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Review

Searching new antifungals: The use of *in vitro* and *in vivo* methods for evaluation of natural compounds

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ABSTRACT

In the last decades, the increased number of immunocompromised patients has led to the emergence of many forms of fungal infections. Furthermore, there are a restricted arsenal of antifungals available and an increase in the development of resistance to antifungal drugs. Because of these disadvantages, the search for new antifungal agents in natural sources has increased. The development of these new antifungal drugs involves various steps and methodologies. The evaluation of the *in vitro* antifungal activity and cytotoxicity are the first steps in the screening. There is also the possibility of antifungal combinations to improve the therapy and reduce toxicity. Despite that, the application of the new antifungal candidate could be used in association with photodynamic therapy or using nanotechnology as an ally. *In vivo* tests can be performed to evaluate the efficacy and toxicity using conventional and alternative animal models. In this work, we review the methods available for the evaluation of the antifungal activity and safety of natural products, as well as the recent advances of new technology in the application of natural products for antifungal therapy.

Contents

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1. Introduction

While the availability of antifungal agents is restricted, the similarity between fungal and mammalian cells is the main reason for the elevated toxicity frequently associated with antifungal therapy (Ostrosky-Zeichner et al., 2010; Pierce et al., 2013). Despite the discovery of new molecules and the new formulation availability to reduce the toxicity and increase bioavailability, the search for new antifungal agents and the characterization of novel targets are a continued need (Agarwal et al., 2008; Martinez-Rossi et al., 2008; Wiederhold and Patterson, 2015). An ideal antifungal agent should have a broad-spectrum and minimum side effects (Carrillo-Muñoz et al., 2006). Plants, animals, and terrestrial or marine microbes are good sources for potential antifungals (Cruz et al., 2007; Rajeshkumar and Sundararaman, 2012; Rojas et al., 2006) because of their extensive biosynthetic capacity. This makes them a precious source of therapeutic compounds (Schmidt et al., 2008). Essential oils, flavonoids, alkaloids, proteins, peptides, glycoproteins (Satya et al., 2005), and tannins are found in plants and can be employed as models for the synthesis of new compounds (Newman and Cragg, 2007). In recent decades, about a quarter of the drugs used worldwide have been discovered in natural products or their derivatives (Balunas and Kinghorn, 2005). Most of the antifungal drugs used today in fact were discovered from natural sources. For example, amphotericin B, a polyene, was discovered in the 1950s from *Streptomyces nodosus* cultures (Trejo and Bennett, 1963). Micafungin is an antifungal drug derived from the fungi *Coleophoma empetri* (Jarvis et al., 2004) and caspofungin is obtained from a fermentation of the fungi *Glarea lozoyensi*. Both are antifungal drugs belonging to the echinocandin class (Abruzzo et al., 2000). In this study, we will review the *in vitro* and *in vivo* methodologies for the evaluation of efficacy and safety of natural products with antifungal potential. Fig. 1 represents all of the stages that a natural product should reach in order to be a candidate for use as an antifungal drug.

2. *In vitro* Methods

The search for natural compounds with antifungal activity has increased, however, the testing methods present many challenges. The diversity of testing methods and the lack of clearly defined testing conditions such as inoculum size and medium type, can lead to low reproducibility. To insure that the selection of compounds with relevant pharmacological action are worthy of following up, it is necessary that the selection and validation of primary screening assays be rapid, simple, and easy to implement. They must also produce quick results at a low cost. In this sense, the standardization of the *in vitro* antifungal susceptibility test has advanced in recent years. The Clinical Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing have set the benchmark methodology by providing laboratory tested, reproducible, and peer-reviewed standards to commercial antifungal drugs (EUCAST, 2008; M27-A3, 2008; M38-A2, 2008). Many authors have followed these documents in their studies (Alastruey-Izquierdo et al., 2015; Cuenca-Estrella et al., 2010; Cuesta et al., 2010; Liu et al., 2007) and were adapted for plant extracts (Scorzoni et al., 2007; Svetaz et al., 2010). Independent of the susceptibility test adopted, it is necessary to use a reference drug to validate the methodology. There are several drugs available for this purpose and the choice depends on the fungi (Cos et al., 2006).

2.1. AGAR diffusion

Agar diffusion technique is a semi-quantitative assay that consists of applying an amount of a sample with a known concentration (the plant extract, fraction of the extract or the pure substance) on the agar surface which previously was inoculated with a standardized amount of fungal cells. The sample can be applied by different techniques, such as disk diffusion, in which disks made of sterile filter paper (6 mm) are impregnated with the sample and then applied to the agar surface. The plate-hole, another agar diffusion technique, which the sample are placed into wells made in the agar medium (Hayhoe and Palombo, 2013; Rios et al., 1988). In both techniques, the sample diffuses into the agar medium, creating a circular concentration gradient. The yeast will then multiply and if the sample has antifungal activity, a growth inhibition zone will appear around the disk. This inhibition zone is measured in millimeters, and some authors classify it into three categories: total inhibition, partial inhibition, or no inhibition (de Souza et al., 2004; Kalemba and Kunicka, 2003).

