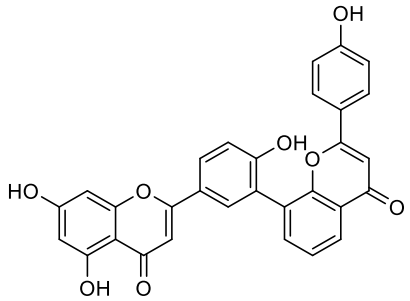
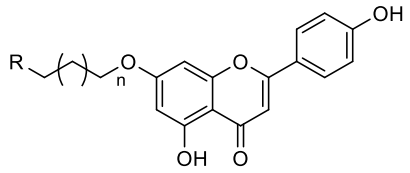
These natural products have been exhaustively explored for their biological activities. Among flavonoids, flavones and chalcones have been considered privileged structures in Medicinal Chemistry.[51-52]

Flavones are a class of flavonoids based on the base structure of 2-phenyl-1-benzopyran-4-one (Figure 10), that are widely distributed in the plant kingdom as secondary metabolites.[53] The various biological activities exhibited by the flavones are dependent on the nature and position of the substituents on the flavone skeleton.[54]

Various natural flavone derivatives, as well as their synthetic analogues, have been synthesized and evaluated for several therapeutic activities like anti-inflammatory, antiestrogenic, anti-allergic, antioxidant, antitumor and antimicrobial.[55]

Flavones with diverse substitution pattern, including those possessing hydroxyl, methoxy, and nitrogen containing flavones have been reported to have considerable antimicrobial activity against a wide range of microorganisms.[56-60] **Table 1** presents some examples of flavones with antimicrobial activity.

Table 1: Examples of flavones with antimicrobial activity.

Flavones	Microorganisms
 $R_5 = R_7 = R_5' = R_4'$= OH (**18**) $R_5 = R_7 = R_4'$= OH; $R_3' = R_5' = OCH_3$ (**19**) $R_6 = R_7 = R_4'$= OH; $R_3' = R_5' = OCH_3$ (**20**) $R_5 = R_5'$= OH; $R_4 = R_2' = R_4' = OCH_3$ (**21**) $R_5 = R_7 = R_4' = OCH_3$ (**22**)	(**18**) - *T. rubrum*: MIC = 250 [56] (**19**) - *S. aureus*: MIC = 500 [57] - *S. lutea*: MIC = 500 [57] - *E. coli*: MIC = 600 [57] (**20**) - *S. aureus*: MIC = 600 [57] - *S. lutea*: MIC = 600 [57] - *E. coli*: MIC = 1000[57] (**21**) - *S. aureus*: MIC = 800 [57] - *S. lutea*: MIC = 1000 [57] - *E. coli*: MIC = 1000 [57] (**22**) - *C. krusei*: MIC = 32 [58]
 **23**	(**23**) - *C. albicans:* MIC = 5 [59] - *S. cerevisiae:* MIC = 5 [59] - *T. beigelii:* MIC = 5-10 [59]
 n = 4; R = Morpholine (**24**) n = 4; R = N-methyl piperizine (**25**)	(**24**) - *S. aureus*: MIC = 12.5 [60] - *K. aerogenes*: MIC = 12.5 [60] (**25**) - *S. aureus*: MIC = 12.5 [60] - *K. aerogenes*: MIC = 12.5 [60]
MIC - Minimum inhibitory concentrations; Results are expressed in µg/mL. *T. rubrum - Trichophyton rubrum; S. aureus - Staphylococcus aureus; S. lutea - Sarcina lutea; E. coli - Escherichia coli; C. krusei - Candida krusei; C. albicans - Candida albicans; S. cerevisiae - Saccharomyces cerevisiae; T. beigelii - Trichosporon beigelii; K. aerogenes - Klebsiella aerogenes*	

Chalcones are secondary metabolites widely distributed in nature, abundant in fruits, vegetables, and edible plants, considered precursors of flavonoids and isoflavonoids.[61] The chalcone family has a wide structural diversity and can be classified roughly into two categories: simple/classic chalcones and hybrid chalcone.[52] Chalcones (1,3-diphenyl-2-propen-1-one) are open-chain flavonoids possessing two aromatic rings (A and B rings) connected by a three-carbon linker.[62] They exist as *E* and *Z* isomers, being the *E* isomer the most thermodynamically stable (Figure 11).[52]

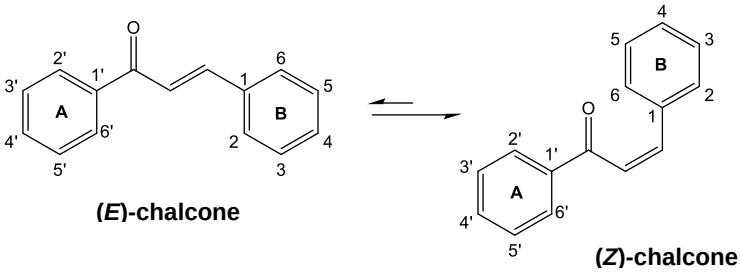
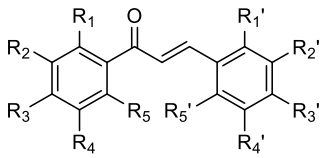


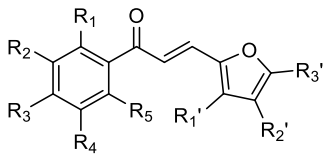
Fig. 11: Structure of (*E*)-chalcone and (*Z*)-chalcone.

This family has attracted much interest not only from the synthetic and biosynthetic perspectives but also from the varied and interesting biological activities. Chalcones were referenced as having anti-inflammatory, analgesic, antipyretic, antibacterial, antifungal, insecticide, antimutagenic, in addition to many others.[61-62]

Some of these compounds showed good antimicrobial activity against a wide range of microorganisms.[63-68] **Table 2** presents some examples of chalcones with antimicrobial activity.

Table 2: Examples of chalcones with antimicrobial activity.