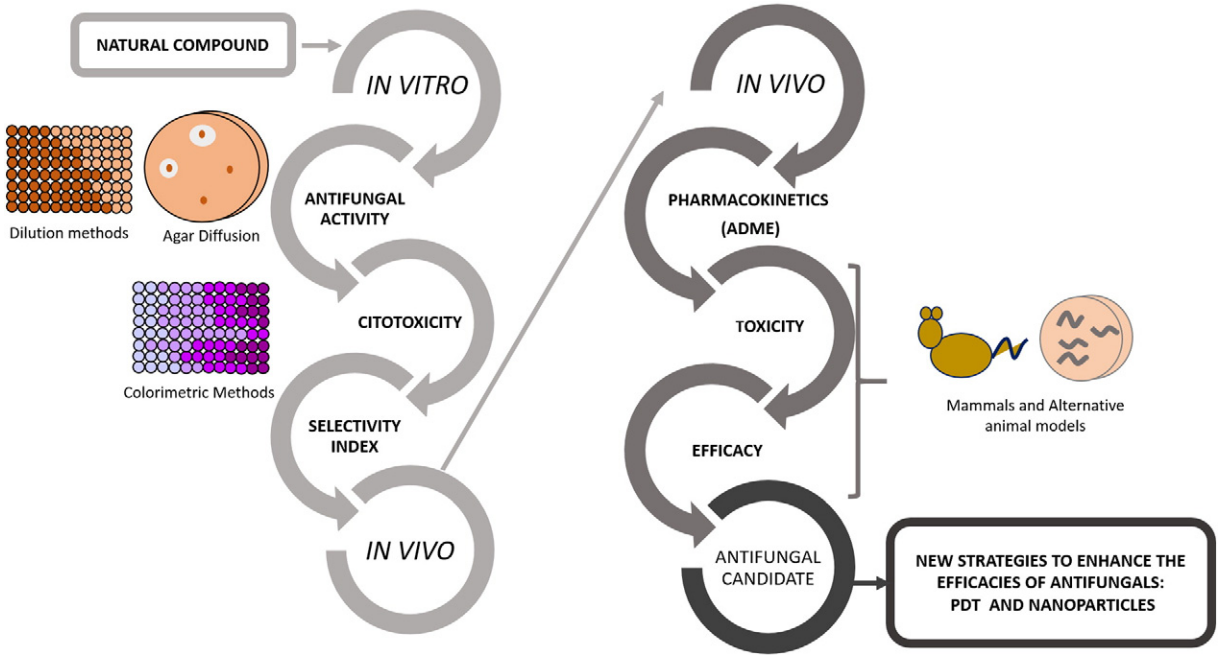

Fig. 1. Representative schema of the steps and assays for an antifungal drug candidate.

Many studies employed agar diffusion during the screening of antifungal activity which give semi-quantitative data, and before using more precise techniques like macrodilution (Kosalec et al., 2013) or microdilution (Ergin and Arikan, 2002).

The disadvantage of agar diffusion methodology is the use of large amounts of plant extracts; moreover, the lipophilicity, volatilization, amount of the compound, the agar media type, pH, and the volume of agar can all strongly affect the inhibition zone (Pauli, 2006). These factors in the agar diffusion technique must be carefully measured for consistency so as to avoid false-positive or negative results. In our group, Scorzoni et al. (2007) evaluated the antifungal activity of crude extracts, fractions, and pure substances from different species of the plant families by two different methodologies: agar diffusion and broth microdilution against *Candida* spp. and *Cryptococcus neoformans*, and observed that the microdilution method was more sensitive than the agar diffusion and moreover provided quantitative data.

2.2. Macrodilution and microdilution assay

The broth dilution methods are the most widely used assays to determine the antifungal activity. The documents standardized to yeasts (M27-A3, 2008) and filamentous fungi (M38-A2, 2008) from CLSI, describes the macrodilution and microdilution susceptibility test to antifungal drugs. These methods provide quantitative data about the Minimum Inhibitory Concentration (MIC) of the compound, which is the lowest compound concentration able to inhibit the antifungal growth.

The microdilution assay is recommended for the screening of natural compounds because of the high throughput potential, considerable savings in media usage, and requirement of a small amount of sample; moreover this method could be applied for different microorganisms (de Melo et al., 2013; Djouossi et al., 2015; Gehrke et al., 2013; Johann et al., 2010; Njateng et al., 2015; Pozzatti et al., 2008). The employment of the macrodilution technique to evaluate natural products as antifungals was reported in different studies (Bouzabata et al., 2013; Cabral et al., 2012; Pinto et al., 2013).

Interestingly, these tests have been standardized to antifungal drugs which suggest that it is necessary to optimize test conditions in order to adapt and standardize these for natural compounds. Our group used the microdilution methodology to demonstrate the antifungal activity of alkyl gallates, which are modified substances derived from natural compounds. The alkyl gallates were tested against several species of pathogenic fungi (*Candida* spp., *Cryptococcus* spp., *Trichophyton* spp., *Aspergillus* spp., *Paracoccidioides* spp., and *Histoplasma capsulatum*). These compounds showed great antifungal activity against most isolates and had a particularly good selectivity index (SI) value, making this compound an excellent candidate for a broad-spectrum antifungal prototype. This also is encouraging for the continuation of subsequent studies for the discovery of its mechanism of action (de Paula e Silva et al., 2014).

2.3. Minimum fungicidal concentration assay

By using both dilution methods, it is possible to determine if the substance is active, however it is not possible to determine if the compound will kill or just inhibit the fungal growth. For this purpose, the minimum fungicidal concentration (MFC) assay is performed. Small aliquots from each of the broth dilution tests are subcultured on a rich solid medium and incubated for a determined time and temperature, depending on the fungal species being tested. According to the documents standardized by the CLSI, the MFC is considered the lowest concentration of the substance in which no visible growth of subculture occurs. Moreover, MFC could give information about fungicide or fungistatic activity. If the MFC is the same as the MIC, it is considered to be a compound fungicide, but if the MFC is higher than the MIC, then it is a fungistatic (M27-A3, 2008). Our group studied the antifungal activity of maytenin and pristimerin against *Candida* spp., *Cryptococcus* spp., *P. brasiliensis*, *H. capsulatum*, *Aspergillus* sp. and *Trichophyton* sp. in which MIC values for the substances ranged from 0.12 to 250 $mg \cdot L^{-1}$. Maytenin demonstrated fungicidal activity for most of the yeasts tested (Gullo et al., 2012).