Chalcones	Microorganisms
 $R_1 = R_3 =$ OH (**26**) $R_1 =$ OH; R_1'= Cl (**27**) $R_1 =$ OH; R_1'= OCH_3; R_4' = OH (**28**) $R_1 =$ OH; $R_3 = R_5 = OCH_3$; R_3' = Br (**29**) $R_1 =$ OH; $R_3 = R_5 = OCH_3$; R_1' = Cl (**30**) $R_1 =$ OH; R_2 = Br; $R_3 = R_5 = OCH_3$; R_3' = Br (**31**) $R_1 =$ OH; R_2 = Br; $R_3 = R_5 = OCH_3$; R_1' = Cl (**32**) R_1' = R_2' = Cl (**33**) R_2' = OCH_3 (**34**) R_3' = OCH_3 (**35**)	(**26**) - *C. albicans*: MIC = 15.6 [63] - *C. glabrata*: MIC = 15.6 [63] - *C. krusei*: MIC = 15.6 [63] (**27**) - *T. rubrum*: MIC_{80} = 4 [64] (**28**) - *T. rubrum*: MIC_{80} = 1 [64] (**29**) - *E. floccosum*: MIC = 50 [65] - *T. rubrum*: MIC = 50 [65] - *M. canis*: MIC = 50 [65] (**30**) - *E. floccosum*: MIC = 100 [65] - *T. rubrum*: MIC = 12.5 [65] - *M. canis*: MIC = 100 [65] (**31**) - *E. floccosum*: MIC = 25 [65] - *T. rubrum*: MIC = 25 [65] - *M. canis*: MIC = 50 [65] (**32**) - *E. floccosum*: MIC = 25 [65] - *T. rubrum*: MIC = 50 [65] - *M. canis*: MIC = 50 [65] (**33**) - *M. canis*: MIC = 12.5 [66] - *T. rubrum*: MIC = 12.5 [66] - *E. floccosum*: MIC = 12.5 [66] (**34**) - *M. canis*: MIC = 3 [66] - *T. rubrum*: MIC = 6.25 [66] - *E. floccosum*: MIC = 1.5 [66] (**35**) - *M. canis*: MIC = 3 [66] - *T. rubrum*: MIC = 12.5 [66] - *E. floccosum*: MIC = 1.5 [66]

Chalcones (Continuation)	Microorganisms (Continuation)
R_3' = CH_3 (**36**) R_1' = R_3' = OCH_3 (**37**)R_3 = CH_3; R_3' = OCH_3 (**38**) R_3 = Br; R_1' = R_2' OCH_3 (**39**) R_3 = Br; R_2' = OCH_3 (**40**) R_3 = R_5 = R_3' = OH (**41**) R_3 = R_5 = R_3' = OH; R_4 = $CH_2CHC(CH_3)_2$ (**42**) R_3 = OCH_3; R_5 = R_3' = OH; R_4 = $CH_2CHC(CH_3)_2$ (**43**) R_1 = R_3 = R_5 = OMe (**44**) R_1 = R_3 = R_5 = OMe; R_2' = F (**45**) R_1 = R_3 = R_5 = OMe; R_3' = F (**46**) R_1 = R_3 = R_5 = OMe; R_1' = R_4' = F (**47**) R_1 = R_3 = R_5 = OH; R_1' = F (**48**) R_1 = R_3 = R_5 = OH; R_1' = R_4' = F (**49**)	(**36**) - *M. canis*: MIC = 3 [66] - *T. rubrum*: MIC = 6.25[66] - *E. floccosum*: MIC = 0.75 [66] (**37**) - *M. canis*: MIC = 3 [66] - *T. rubrum*: MIC = 6.25 [66] - *E. floccosum*: MIC = 3 [66] (**38**) - *M. canis*: MIC = 3 [66] - *T. rubrum*: MIC = 50 [66] - *E. floccosum*: MIC = 0.5 [66] (**39**) - *M. canis*: MIC = 25 [66] - *T. rubrum*: MIC = 3[66] - *E. floccosum*: MIC = 3 [66] (**40**) - *M. canis*: MIC = 25 [66] - *T. rubrum*: MIC = 3 [66] - *E. floccosum*: MIC = 0.5 [66] (**41**) - *S. aureus*: MIC = 62.5 [67] (**42**) - *S. aureus*: MIC = 31.2 [67] (**43**) - *S. aureus*: MIC = 7.8 [67] (**44**) - *C. albicans*: MIC = 15.62 [68] - *S. aureus*: MIC = 31.25 [68] - *E. coli*: MIC = 31.25 [68] (**45**) - *C. albicans*: MIC = 125 [68] - *S. aureus*: MIC = 125 [68] - *E. coli*: MIC = 125 [68] (**46**) - *C. albicans*: MIC = 62.5 [68] - *S. aureus*: MIC = 62.5 [68] - *E. coli*: MIC = 125 [68] (**47**) - *C. albicans*: MIC = 125 [68] - *S. aureus*: MIC = 15.6 [68] - *E. coli*: MIC = 62.5 [68] (**48**) - *C. albicans*: MIC = 15.62 [68] - *S. aureus*: MIC = 125 [68] - *E. coli*: MIC = 125 [68] (**49**) - *C. albicans*: MIC = 125 [68] - *S. aureus*: MIC = 62.5 [68] - *E. coli*: MIC = 125 [68]
 R_1 = OH; (**50**) R_1 = OH; R_4'= OH; (**51**) R_1 = OH; R_3 = R_5 = OCH_3; (**52**)	(**50**) - *T. rubrum*: MIC_{80} = 4 [64] (**51**) - *T. rubrum*: MIC_{80} = 4 [64] (**52**) - *T. rubrum*: MIC = 250 [65] - *M. canis*: MIC = 250 [65]
MIC - Minimum inhibitory concentrations; Results are expressed in µg/mL. *C. albicans - Candida albicans; C. glabrata - Candida glabrata*; *C. krusei - Candida krusei*; *T. rubrum - Trichophyton rubrum*; *E. floccosum - Epidermophyton floccosum; M. canis - Microsporum canis; S. aureus - Staphylococcus aureus; E. coli - Escherichia coli.*	

1.4.2.2. Xanthones

Xanthones are secondary metabolites commonly found in families of higher plants, fungi and lichens. They are heterocyclic compounds with a dibenzo-γ-pyrone framework (Figure 12).[69]

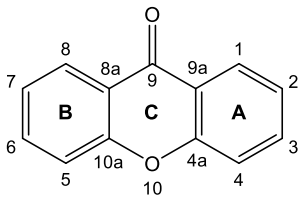


Fig. 12: The structure of xanthone.

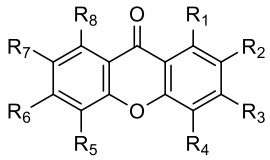
In contrast with flavonoids that are easily found in nature, xanthones are found in a limited number of species. Some of the plants that contain xanthones include species of *Artocarpus, Bersama, Blackstonia, Calophyllum, Cudrania, Eustoma, Frasera, Garcinia, Gentianella, Hypericum, Orphium, Peperomia, Rheedia, Rhus, Schultesia,* and *Tripterospermum.*[70] These secondary metabolites have also been isolated from several terrestrial and marine fungi such as *Aspergillus* sp., *Emericella* sp*.*, and *Penicillium* sp.[70]

Naturally-occurring xanthones, with nearly one thousand known members[69] contain different types of substituents in different positions, being classified into six groups according to their substitution pattern: simple oxygenated xanthones, xanthone glycosides, prenylated xanthones, xanthonolignoids, bisxanthones and miscellaneous xanthones.[71] These diversity of substitution pattern is responsible for the large variety of biological activities, such as antioxidant, hypoglycemic, antiallergenic, anti-inflammatory and antimicrobial activities.[69, 72] Moreover, these natural products have been reported for their stimulatory effect on CNS, and inhibitor properties on reverse transcriptase DNA polymerase activity of HIV.[36,71]

The xanthones of synthetic origin can have simple groups such as hydroxyl, methoxy, methyl, carboxyl, as well as more complex substituents such as epoxide, azole, methylidenebutyrolactone, aminoalcohol, sulfamoyl, methylthiocarboxylic acid, and dihydropyridine in their scaffold.[71]

Some of these compounds showed interesting antimicrobial activity against a wide range of microorganisms.[73-76] **Table 3** presents some examples of xanthones with antimicrobial activity.

Table 3: Examples of xanthones with antimicrobial activity.

Xanthones	Fungi
 R_4 = OH (**53**) R_1 = R_2 = OH (**54**) R_3 = R_4 = OH (**55**) R_1 = OCH_3 (**56**) R_3 = OH; R_4 = OCH_3 (**57**) R_3 = R_5 = OCH_3 (**58**) R_1 = R_6 = OH; R_3 = OCH_3; R_8 = prenyl (**59**) R_1 = R_6 = OH; R_2 = R_8 = prenyl; R_3 = OCH_3; (**60**) R_1 = R_3 = R_6 = OH; R_2 = R_8 = prenyl (**61**) R_1 = R_3 = R_6 = OH; R_2 = R_8 = prenyl; R_7 = OCH_3 (**62**) R_2 = R_3 = OCH_3 (**63**)	(**53**) - *C. neoformans*: MIC = 31.3 [73] - *A. fumigatus*: MIC = 62.5 [73] - *M. canis*: MIC = 15.6 [73] - *E. floccosum*: MIC = 7.8 [73] - *T. rubrum*: MIC = 31.3 [73] (**54**) - *C. albicans*: MIC = 31.3 [73] - *A. fumigatus*: MIC = 31.3 [73] - *M. canis*: MIC = 15.6 [73] - *E. floccosum*: MIC = 15.6 [73] - *T. rubrum*: MIC = 31.3 [73] (**55**) - *M. canis*: MIC = 15.6 [73] - *E. floccosum*: MIC = 7.8 [73] - *T. rubrum*: MIC = 31.3 [73] (**56**) - *M. canis*: MIC = 31.3 [73] - *E. floccosum*: MIC = 31.3 [73] - *T. rubrum*: MIC = 62.5 [73] (**57**) - *E. floccosum*: MIC = 31.3 [73] (**58**) - *M. canis*: MIC = 125 [73] - *E. floccosum*: MIC = 62.5 [73] - *T. rubrum*: MIC = 125 [73] (**59**) - *S. aureus*: MIC = 64 [74] - *E. coli*: MIC = 64 [74] (**60**) - *S. aureus*: MIC = 64 [74] (**61**) - *S. aureus*: MIC = 64 [74] - *E. coli*: MIC = 64 [74] (**62**) - *S. aureus*: MIC = 128 [75] - *E. coli*: MIC = 128 [75] (**63**) - *C. albicans*: MIC = 31.3 [76] - *S. aureus*: MIC = 156.2 [76] - *E. coli*: MIC = 125 [76]

MIC - Minimum inhibitory concentrations; Results are expressed in µg/mL.
C. neoformans - Cryptococcus neoformans; C. albicans - Candida albicans; C. glabrata - Candida glabrata; *C. krusei - Candida krusei*; *T. rubrum - Trichophyton rubrum*; *E. floccosum - Epidermophyton floccosum; M. canis - Microsporum canis; S. aureus - Staphylococcus aureus; E. coli - Escherichia coli.*

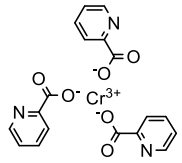
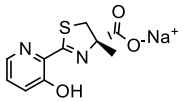
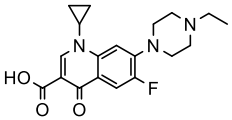
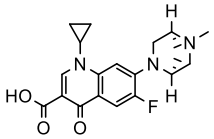
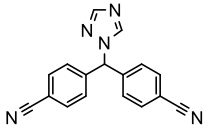
2. Results and Discussion

In the present research work the antimicrobial activity of commercially available bioactive compounds were evaluated, including the iron chelating agents chromium picolinate (**64**) and desferrithiocin (**65**), the antibacterial agents enrofloxacin (**66**) and danofloxacin (**67**), and the aromatase inhibitor letrozole (**68**). In addition to these compounds, a small library of flavonoids, namely the natural flavones scutellarein (**69**) and tangeretin (**70**), as well as structure related flavones (**71** and **72**) and chalcones (**73-99**) and some xanthones (**100-104**), previously synthesized in LQOF and CIIMAR research group, were evaluated for their antimicrobial potential. These effects were evaluated on a yeast (*Candida albicans*), a non-dermatophyte filamentous fungus (*Aspergillus fumigatus*), three dermatophyte filamentous fungi (*Trichophyton rubrum*, *Microsporum canis, Epidermophyton floccosum*), and two bacteria (*Escherichia coli* and *Staphylococcus aureus*) strains.

2.1. Evaluation of the antimicrobial activity of commercial compounds 64-68

In order to investigate the antimicrobial activity of commercial bioactive compounds, their growth inhibitory effect was evaluated, by obtaining minimum inhibitory concentrations (MICs) and minimum lethal concentrations (MLCs) values, against *C. albicans*, *A. fumigatus*, *T. rubrum*, *M. canis, E. floccosum, E. coli* and *S. aureus.* The results are presented in **Table 4**.

Table 4: Antimicrobial activity of compounds[a] 64-68

Compound	*C. albicans*		*A. fumigatus*		*T. rubrum*		*M. canis*		*E. floccosum*		*E. coli*		*S. aureus*	
	MIC	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**
 **64**	NI	NI	NI	NI	NI	NT	NT	NT	NT	NT	NI	NT	NI	NT
 **65**	**256**	**> 512**	NI	NI	**256**	**> 512**	**128**	**> 512**	**128-256[b]**	**> 512**	**256-512[b]**	**> 512**	**256**	**> 512**
 **66**	NI	NI	NI	NI	NI	NT	NT	NT	NT	NT	**32**	NT	**32**	NT
 **67**	NI	NI	NI	NI	NI	NT	NT	NT	NT	NT	**32**	NT	**32**	NT
 **68**	NI	NI	NI	NI	NI	NT	NT	NT	NT	NT	NI	NT	NI	NT

[a] Results are expressed as MIC and MLC in µg/mL and show averages of at least three independent assays made in duplicate.
[b] Display of interval of values, due to variation of MIC values.
MIC - Minimum inhibitory concentrations; MLC - Minimum lethal concentrations; NI - No growth inhibition (MIC>512 µg/mL); NT – Not tested.