2.4. Colorimetric assays

The microdilution technique is recommended since that is less expensive (uses less media, inoculum, and less of the compound), and provides highly reproducible results. Fungal biomass is assessed visually by grading turbidity, or spectrophotometrically by measuring optical density. However, some species do not generate enough fungal biomass. Alternatively, biomass can be determined colorimetrically in which the fungal metabolic activity and the fungal biomass can be estimated using a colorimetric substance (Meletiadis et al., 2001a, 2001b). In this sense, colorimetric microdilution has been used to screen compounds with antifungal activity, as it was published by Liu et al. (2007) and Monteiro et al. (2012).

Meletiadis et al. (2001a) used the heterocyclic organic compounds, tetrazolium salts MTT (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide; thiazolyl blue) and the XTT (sodium3′-[1-[(phenylamino)-carbony]-3,4-tetrazolium]-bis(4-methoxy-6-itro)benzene-sulfonic acid hydrate) for antifungal susceptibility testing. These salts can penetrate rapidly into the cells and the dehydrogenase activity reduces to violet formazan crystals. This reduction is made by receiving electrons enzymatically from substances of the hydrogen transport system or non-enzymatically from artificial electron transporters (phenazine methosulfate and menadione). The amount of these crystals is quantified by spectrophotometrically estimating the number of living cells in the sample (Denizot and Lang, 1986; Freimoser et al., 1999).

The use of MTT results in non-water-soluble crystals and XTT is converted into a water-soluble crystal. However it needs the presence of an electron-coupling agent (Kuhn et al., 2003). Due to the product from XTT being water soluble, it is possible to study the biofilm metabolic activity with or without treatment measuring the supernatants (Pierce et al., 2008). The MTT method can be exemplified in *Aspergillus niger*, *Candida albicans* and *Trichophyton rubrum* studies (Fu et al., 2010). The XTT method is commonly used in biofilm formation and anti-biofilm activity of *Candida* spp. (Ansari et al., 2013; Ramage et al., 2012; Saharkhiz et al., 2012).

Another colorimetric method is the MABA (Microplate Alamar Blue Assay), alamarBlue®, in which resazurin is the active substance. For example, Liu et al. (2007) and Monteiro et al. (2012) studies demonstrate the importance of this method in the screening of antifungal natural compounds. Antifungal susceptibility tests to *Paracoccidioides* spp. were evaluated using the microdilution technique according to the document M27-A3 (2008). MABA was necessary in order to have reliable, reproducible, quick, and highly accurate routine laboratory tests (de Paula e Silva et al., 2013).

2.5. Cytotoxicity assay

Once the antifungal activity of a compound of interest is established, it is necessary to know how much of this compound can influence the viability of mammalian cells using an *in vitro* cytotoxicity assay. Cellular death, changes in cell membrane permeability, and metabolism inhibition are characteristics of cellular toxicity. Cell viability tests can be easily performed with *in vitro* cellular systems and vital dyes. Some of these systems were described in the above sections (Rogero et al., 2000). The selection of a cytotoxicity assay relies on the performance and quality of the data produced. The alamar blue and MTT assays are high quality and are suitable to identify cytotoxic compounds; however, the alamar blue assay is homogenous and provides the advantage of higher sensitivity through which it may detect low cell densities. It is

recommended to carefully consider the possibility of false positives and negatives due to the inhibition or induction of drug metabolizing enzymes, as this could lead to a misinterpretation of the data (Hamid et al., 2004).

Sulforhodamine B is another method widely used whose mechanism is based on the labeling of overall protein content within the cells that were not killed with the substance tested (Rubinstein et al., 1990). The amount of dye from pigmented cells is directly proportional to the total mass of protein and thus is correlated with the number of cells (Papazisis et al., 1997; Vichai and Kirtikara, 2006). Some compounds interfere in the MTT reduction with no effect in cell viability. This does not occur with sulforhodamine B which is based on cell density determination unlike MTT which requires cellular metabolic activity to convert colorless tetrazolium to purple colored formazan dye. In addition, another benefit of sulforhodamine B is its linearity.

Many studies use the MTT method to perform cytotoxicity with medicinal plants like *Breonadia salicina* (Mahlo et al., 2013), *Maytenus undata* (Mokoka et al., 2013); *Retama raetam* (Edziri et al., 2012); *Ricinus communis* (Zarai et al., 2012); *Asteraceae* plant family (Zapata et al., 2010), and *Tanacetum balsamita* (Yousefzadi et al., 2009). Other studies use sulforhodamine B, like *Rudbeckia laciniata* (Lee et al., 2014) and *Pleurospermum kamtschaticum* (Lee et al., 2012).

The IC50 can be defined as a substance concentration that can inhibit 50% of the cells. This value can be used to find also the selectivity index (SI), which is the ratio between the IC50 and MFC values. The most important index is the value of SI: the activity of the compound against the organism is better and presents lower cytotoxicity, thus, the safety of the compound is greater (Protopopova et al., 2005). These parameters were evaluated in many studies in the search of antifungal activity (Ghoneim et al., 2013).