From the results shown in **Table 4**, it was found that among the tested compounds only the iron chelating agent desferrithiocin (**65**) showed some antifungal activity. Compound **65** exhibited a broad spectrum of activity, being active against all the tested strains except non-dermatophyte filamentous fungus *A. fumigatus*, with MIC values ranging from 128 to 512 µg/mL, while compounds **64**, **66**-**68** were found to be inactive against all the tested organisms (MIC > 512 µg/mL). Moreover, desferrithiocin (**65**) showed to be more active against the three dermatophyte filamentous fungi (MIC range of 128-256 µg/mL) than against the yeast *C. albicans*, and the bacteria *E. coli* and *S. aureus* (MIC ranging from 256 to 512 µg/mL). Nevertheless, only a fungistatic effect was observed for all strains treated with compound **65** (MLC > 512).

In microorganisms, iron acts as a global regulator for many cellular, metabolic and biosynthetic processes, such as DNA synthesis, electron transport system, enzyme cofactor, oxygen transport, ATP synthesis, among others.[77-78] Iron is also essential for biofilm formation, as it regulates surface motility and stabilizes the polysaccharide matrix.[79-80] Under conditions of iron deficient growth, the hydrophobicity of the microbial surface decreases, altering the surface protein composition, leading to a deficient biofilm formation.[81]

Iron limitation can inhibit microbial growth, and iron chelators have been shown to inhibit fungal pathogens.[78] Lactoferrin, ciclopirox, and deferiprone inhibit the growth of *A. fumigatus*, being this effect related with iron depletion.[82] Moreover, the topical use of ciclopirox for superficial fungal infections suggest that inhibition of fungal iron acquisition could be an interesting strategy to discover new antifungal agents.[83] Furthermore, transfusional siderosis and iron overload in malignancy and liver and stem cell transplantation are associated with an increased risk of aspergillosis.[84-86] Therefore, it is not surprising that iron chelating agents, such as desferrithiocin (**65**) can have some antifungal activity.

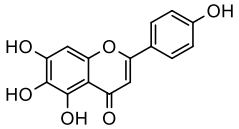
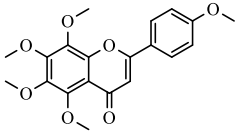
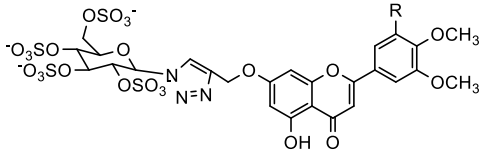
Concerning antibacterial activity, in addition to desferrithiocin (**65**), enrofloxacin (**66**) and danofloxacin (**67**) revealed to be quite potent against the growth of *S. aureus* and *E. coli*, showing a MIC value of 32 µg/mL, as expected considering their application as antibacterial agents. Nevertheless, no antifungal activity was observed for these compounds, in contrast with it is described for other quinolones. In fact, several commercially available fluoroquinolones used for the treatment of bacterial infections are active against other non-bacterial infectious agents, namely pathogenic fungi.[87-88] This extensive range of anti-infective activities may be due to a common mode of action, namely the topoisomerase II inhibition.[89] The idea that topoisomerase inhibitors may be potentially useful antifungal compounds has been reported in the past by Shen and Fostel.[90]The absence of antifungal activity observed for enrofloxacin (**66**) and danofloxacin (**67**) suggests

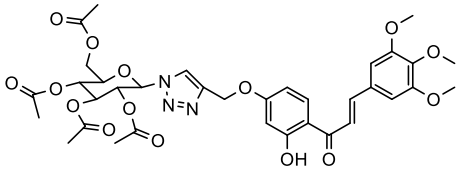
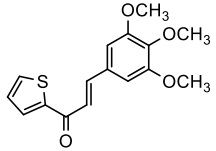
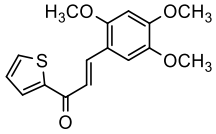
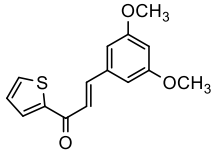
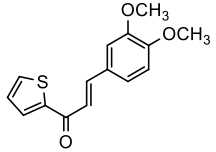
that the topoisimerase II inhibitory effect should not be essential for the inhibitory effect on *C. albicans*, *A. fumigatus*, *T. rubrum*, *M. canis,* and *E. floccosum.*

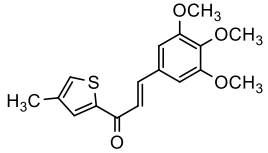
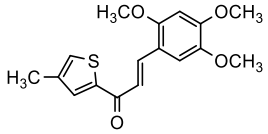
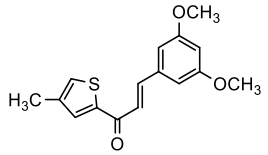
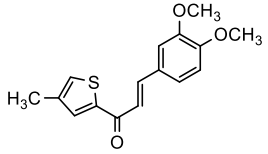
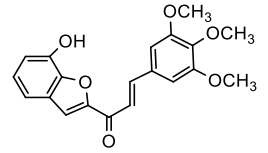
2.2. Evaluation of the antimicrobial activity of flavonoids 69-99 and xanthones 100-104

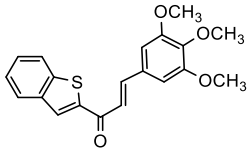
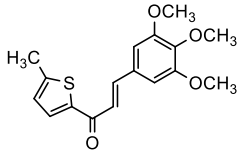
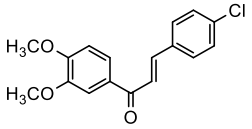
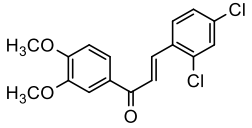
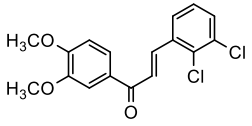
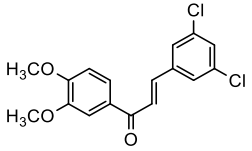
The results obtained on the evaluation of the antifungal and antibacterial activity of the flavonoids and xanthones are present in **Table 5**.

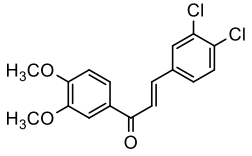
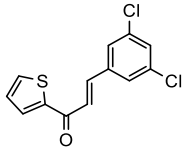
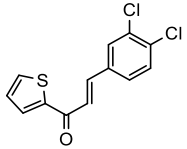
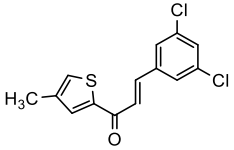
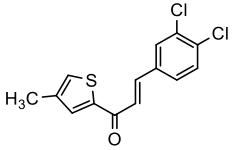
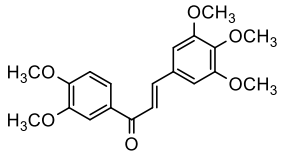
Table 5: Antimicrobial activity of flavonoids[a] 69-99 and xanthones[a] 100-104

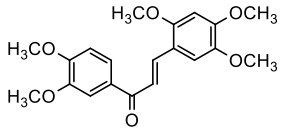
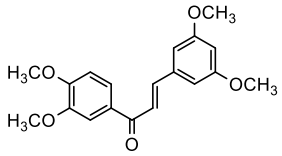
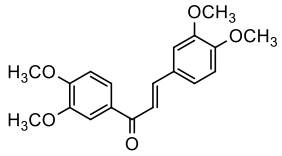
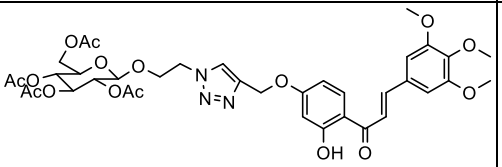
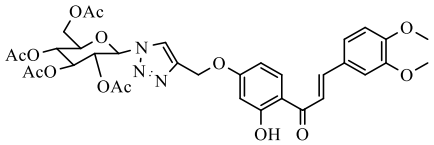
Compound: Flavones	*C. albicans*		*A. fumigatus*		*T. rubrum*		*M. canis*		*E. floccosum*		*E. coli*		*S. aureus*	
	MIC	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**
 **69**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **70**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **71 – R = H** **72 – R = OCH_3**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI

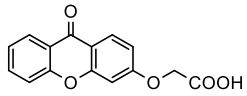
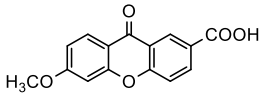
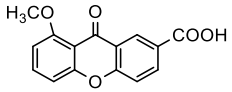
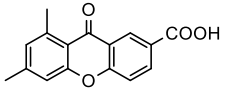
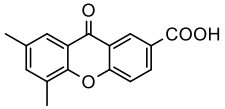
Compound: Chalcones	*C. albicans*		*A. fumigatus*		*T. rubrum*		*M. canis*		*E. floccosum*		*E. coli*		*S. aureus*	
	MIC	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**
 **73**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NT	NT	NT	NT
 **74**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **75**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **76**	NI	NI	NI	NI	**16**	**64**	**16**	**32**	**16**	**16**	NI	NI	NI	NI
 **77**	NI	NI	NI	NI	**16**	**>64**	**16**	**>64**	**16**	**>32**	NI	NI	NI	NI

Compound: Chalcones (Continuation)	*C. albicans*		*A. fumigatus*		*T. rubrum*		*M. canis*		*E. floccosum*		*E. coli*		*S. aureus*	
	MIC	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**
 **78**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **79**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **80**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **81**	NI	NI	NI	NI	**64**	**>128**	**>128**	NT	**>128**	NT	NI	NI	NI	NI
 **82**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI

Compound: Chalcones (Continuation)	*C. albicans*		*A. fumigatus*		*T. rubrum*		*M. canis*		*E. floccosum*		*E. coli*		*S. aureus*	
	MIC	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**
 **83**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **84**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **85**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **86**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **87**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **88**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI

Compound: Chalcones (Continuation)	*C. albicans*		*A. fumigatus*		*T. rubrum*		*M. canis*		*E. floccosum*		*E. coli*		*S. aureus*	
	MIC	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**
 **89**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **90**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **91**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **92**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **93**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **94**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI

Compound: Chalcones (Continuation)	*C. albicans*		*A. fumigatus*		*T. rubrum*		*M. canis*		*E. floccosum*		*E. coli*		*S. aureus*	
	MIC	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**
 **95**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **96**	NI	NI	NI	NI	**256**	**>512**	**>512**	NT	**>512**	NT	NI	NI	NI	NI
 **97**	NI	NI	NI	NI	**256**	**>512**	**>512**	NT	**>512**	NT	NI	NI	NI	NI
 **98**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **99**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI

Compound: Xanthones	*C. albicans*		*A. fumigatus*		*T. rubrum*		*M. canis*		*E. floccosum*		*E. coli*		*S. aureus*	
	MIC	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**
 **100**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **101**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **102**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI
 **103**	NI	NI	NI	NI	**256**	**256**	**128**	**128**	**64**	**64**	NI	NI	NI	NI
 **104**	NI	NI	NI	NI	NI	NI	NT	NT	NT	NT	NI	NI	NI	NI

[a] Results are expressed as MIC and MLC in µg/mL and show averages of at least three independent assays made in duplicate.
MIC - Minimum inhibitory concentrations; MLC - Minimum lethal concentrations; NI - No growth inhibition (MIC>512 µg/mL); NT – Not tested.

From the results presented in **Table 5**, chalcones **76**, **77**, **81**, **96**, **97** and xanthone **103** were found to inhibit the growth of dermatophyte filamentous fungi, whereas flavones **69-72**, chalcones **73-75, 78-80, 82-95, 98** and **99,** and xanthones **100-102**, and **104** were inactive against all tested organisms (MIC >512 µg/mL). Among the active compounds, chalcones **76** and **77** exhibited the most potent inhibitory effects (MIC=16 µg/mL). Xanthone **103** showed also an interesting effect, being more active against *E. floccosum* (MIC=64 µg/mL) than *T. rubrum* (MIC=256 µg/mL) and *M. canis* (MIC=128 µg/mL). None of the compounds tested was active against *C. albicans* and *A. fumigatus.*

Considering the antibacterial activity, none of the screened compounds presented activity against *E. coli* and *S. aureus* (MIC >512 µg/mL).

The study of fungicidal or fungistatic activity, by MLC determination, was performed for all compounds. Compound **76** was found to be fungicidal, being MLC equal to MIC for *E. floccosum* (16 µg/mL). However, for *M. canis* and *T. rubrum* the MLC was one or two dilutions higher than MIC (MIC/MLC=16/32 µg/mL for *M. canis* and MIC/MLC=16/64 µg/mL for *T. rubrum*). Furthermore, compound **103** was found to be fungicidal for *T. rubrum* (MIC/MLC=256 µg/mL), *M. canis* (MIC/MLC=128 µg/mL) and *E. floccosum* (MIC/MLC=64 µg/mL).

2.2.1. Structure-activity relationship (SAR) studies

In order to perform some SAR considerations, the library of compounds selected to be studied included several structure related flavones, chalcones and xanthones possessing different substitution patterns, including substituents with electron withdrawing (chlorine, and carboxylic acid), and electron donating (methyl, and methoxy) effects. In addition to chalcones with phenyl A and B rings, chalcones with other heterocyclic rings such as thiophenyl, benzofuranyl and benzothiophenyl were also used.