2.6. Combination of substances

The combination of substances is a promising way to increase the percentage of successful treatments (Shao et al., 2007). Combinations of two or more antifungals and combinations of antifungal drugs with natural products have been evaluated in several studies by an *in vitro* method called “checkerboard” that is widely used (Greco et al., 1995; Odds, 2003).

The checkerboard test was based on the procedure established by CLSI for the broth microdilution technique for antifungal susceptibility testing. Two compounds are combined in 96 plate wells, placing one of them in descending concentration in rows and the other in columns, forming the combination of the compounds. To check the combinatorial effect of the compound, the fractional inhibitory concentration index (FICI) was adopted, which can be synergistic, additive, indifferent, or antagonistic. The FICI is calculated as the sum of the fractional inhibitory concentration (FIC) of the compound 1 + FIC of compound 2. The FIC of compound 1 is obtained by dividing the MIC when it is used in combination by the MIC of one compound by itself. The same calculation is performed for compound 2. The combination is defined as synergistic when the FICI is equal or less than to 0.5; additive FICI is greater than 0.5 and smaller/equal to 1.0; FICI is indifferent when greater than 1.0 and smaller/equal to 4.0; and antagonistic if FICI is greater than 4.0 (Clancy and Nguyen, 1997; Johnson et al., 2004; Odds, 2003; Patterson et al., 2000). The combination of antifungal drugs and natural compounds is described for pathogenic fungi (Menezes et al., 2012; Rodero et al., 2000; Venturini et al., 2011). In a study conducted in our laboratory evaluating the alkyl protocatechuates combined with fluconazole against *T. rubrum* and *Trichophyton mentagrophytes*, the heptyl protocatechuate showed a synergistic activity, reducing the MIC of fluconazole in fourfold (Soares et al., 2014).

3. *In vivo* models

3.1. Toxicology

To develop new antifungal compounds it is necessary to take into consideration its safety. For that, it should be considered for *in vivo* tests in mammals, lower vertebrates and invertebrates. Vertebrate models are usually used instead of invertebrates due to their closer resemblance of their bodily structure, function, and metabolism to that of humans. However, invertebrate models have been utilized due to low rearing costs, well-defined genetic backgrounds, and because they are easy to manipulate (Dolganiuc and Szabo, 2009).

The toxicity analysis in mammals such as mice and rats include the administration of certain dosages of the compound studied and lethal dose (LD50) can be calculated. Furthermore, loss of body weight and other signs of significant toxicity are continuously monitored. Finally, histological analysis of damage to organs and biochemical liver and kidney function tests can be performed. Concerning this, some studies have evaluated the acute and/or subchronic toxicity of natural products with potential antifungal activity. In one study of acute toxicity, Li et al. (2012) showed that the LD50 of a natural product isolated from *Pogostemon cablin* was much higher than the daily dose administered for the efficacy experiments for the treatment of *Candida* infections. Fontenelle et al. (2007) performed another experiment of acute and subchronic toxicity of the essential oil of *Lippia sidoides* in rats in which they observed no alterations in the organs, serum biochemical parameters, or body weight. Moreover, the administration of the essential oil of *Psidium cattleianum* at doses of 100, 200, and 500 mg.·kg^{-1} did not cause alterations in body weight or death of any animal (Castro et al., 2015). On the other hand, the acute and subacute oral treatment of a hexane extract of *Pterocaulon polystachyum* was relatively toxic to the liver and kidney (Regner et al., 2011).

The use of alternative animal models was also used in toxicology. Regarding the lower vertebrates, the zebrafish (*Danio rerio*) has been used as a toxicity model. Zebrafish should be used in studies of acute and chronic systemic toxicity and specific organ toxicities (Scholz, 2013). For the assay, the zebrafish embryos are incubated in 48-plate wells containing 1 ml of test medium per well and are exposed to different concentrations of compounds. From this data, histological analysis can be conducted (Driessen et al., 2013). Zebrafish embryos were used as a toxicity model for pahayokolide A, a compound from *Lyngbya* which has antifungal and antibacterial activities. However, the compound was acutely toxic to zebrafish embryos, killing 100% of embryos at concentrations of 5 mg·L^{-1} or higher (Berry et al., 2004).

In modern studies, in the evaluation of toxicity of new compounds, invertebrate models also show suitable application for pre-screening. A preliminary assessment of toxicity in *Galleria mellonella* reduces the probability of an agent with *in vitro* activity advancing to mammalian models and failing. This alternative model could detect the toxic effect of the compound earlier saving time and money while reducing the number of mammals required (Desbois and Coote, 2012). The nematode *Caenorhabditis elegans* (Boyd et al., 2012) also should be used for screening toxicity of new antifungal compounds. Breger et al. (2007) observed that in the *C. albicans–C. elegans* model, fluconazole was effective in prolonging survival of nematodes up to a concentration of 32 mg·L^{-1}; however at higher concentrations (100 mg·L^{-1}) the drug was toxic to the nematode.

3.2. Pharmacokinetics

After having observed that the natural compound has satisfactory antifungal activity *in vitro*, it is necessary to conduct tests to evaluate the processes of absorption, distribution, metabolism, and excretion (ADME) of the compound in the organism. These processes are investigated by pharmacokinetics, which include *in silico*, *in vitro*, and *in vivo* strategies.

The *in silico* models use computational tools such as VolSurf, which predict human intestinal absorption, protein binding, blood–brain barrier permeation, drug solubility, metabolic stability, and volume of distribution (Koukoulitsa et al., 2005). In this context, the VolSurf computational method was used to investigate the pharmacokinetic profile of antimicrobial compounds (Karioti et al., 2011).

Recently, *in vitro* studies with ADME have been incorporated earlier in the drug discovery phase. In assays for intestinal drug permeability, Caco-2 cells, human colon carcinoma cell line, is a widely used model. The experiment is performed in a 24-well transwell, in which measurements are made of the uptake of compounds from the lumen (inside the transwell) to the blood (outside the transwell). Additionally, the interaction of the compound with efflux pumps and metabolizing enzymes could be evaluated in the Caco-2 cells. Drug metabolism has also been estimated for *in vitro* systems, which include microsomes and hepatocytes. Microsomes are endoplasmic reticulum membrane vesicles prepared from the liver, which contains the P450 isoforms and one of the phase II conjugating enzymes, UDP-glucuronosyltransferase (UGT). Hepatocytes have all the hepatic drug-metabolizing enzymes and cofactors. Therefore, they are used to study both phase 1 and 2 liver drug metabolism pathways. Drug screening using microsomes or hepatocytes involves the incubation of the drug candidate in a 96-well plate, followed by quantification of the amount of the parent compound that remains after metabolism. Moreover, test compounds are incubated with microsomes or hepatocytes in the presence of various isoform-specific substrates. Thus, it is possible to identify the cytochrome P450 (CYP) isoforms that are involved in the metabolism of the compound, as well as determining whether the metabolites generated are more active or more toxic than the compound. Furthermore, it is possible to evaluate natural compound–drug interactions (Li, 2001; Li, 2005; Mazzari and Prieto, 2014).

The effect of antifungal ketoconazole was studied in Caco-2 cells, as well as the involvement of P-glycoprotein with this drug. The basal-to-apical transport of rhodamine 123, a P-glycoprotein substrate, was inhibited by ketoconazole, yet inversely, the apical-to-basal transport of rhodamine 123 was increased by this drug. Therefore, ketoconazole could interact with P-glycoprotein mediated transport. Moreover, it was demonstrated that P-glycoprotein was not involved in the transport of ketoconazole in the intestinal epithelia (Takano et al., 1998).

With regard to the *in vitro* metabolic profile, an antifungal piperazine propanol derivative was evaluated by incubation with microsomes of several species and with rat hepatocytes. In hepatocyte incubations, several epoxide- and hydroxylated metabolites could be observed. Additionally, these metabolites formed several glucuronides. From the analysis of the metabolic pattern in microsomes, products of carbamate hydrolysis were characterized. This hydrolysis was highly species dependent (Wind et al., 2009).

The pharmacokinetic profile also could be investigated in the animal models. These studies may be done by the administration of different dosages and in a second step, the quantification of the compounds and their metabolites by high performance liquid chromatography. Thus, parameters such as maximum serum concentration (Cmax), area under the curve of plasma concentration versus time (AUC0–t), and half-life (t1/2) can be determined. In this context, Li et al. (2012) administered a natural product isolated from *P. cablin* in mice and a pharmacokinetics profile was determined. The compound was effective against *Candida* spp. *in vitro* and *in vivo* and the pharmacokinetic parameters of the compound revealed that it was easily absorbed after oral administration in mice with the Cmax values higher than the corresponding MIC. Moreover, the mean AUC increased proportionally with the dosage, which was consistent with the dose-dependent effect of the natural product on the fungal load in the vagina. However, the compound suffers from a rather short T1/2 (approximately 51–53 min), which indicates that more frequent administration is required to obtain the optimum therapeutic response. The results suggest that a natural product might be a good candidate for the development of new anti-*Candida* agents.

The comprehension of the cited models should be considered, because in addition to pharmacological properties, good pharmacokinetic and toxicological profiles are crucial determinants of the ultimate clinical success of natural products as new antifungals.

3.3. Alternative animal models

In vitro experiments give us the basic knowledge about the fungal cells directly in contact with the compound. However, after determining the antifungal activity of the compound, it is necessary to understand if this compound has the same activity when it is submitted to complex systems, where degradation and/or modification of the compounds can occur and change its potential. Considering this, *in vivo* experiments are crucial for the confirmation of in vitro data regarding the antifungal compound candidate.

Despite the need for using animal models to perform studies, currently the use of mammalian animal models is highly regulated with much dependence on ethical committees and good laboratory practices. In 1959, Russell and Burch proposed the theory of the three Rs (Reduction, Refinement and Replacement) aiming to make the world aware of this scientific issue in order to compel them to search for alternatives to the use of traditionally used (but highly regulated) experimental animal models (Arora et al., 2011).

The moth, *G. mellonella* and the nematode worm, *C. elegans* are good examples of such alternative animal models (Desalermos et al., 2012; Fuchs and Mylonakis, 2006; Mylonakis et al., 2007). The possibility to incubate larvae at temperatures ranging from 25 to 37 °C can simulate conditions of fungal infection in mammals while allowing the possibility to administer the exact quantities of pathogens/drugs. These are important advantages of *G. mellonella* as a fungus-host model. Moreover, *G. mellonella* has six types of cells, some of which have phagocytic capacity. This plays an important role in their defense system. Furthermore, cell density in the haemolymph changes during infection and can be easily measured and used as a parameter of the response of larvae after exposure to pathogens (Desalermos et al., 2012; Fuchs and Mylonakis, 2006; Fuchs et al., 2010).