Comparing the results obtained for chalcones with a thiophenyl A ring (**74-77**, **90**, **91**), only compounds **76** and **77** revealed activity, showing the same potency (MIC=16 µg/mL), being the first fungicidal and the second fungistatic. This group of chalcones possess the same A ring but differ from each other in the number and position of the methoxy or chlorine substituents in B ring. Compounds **76** and **77** have a 3,5- or a 3,4-dimethoxyphenyl B ring, and **74** and **75** have a 3,4,5- or 2,4,5-trimethoxyphenyl B ring, while **90** and **91** have a 3,5- or a 3,4-dichlorophenyl B ring. Taking this into account, it can be suggested that the substituents in B ring appear to be a factor responsible for the activity, being more favourable for the activity the presence of electron donating substituents at positions 3,4- or 3,5-.

Considering chalcones with a 3-methyltiophene A ring (**78-81**, **92**, **93**), only compound **81** revealed activity against *T. rubrum* (MIC=64 µg/mL). This group of chalcones differs from the group previously described (compounds **74-77**, **90**, **91**), in the presence of a methyl group in position 3, of the thiophene A ring. Their structure show variations in the number and position of the methoxy or chlorine substituents, associated with the phenyl B ring. Compounds **78** and **79** are 3,4,5- or 2,4,5-trimethoxylated, while **80** and **81** are 3,5- or 3,4-dimethoxylated, and **92** and **93** are 3,5- or 3,4-dichlorinated. When comparing the results of chalcones **76** and **80** with those obtained for chalcones **77** and **81** respectively, it can be seen that the presence of a methyl group at the position 3 of the thiophene ring triggered the loss of activity for the compound with a 3,5-dimethoxyphenyl B ring and a marked reduction in the fungistatic activity of compound with a 3,4-dimethoxyphenyl B ring. Interestingly, when comparing the results for compounds **80** and **81**, both with the presence of a methyl group at C-3 of the thiophenyl ring, differing only in the B ring substitution pattern, we can reaffirm the importance of the B ring substitution pattern for the activity. Once again, the presence of electron donating substituents at positions 3,4- (such as for compound **81**) is more favourable for activity than the presence of electron withdrawing groups (such as for compound **93**).

In addition to the 3,4,5-trimethoxychalcones with a thiophenyl A ring **74** and **78**, other chalcones with the same B ring substitution pattern possessing different A rings were also evaluated, namely compounds **82**, **83**, and **84** with a benzofuranyl, benzothiophenyl and 4-methyltiophenyl rings, respectively. As for compounds **74** and **78**, none of these compounds had activity.

Among chalcones with a 3,4-dimethoxyphenyl A ring (**85-89**, and **94-97)**, only the chalcones with a 3,4- and 3,5-dimethoxyphenyl B ring (compounds **96** and **97**) showed some activity. This group of chalcones differ from each other in the substitution pattern of B ring, including a different number and position of chlorine and methoxy substituents in B rings. When comparing the results of B ring dimethoxylated chalcones **96** and **97** with dichlorinated chalcones **88** and **89**, respectively, we can reaffirm that the presence of a dimethoxy B ring is associated with the appearance of activity against the dermatophyte *T. rubrum* (MIC = 256 µg/mL).

Among the tested xanthones, compound **103** was the only one to have activity. In addition to the 9H-xanthen-9-one base structure, xanthones **101**, **102** and **103** have in common the presence of a carboxyl group at the position 2. In the phenyl B ring of **103** there are two methyl substituents at positions 6 and 8, while **104**, the only one having exactly the same substituents, has the two methyl substituents at positions 5 and 7. Comparing the results of xanthone **103** with structure related xanthones **101**-**104** it seems that the presence

of two methyl groups at C-6 and C-8 are associated with an improvement of the antifungal activity.

2.3. Evaluation of the effect of compounds with antifungal activity on the biosynthesis of ergosterol

Considering that, the major families of antifungal agents act in the cytoplasmic membrane, by linkage to ergosterol (polyenes), or by ergosterol synthesis inhibition (azoles and allylamines), in the present work we decided to evaluate the effect of the compounds **76**, **77**, and **81**, showing lower MIC values in *T. rubrum*, in the ergosterol content. Unfortunately, only one of the three performed experiments was accepted. Sub-inhibitory concentrations of the chalcones selected, as well as fluconazole, were used in the cells preparation and the ergosterol quantification is presented in figure 13.

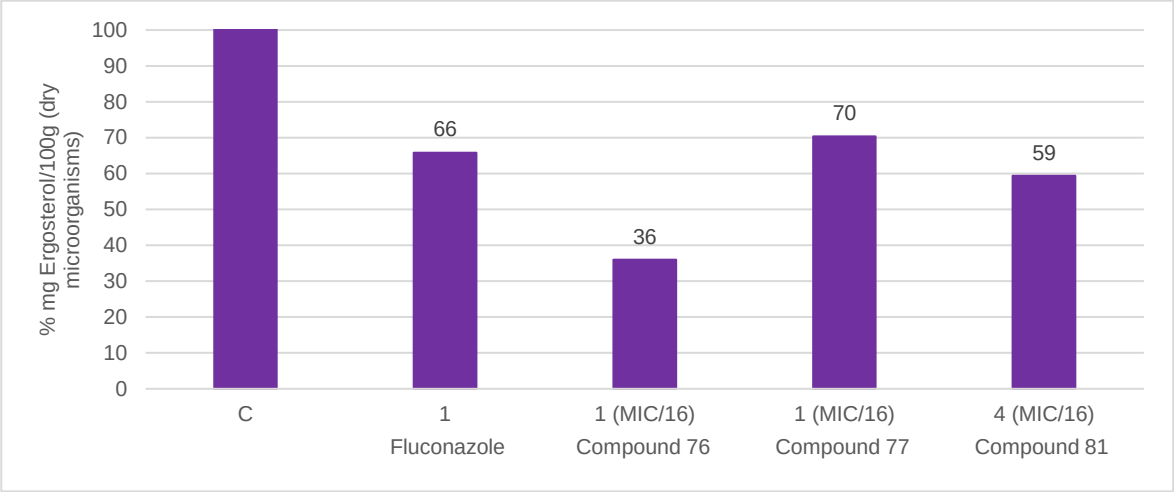


Fig. 13: % of Ergosterol concentration in *T. rubrum* cell without any treatment (C) and treated with different concentration (µg/mL) of fluconazole, compounds **76**, **77** and **81**.

Values obtained from the quantification of ergosterol from *T. rubrum* without any treatment appear as the control. The investigated dermatophyte showed to be sensitive to fluconazole, as expected, since its activity affects the synthesis of this important membrane component, having a reduction of 34% in its levels.

The concentration of MIC/16 of the three tested chalcones affected the amount of ergosterol, being observed a decrease of 64%, 30% and 41% for compounds 76, 77, and 81, respectively.

The results obtained from the determination of ergosterol seem to support the idea that chalcones act as azole, by inhibiting ergosterol biosynthesis. However, more repetitions are necessary to confirm these data and to evaluate the meaning of these results.