Concerning *G. mellonella*, the efficacy of antifungal drugs show a good relation between *in vitro* test and *in vivo* treatment of the larvae infected with *C. albicans*, *C. krusei* and *C. tropicalis* (Mesa-Arango et al., 2013; Scorzoni et al., 2013). The efficacy of anthraquinones from a Rubiaceae plant, *Morinda tomentosa*, against *C. albicans* was proven in the *G. mellonella* model, however these compounds did not show significant antifungal activity in the model (Favre-Godal et al., 2014).

Regarding *C. elegans*, genomic knowledge enables an understanding of fields not well elucidated. This is one of the main advantages of this model (Pukkila-Worley et al., 2011). *C. elegans* was suitable to evaluate libraries of chemical compounds for antifungal activities for *C. albicans* and also for screening toxic compounds (Breger et al., 2007; Pukkila-Worley et al., 2009). Recently, the antifungal activity of magnolol and honokiol from the plant *Magnolia officinalis* were also evaluated *in vivo* against *C. albicans* using *C. elegans* (Sun et al., 2015). Thus, the use of alternative animal models is useful in the search for new natural antifungal compounds while avoiding obstacles in current antifungal research such as cost and time constraints.

3.4. Efficacy in mammalian model

The mammalian animal models are very important in the search for new treatments with mice being the most widely used models. The choice of animal species along with the route of inoculation of the organism depends on the desired goal. The intraperitoneal route is widely used due to the ease of manipulation. The intravenous route is a standardized technique and provides answers about the spread of the organism to different organs. The intracerebral approach allows

for the study of the proliferation of the yeast in the central nervous system. Intranasal and intratracheal routes are best utilized to mimic natural respiratory infections while also allowing for the evaluation of the ability of the organism to spread from the lungs. The infections in these animals may be subclinical or lethal depending on the size of the inoculum, the route of inoculation of the strain in question, as well as the strain and immune status of the animal (Casadevall and Perfect, 1999; Dixon and Polak, 1986).

Using mammal models, it is possible to evaluate the survival rate or death of animals along with their fungal burden in different organs. Consequently, it is possible to analyze the effectiveness of the immune system of the host, the virulence of the microorganism, the effectiveness of treatment with antifungal drugs, and comparing the host response to different strains of the microorganism (Chrétien et al., 2002).

In vitro and *in vivo* studies to evaluate the synergistic effect of the combination between new compounds and antifungal drugs have also been performed. The combination of berberine and amphotericin B allowed the survival of mice with disseminated candidiasis for 22 more days compared with the amphotericin B treatment alone (Han and Lee, 2005). Additionally, the combination of amphotericin B with grape seed extract has promising results as shown by Han (2007). The antifungal activity of the extracts of the *Combretum* and *Terminalia* species against *C. albicans*, *C. neoformans*, *Microsporum canis* and *Sporothrix schenckii* was evaluated by Masoko et al. (2010) and verified activity in mice with wounds infected with fungi by topical administration. Eight saponin isolates from *Tribulus terries* were also tested by Zhang et al. (2005) against *Candida* species and *in vivo* results showed potential antifungal activity. The activity of pogostone, a substance isolated from *P. cablin*, was evaluated by Li et al. (2012) *in vitro* and *in vivo* against clinical isolates of *Candida* spp.

4. New strategies to enhance the efficacies of antifungals

4.1. Photodynamic therapy (pdt) using natural compounds

In photodynamic antimicrobial chemotherapy (PACT), antimicrobial photodynamic inactivation (aPI), or photodynamic therapy (PDT), a combination of a sensitizing drug or compound and visible light, cause selective destruction of microbial cells (Donnelly et al., 2008). This therapy combines a pharmacologically inert chromophore, termed as photosensitizer (PS), with a light that corresponds to the chromophore's specific absorption wavelength. The chromophore's exposure to its specific light wavelength in the presence of oxygen induces the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) radicals, including singlet oxygen and hydroxyl radicals (Silva et al., 2015). The generation of nitrosative and oxidative stresses by the process reduces the probability of the selection of resistant strains because they have diverse cellular targets. Also, these radicals cause alterations in the structure of the membrane and the fungal cell wall, providing penetration of the PS into the cell. At the same time, the radicals cause damage in cytoplasmatic organelles and nucleic acids, inducing cell death by apoptosis, necrosis, or autophagy (Baltazar et al., 2015b).

PDT was initially developed as a cancer treatment, but has also been investigated as a treatment for choroidal neovascularization secondary to age-related macular degeneration, localized infections, and for disorders related to dermatology and immunology. Many different types of photoactivable molecules have been synthesized and tested as PDT agents, including porphyrins, chlorins, bacteriochlorins and phthalocyanines (Agostinis et al., 2011).

For PDT to be both effective and safe, the PS should be delivered in therapeutic concentrations to the target cells and simultaneously be absorbed in only small quantities by non-target cells, minimizing undesirable side effects in healthy tissues (Huang et al., 2012).

In vitro methods using PDT have been widely performed against several species of microorganisms, including bacteria, viruses, protozoa, and fungi (Arboleda et al., 2014; Brooks et al., 1994; Choi et al., 2014; Lim et al., 2012; Lyon et al., 2011; Makarov et al., 2014; Morton et al., 2014; Rossoni et al., 2014; Sabino et al., 2015; Soares et al., 2011; Song et al., 2011; Takahashi et al., 2014). Silva et al. (2015) report that after cancer, infections represent the next most frequent application of PDT. Some natural compounds are being described as well-established photosensitizers. Gasparetto et al. (2010) evaluated the extracts of *Althernanthera maritima* (an herbaceous plant commonly found in the sandy beaches of the Brazilian coast), as a photosensitizer against *Candida dubliniensis*. It was concluded that hexane and ethanol extracts of *A. maritima* aerial parts, by laser irradiation at 685 nm, produced an antifungal effect against *C. dubliniensis*. Hypericin is a natural pigment of hypericum plants and is prominent among photosensitizers, exerting phototoxicity through several mechanisms (Theodossiou et al., 2009). The photodynamic effect of this natural pigment was evaluated against a species of *C. albicans*, *Candida parapsilosis*, *Candida krusei* (Rezusta et al., 2012) and also against the dermatophytes *T. rubrum* and *T. mentagrophytes* (Paz-Cristobal et al., 2014). The hypericin aPI was fungicidal against the dermatophytes when tested *in vitro*. Regarding *Candida* spp., only *C. albicans* could be effectively killed without damage to normal skin cells.

Previous studies reported the use of PTD in animal models against several species of fungi (Baltazar et al., 2015c; Chibebe Junior et al., 2013; Dai et al., 2011; Machado-de-Sena et al., 2014; Martins Jda et al., 2011; Mima et al., 2010; Mitra et al., 2011; Oberste-Lehn and Plempel, 1977). However, there are just a few reports of PDT in fungal infections in animal models using natural photosensitizers. Regarding natural compounds, Dovigo et al. (2013) described the photoinactivation of *C. albicans* in a murine model of oral candidiasis using curcumin as a PS. It was concluded that a single application of PDT was sufficient to eliminate *C. albicans* from mice that received 80 μM of curcumin and 37.5 J/cm of light.

4.2. The development of antifungal natural products using nanoparticle technology

The development of new formulations for antifungal compounds has been widely studied with the aim of reducing toxicity and increasing the bioavailability and consequently the efficacy. Nanotechnology is an alternative formulation providing interesting results with traditional and new antifungal compounds. Amphotericin B nanoparticles reduced the toxicity and maintained or increased the antifungal activity against *Candida* spp. (Casa et al., 2015; Tang et al., 2015; Tang et al., 2014). Similar results were observed with itraconazole nanoparticle (Qiu et al., 2015), fluconazole nanoparticles (Longhi et al., 2015), and miconaloze (Kumar and Poornachandra, 2015). Nanotechnology was also used for new natural antifungal candidates. Curcumin is known by a high diversity of biological activity, however the low aqueous solubility and poor bioavailability affects its use. The use of nanoparticles of Curcumin increase the antimicrobial activity of this compound against different microorganisms, including *Penicillium notatum* and *Aspergillus niger* (Bhawana et al., 2011). The allicin (active principle of garlic) in nanoparticle formulation showed significant reduction in the MIC for yeast and filamentous fungi (Luo et al., 2009). Copaiba oil has its antifungal activity enhanced after nanoencapsulation and was considered an alternative treatment for cutaneous infections caused by yeasts and dermatophytes (Svetlichny et al., 2015). *In vivo* study using a model of oral candidiasis, compared eugenol loaded in nanoparticles and liquid eugenol. The eugenol nanoparticles demonstrated higher capacity to reduce the log cfu (colony-forming unit) value than the liquid eugenol, showing an enhancement in antifungal activity when eugenol was formulated in nanoparticles (Garg and Singh, 2011).

4.3. Combination of PDT and nanoparticles of natural compounds against antifungal infection

The combination of nanotechnology and PDT is also a new feature to enhance the efficacies of antifungals. Lipid and detergent nanostructures like liposomes and micelles were routinely used in PDT before nanotechnology became a separate area of specialization. Today, the revolution of nanotechnology has provided many examples of nanoscale drug-delivery platforms, including liposomes, lipoplexes, nanoemulsions, micelles, polymer nanoparticles, silica nanoparticles, and fullerenes among others, that have been applied to PDT as described by Huang et al. (2012). Natural polymers such as proteins and polysaccharides have been studied for use in PDT. Furthermore, the use of chitosan, a biopolymer derivative from

Table 1
In vitro and *in vivo* methods and applications for screening of natural antifungal compounds.