3. Material and Methods

In this work, a small library composed by 7 commercial compounds and 34 new synthetized compounds were screened for their potential antimicrobial activity against several fungi and bacteria strains.

3.1. Evaluating the antifungal and antibacterial activity

3.1.1. Commercial compounds and new synthetized compounds

The commercial compounds were obtained from different suppliers (TCI and Sigma) and all the new synthetized compounds were previously synthesized in LQOF and CIIMAR research group. Gentamicin (Sigma) and voriconazole (Pfizer) were used in quality control.

3.1.2. Microorganism strains and media

The antifungal activity of the compounds was evaluated against five fungi strains: a yeast reference strain, *C. albicans*-ATCC 10231; four filamentous fungi, one reference strain of *A. fumigatus*-ATCC 204305 and three clinical dermatophyte strains (*T. rubrum*-FF5, *M. canis*-FF1 and *E. floccosum*-FF9). *Candida krusei*-ATCC 6258 was used for the quality control.

The antibacterial potential of almost all the compounds was evaluated against two reference bacteria strains: *E. coli*-ATCC 25922, Gram-negative bacteria, and *S. aureus*-ATCC 25923, Gram-positive bacteria.

To guarantee the purity, viability and optimal growth, all the strains were sub-cultured before each assay: *C. albicans* and *A. fumigatus* on Sabouraud dextrose agar (SDA, BioMérieux) and incubated for 24 h and 48 h, respectively, at 35 ºC; dermatophytes on Mycobiotic agar (MYC, Becton Dickinson) and incubated for 5-7 days, at 25 ºC; *S. aureus* and *E. coli* on Mueller-Hinton agar (MHA, Liofilchem) and incubated for 24 h, at 35 ºC.

RPMI-1640 broth medium, with L-glutamine and without bicarbonate (Biochrom), used on the evaluation of antifungal activity, was buffered with 0.165 mol/L of 3-(N-morpholino)-propanesulfonic acid (MOPS, Sigma-Aldrich), and pH was adjusted to 7.0 ± 0.2 with 1 mol/L sodium hydroxide.

Mueller-Hinton broth 2 (MHB2, Becton Dickinson) was used on the evaluation of antibacterial activity.

3.1.3. Antimicrobial susceptibility testing by broth microdilution

The MICs and MLCs was used for determining the antimicrobial activity of each compound, in accordance with the recommendations of the Clinical and Laboratory Standards Institute (CLSI) reference documents, with minor modifications: M27-A3[91] and M38-A2[92] for yeasts and filamentous fungi, respectively; M7-A10[93] and M100-A27[94] for bacteria.

25.6 mg/mL stock solutions of all the above-mentioned compounds were prepared in dimethylsulfoxide (DMSO, Sigma-Aldrich). Two-fold serial dilutions of the compounds were prepared within the concentration range of 8-512 µg/mL, in MHB2 for bacteria and RPMI for fungi. The highest concentration tested was chosen in order to maintain DMSO in-test concentration 2% (v/v), as recommended by the CLSI.[91-93] At this concentration, DMSO did not affected bacterial/fungal growth.

Microtiter plates, 96 flat bottom wells, sterile, and disposable were used to evaluate susceptibility of the microorganisms to the compounds. Equal volumes of cell suspension and compound dilution were added in the wells of the microplate.

Three controls were performed: a sterility control - addition to the wells 200 µL of RPMI, or 100 µL of MHB2; a growth control - addition to the wells of 98 µL of RPMI, with 2 µL of DMSO, and 100 µL of fungus suspension, or 49 µL of MHB2, whit 1 µL of DMSO, plus 50 µL of bacterial suspension; a quality control, performed with an ATCC reference strain and a commercial antimicrobial compound.

All the compounds were tested in duplicate and independently three times.

Antibacterial susceptibility testing

Inoculum suspension preparation: cell suspensions were prepared from pure cultures on MHA/24 h in 2 mL of sterile saline solution. The suspension was vortexed for a few seconds and cell density was analysed with a spectrophotometer and adjusted to obtain a MacFarland standard of 0.5 at 530 nm, corresponding to 1-5 x 10^6 cells/mL. This initial suspension was diluted of 1:100 in MHB2, corresponding to an inoculum suspension of 1-5 x 10^4 CFU/mL. The cell suspension was diluted 1:1 in the test plate (50 µL + 50 µL of compound solutions) resulting in density of 0.5-2.5 x 10^4 CFU/mL, the desired size of the final inoculum.[93]

Gentamicin ranging between 0.25-1 µg/mL was used as a quality control, with *E. coli*-ATCC 25922.[93-94] The results obtained were within the recommended limits.

The plates were incubated aerobically at 35ºC for 24 h.

The MIC was determined as the lowest concentration at which no visible growth was observed.

The MLC was assessed by spreading 10 µL of culture collected from wells showing no visible growth on MHA plates. The MLC was determined as the lowest concentration at which no colonies grew after 16-18 h incubation at 35 ºC.

Antifungal susceptibility testing

The antifungal activity of the test compounds was evaluated against *C. albicans*, *A. fumigatus* and *T. rubrum*. For the compounds showing any activity against *T. rubrum*, *M. canis* and E*. floccosum* were also tested.

Inoculum suspension preparation: yeast cells suspensions were prepared from pure cultures on SDA/24 h in 2 mL of sterile saline solution and adjusted to MacFarland standard of 0.5 at 530 nm, as for bacteria. This procedure produces an initial suspension of 1-5 x 10^6 cells/ml. This initial suspension was diluted of 1:50 in RPMI, and then 1:20 in RPMI corresponding to an inoculum of 1-5 x 10^3 CFU/mL. Finally, the cell suspension was diluted 1:1 in the test plate (100 µL + 100 µL of compound solutions) resulting in density of 0.5-2.5 x 10^3 CFU/mL, the desired size of the final inoculum.[91] For filamentous fungi a spore suspension is prepared from pure culture with spores, in SDA (*A. fumigatus*) or MYC (dermatophytes), in approximately 1 mL of sterile saline added of one drop (approximately 0.01 mL) of Tween 20, to facilitate the spore separation. The cell density was adjusted by the spore count in the range of 150-180 spores, followed a dilution of 1:50 in RPMI for *A. fumigatus* (0.4-5 x 10^4 CFU/mL) and in the range of 20-60 spores, followed a dilution of 1:100 in RPMI for dermatophytes (1-3 x 10^3 CFU/mL). The cell suspension was diluted 1:1 in the test plate (100 µL+100 µL of compound solutions) resulting in density of 0.2-2.5 x 10^4 CFU/ml for *A. fumigatus* and 0.5-1.5 x 10^3 CFU/mL for dermatophytes, the desired size of the final inoculum.[92]

Voriconazole ranging between 0.25-1 µg/mL was used as a quality control, with *C. krusei*-ATCC 6258.[91] The results obtained were within the recommended limits.

The plates were incubated aerobically at 35 ºC during 24 h for yeasts, 48 h for *A. fumigatus* and at 25 ºC for 5-7 days considering dermatophytes.

MICs were determined as the lowest concentrations resulting in 100% growth inhibition, in comparison to the compound-free controls.

The MLC was assessed by spreading 10 µL of culture collected from wells showing no visible growth on SDA plates. The MLC was determined as the lowest concentration at which no colonies grew after 48 h incubation at 35 ºC for *C. albicans* and *A. fumigatus* and 7 days at 25ºC for dermatophytes.

3.1.4. Sterol extraction

Sterols were extracted from cultures of the dermatophyte *T. rubrum* and analysed by normal-phase High-performance liquid chromatography (HPLC).

This method was applied to the analysis of the ergosterol content from fungal cells of the dermatophyte *T. rubrum* and treated with different concentrations of chalcones **76**, **77** and **81**, and fluconazole, a well-known inhibitor of ergosterol biosynthesis, although its activity against dermatophytes is not elevated.

Spores suspensions were prepared in 0.9% NaCl with subsequent adjustment to 6.0 x 10^5 spores/mL for *T. rubrum*. Fifty microliters of the spores suspension were diluted in RPMI-1640 medium. Several twofold dilutions of the **76, 77, 81** and fluconazole compounds were prepared and added to the cell suspensions. Cultures without any compound were used as a negative control and fluconazole as a positive control. Cultures were incubated at 25ºC for 7 days.

The sterol extraction was adapted from Arthington-Skaggs et al.[95] Briefly, fungal cells were harvested by centrifugation at 4000 rpm for 10 minutes, and the pellets were washed twice with sterile distilled water. The pellet was dried and weighted. Three mL of 25% alcoholic potassium hydroxide solution was added, followed by a vigorous agitation in a vortex for 1 min. Cell suspensions were incubated in a water bath at 85ºC during 60 min. After incubation the tubes were left to cool at room temperature. Sterols were then extracted by addition of 1 mL of sterile distilled water and 3 mL of n-heptane (Romil Chemicals, Leics., England) to each tube, followed by a vigorous vortex agitation for at least 5 min. The organic phase (n-heptane) was then transferred to a clean glass tube, and the n-heptane was evaporated to dryness, under a nitrogen stream. The extracted sterols were redissolved in 0.5 mL of dichloromethane (Merck) prior to HPLC with ultraviolet (UV) detection analysis.

3.1.5. Sterol analysis

Separations of sterols were optimized for the described experimental conditions used in the validation procedure.[73]

Ergosterol was analysed by HPLC-UV based on the method proposed by Peacock and Goosey.[96] HPLC analysis was performed on a Dionex Ultimate 3000 (Thermo Fisher Scientific, USA) equipped with a 3000 quaternary pump, a 3000 autosampler, and a 3000 Variable UV/Vis detector. Chromeleon software version 7.2 Ultimate (Thermo Fisher Scientific, USA) was used to manage chromatographic data. The column was stainless steel (250 x 4.6 mm), packed with Hypersil silica 3 µm (Hichrom). The mobile phase was a solution of methanol (Merck) in dichloromethane 0.025% (*v/v*), and the flow rate was 1.5

mL/min with an injection volume of 40 µL and a race time of about 30 min. Detection was performed at 282 nm. Methanol and dichloromethane were HPLC grade. Ergosterol (E) (Sigma) were analytical grade.

Ergosterol (E, tR 19.0–20.8 min) were detected at their maxima absorption (282 nm), and compounds were identified by coinjection with real standards. Ergosterol was well separated, without interferences, in all samples. A model of chromatograms is presented in Figure 14.

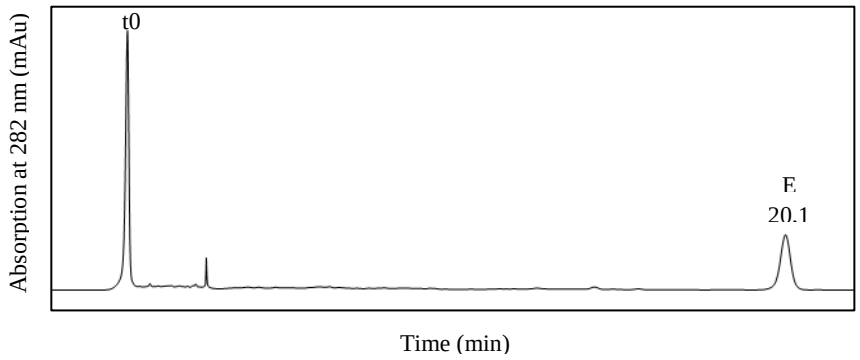


Fig. 14: HPLC chromatogram obtained from an extract of untreated *T. rubrum* cells. E = ergosterol. Conditions: silica: methanol in dichloromethane 0.025% (*v*/*v*), 1.5 mL/min.

4. Conclusion and Future Work

A total of 41 compounds, including 7 commercial available compounds and 34 new synthetic compounds, including flavones, chalcones and xanthones, were studied for their antimicrobial activity. Among these compounds, the iron chelating agent desferrithiocin (**65**), as well as synthetic chalcones **76**, **77**, **81**, **96**, and **97** and xanthone **103** showed antifungal activity. Desferrithiocin (**65**) exhibited some antimicrobial activity not only on fungi tested strains *C. albicans*, *T. rubrum*, *M. canis*, and *E. floccosum*, but also on bacteria strains *E. coli* and *S. aureus*. Nevertheless, no activity was observed for this compound against the non-dermatophyte *A. fumigatus* at the maximum tested concentration of 512 µg/mL. Chalcones **76**, **77**, **81**, **96**, **97** and xanthone **103**, although revealing a reduced spectrum of activity, limited only to dermatophytes, presented greater activity. Chalcones **76** and **77** showed the most potent inhibitory effect, being chalcone **76** fungicidal.

Furthermore, a HPLC method was used to study the effect of the active chalcones **76**, **77**, **81** on the biosynthesis of ergosterol of *T. rubrum*. The results suggested that these chalcones may act by inhibiting ergosterol biosynthesis. Nevertheless, the results using this methodology were not completely enlightening. In fact, the difficulty in quantifying the mass values of the fungus extract made it difficult to interpret the results. Therefore, changes to the adopted procedure should be implemented in the future in order to overcome this problem and to clarify the effect of chalcones **76**, **77**, and **81** on sterol biosynthesis.

In addition, despite the use of the broth microdilution susceptibility test, flow cytometry methods could be used to evaluate the activity of some antifungal agents. The quantification of ergosterol by near-infrared (NIR) spectroscopy, also appears as a possibility to be applied in the future, instead of HPLC.

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