Objective	Methods/models	Application-reference	Natural compound/drug
In vitro efficacy	Agar diffusion	Dzoyem et al. (2011)	*Diospyros canaliculata* extracts
		Palá-Paúl et al. (2012)	*Chamaecyparis lawsoniana* essential oils
		Galán et al. (2013)	Isoquinoline derivatives
	Macrodilution	Cabral et al. (2012)	*Juniperus communis* essential oils
		Pinto et al. (2013)	*Ferulago capillaris* essential oil
		Bouzabata et al. (2013)	*Myrtus nivellei* essential oil
		Gullo et al. (2012)	*Maytenus ilicifolia* substances
	Microdilution	De Melo et al. (2013)	*Lippia gracilis* essential oils
		Gehrke et al. (2013)	Compounds of *Schinus lentiscifolius*
		Djouossi et al. (2015)	Flavonoids
		Njateng et al. (2015)	Terpernoid saponin and other compounds
		De Paula e Silva et al. (2014)	Alkyl gallates
		Pozzatti et al. (2008)	Essential oils of different plants
		Johann et al. (2010)	Schinol and biphenyl compound of *Schinus terebinthifolius*
	Colorimetric assays (MTT, XTT and MABA)	Ansari et al. (2013)	Zizyphus spina-christi (Jujube honey)
		Fu et al. (2010)	Series of caffeic acid amide
		Liu et al. (2007)	Extracts of *Combretum* and *Terminalia*
		Monteiro et al. (2012)	Different natural extracts
		Saharkhiz et al. (2012)	*Mentha piperita*
		Ramage et al. (2012)	Tea tree oil and its derivate
	Synergism	Han and Lee (2005)	Berberin + AMB
		Han (2007)	Grape seed extract + AMB
		Menezes et al. (2012)	Simvastatin + Fluconazole
		Rodero et al. (2000)	AMB + 5FC, AMB + Rifampicin, FCZ + 5FC
		Venturini et al. (2011)	Amphotericin + Voriconazole
In vivo efficacy	Mice, rats: death curve, fungal burden, histopathology	Li et al. (2012)	Pogostone from *Pogostemon cablin*
		Han and Lee (2005)	Berberin + AMB
		Han (2007)	Grape seed extract + AMB
		Masoko et al. (2010)	Extracts of *Combretum* and *Terminalia* species
		Zhang et al. (2005)	Saponins from *Tribulus terries*
	G. mellonella: death curve, fungal burden, histology	Favre-Godal et al. (2014)	Anthraquinones from *Morinda tomentosa*
	C. elegans: death curve, fungal burden	Breger et al. (2007)	Different natural compounds
		Coleman et al. (2010)	Saponins
		Zhao et al. (2013)	Tetrandrine alkaloid
		Sun et al. (2015)	Neolignans of *M. officinalis*
In vitro toxicity	Colorimetric assays (MABA, MTT, XTT, sulforhodamine B)	Edziri et al. (2012)	*Retama raetam*
		Lee el al. (2012)	*Pleurospermum kamtschaticum*
		Lee et al. (2014)	*Rudbeckia laciniata*
		Mahlo et al. (2013)	*Breonadia salicina*
		Mokoka et al. (2013)	*Maytenus undata*
		Yousefzadi et al. (2009)	*Tanacetum balsamita*
		Zapata et al. (2010)	*Asteraceae* plant family
		Zarai et al. (2012)	*Ricinus communis*
In vivo toxicity	Mammals models- mice, rats	Fontenelle et al. (2007)	Essential oil from *Lippia sidoides*
		Regner et al. (2011)	Extract of *Pterocaulon polystachyum*
		Li et al. (2012)	Pogostone from *Pogostemon cablin*
		Castro et al. (2015)	Essential oil from *Psidium cattleianum*
	Zebrafish	Berry et al. (2004)	Pahayokolide A from *Lyngbya*
	G. mellonella	Gibreel and Upton (2013)	Epidermicin
	C. elegans	Pukkila-Worley et al. (2009)	Fluconazole
In silico Pharmacokinetics	Computational tool VolSurf	Djeddi et al. (2008)	Sesquiterpene lactone of *Centaurea pullata*
		Karioti et al. (2011)	Proanthocyanidins
In vitro Pharmacokinetics	Caco-2 cells	Takano et al. (1998)	Ketoconazole
	Microsomes and hepatocytes	Wind et al. (2009)	Piperazine propanol derivative
In vivo Pharmacokinetics	Mice, rats	Li et al. (2012)	Compound of *Pogostemon cablin*
New application	PDT	Gasparetto et al. (2010)	Extracts of *A. maritima*
	PDT	Rezusta et al. (2012)	Hypericin
	PDT	Paz-Cristobal et al. (2014)	Hypericin
	PDT *in vivo*	Dovigo et al. (2013)	Curcumin
	Nanotechnology	Bhawana et al. (2011)	Curcumin
	PDT and nanotechnology	Luo et al. (2009)	Allicin
		Svetlichny et al. (2015)	Copaiba oil
		Baltazar et al. (2015a)	Curcumin
		Chen et al. (2012)	Chitosan nanoparticules loaded with erythrosine
		Garg and Singh. (2011)	Eugenol nanoparticules

chitin, has been used in the manufacture of nanoparticles to improve drug delivery. The use of nanoparticles of chitosan loaded with erythrosine were active against *C. albicans* (planktonic and biofilm) after PDT application (Chen et al., 2012). Curcumin is a yellow-orange polyphenol compound produced by the rhizome of *Curcuma longa* plants with a wide range of pharmacological activities (Martins et al., 2009). Baltazar et al. (2015a) evaluated *in vitro* the activities and the mechanism of action of a photoactivated free and encapsulated curcumin against a clinical strain of *T. rubrum*. The results showed that curcumin aPI is an effective alternative for the treatment of *T. rubrum* infection, inducing the increase of nitric oxide (NO) and promoting apoptosis of fungal cells. According to many authors it still needs more studies to assert that nanotechnology may enhance photodynamic therapy. Despite the many reports in the literature of the use of both photodynamic therapy and nanotechnology for the treatment of cancer, there are very few studies on the use of both for antifungal treatment.

Table 1 provides examples of studies applying the methodologies discussed in this review.

5. Conclusion

Recently, the need for search and development of new compounds with antifungal activity has increased. The therapeutic failure, development of resistant strains, and low availability of antifungal drugs has led to natural compounds to be widely studied. Furthermore, choosing the correct assays as well as considering the compound and the fungi is important. A sequence of *in vitro* methods followed by *in vivo* models to assess the pharmacology and toxicity is generally used for compounds against fungi in a preclinical stage. To evaluate the antifungal activity of compounds, isolated or combined, macro- and micro-dilution, agar diffusion and colorimetric methods are available for the verification of cell viability. Cytotoxicity should also be performed, being essential before initiating animal testing. Then, the toxicology, efficacy, and pharmacokinetics studies should be employed, starting with alternative models and followed by mammalian models. Finally, photodynamic therapy and nanotechnology have been a great ally in the pursuit of new strategies to combat fungal infections.

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