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# Current Research and New Perspectives in Antifungal Drug Development

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**Abstract**

In recent times, fungi are becoming more and more active as causal agents of human infections, which is primarily determined by the growing number of people with severe immunosuppression. Thus, the problems of elucidating the mechanisms of action of antifungal preparations, highlighting ways to obtain resistance to their action and research strategies aimed at discovering new compounds with antifungal properties remain the focus of contemporary biomedicine and pharmaceutics. This paper reviews the recent achievements in antifungal drug development and focuses on new natural antifungal remedies with a noticeable effect on pathogens with minimal adverse effects on the host organism.

**Keywords**

Drug resistance · Natural antifungals · Oxidative stress · Pathogenic fungi · Spirulina extracts

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## 1 Introduction

In recent times, fungi are becoming more and more active as causal agents of human infections, which is primarily determined by the growing number of people with severe immunosuppression. The list of this type of patients includes people suffering from haematological and autoimmune diseases, have undergone organ transplantation, or have a compromised immune status (Debourgogne et al. 2016). Even in the case of appropriate treatment, most invasive fungal infections are associated with high mortality rates of over 50% (Brown et al. 2012; Chowdhary et al. 2016). This explains the major interest towards the role of fungi in the development of various pathological conditions (Caggiano 2012). Although, much work is being done to develop more active and less toxic antifungal drugs and preventive measures are applied virtually worldwide, mycoses (especially deeply invasive ones) continue to be some of the most serious infectious complications that cause an unacceptable high mortality rate (Lehrnbecher et al. 2010). Fungal diseases may take the form of superficial (dermatophyte), invasive (systemic) and opportunistic infections. Skin fungal infections are caused by dermatophytes that live in the upper most keratin layer, the nail or the hair shaft. This concept is actually based on the specific location of affections and not on the systematic belonging of causal agents, although most dermatophytes belong to three genera *Microsporum*, *Trichophyton*

and *Epidermophyton*. It is worth mentioning that dermatomycoses are widespread among immunocompetent patients and are associated with a very high morbidity. According to some authors, approximately a quarter of the world's population is infected with these pathogens (Brown et al. 2012; Havlickova et al. 2008).

Invasive (systemic) fungal infections have a much lower incidence than superficial infections, but cause a greater concern due to an unacceptable high mortality rate. These infections annually take the life of about one and a half million people (Harrison et al. 2014). This type of infection occurs following the inhalation of spores that cause fungal pneumonia, which cannot be transmitted from person to person, but can also occur in healthy individuals. Many microorganisms that cause systemic fungal infections are characterized by specific geographic locations with favorable climate for their proliferation. Among the most common diseases of this type are coccidioidomycosis (*Coccidioides immitis*), histoplasmosis (*Histoplasma capsulatum*) and blastomycosis (*Blastomyces dermatitidis*). In Canada, for example, Ontario is an important region of endemicity for blastomycosis and histoplasmosis, and in USA- histoplasmosis and blastomycosis incidence is highest in the Midwest, but coccidioidomycosis incidence rate is highest in the West (Baddley et al. 2011; Brown et al. 2018). Nearly 90% of *fungal infections resulting in deaths are* caused by pathogenic fungi from the genera *Cryptococcus*, *Candida, Aspergillus* and *Pneumocystis* (Velayuthan et al. 2018).

Fungi causing opportunistic infections (*Candida albicans*, *Cryptococcus neoformans*, *Aspergillus* sp., *Murcor* sp., etc.) are not hazardous to healthy people. Usually, these fungi affect individuals with a weakened immune system, causing serious infections such as candidiasis, cryptococcal meningitis, aspergillosis, mucormycosis, etc. Patients *especially susceptible* to these infections include people with diabetes, leukemia, cancer, HIV and other types of immunodeficiencies (Cowen et al. 2014; Oltu and Rudic 2016). The *development* of medical practices with the introduction of new therapeutic procedures, such as the use of some aggressive chemotherapies or new immunosuppressive drugs, such as tumor necrosis factor (TNF) antagonists, anti-CD52 antibody (alemtuzumab) and interleukin-2 receptor antagonist (basiliximab), favored an increased incidence of invasive fungal infections (Debourgogne et al. 2016).

The number of patients susceptible to invasive infections caused by filamentous fungi has reached alarming proportions and continues to grow steadily. This group of microorganisms is ubiquitous in different natural habitats such as soil and various organic substrates. The most common etiologic agents of invasive mycoses are part of the genera *Aspergillus* and *Mucor*. In recent years, this list has been supplemented with less frequent filamentous fungi in the environment, such as *Fusarium spp.* and *Penicillium spp*. Nonetheless, the medical term for fungal invasion has also extended its application limits from invasive disease to less recognized entities previously, such as severe asthma with fungal sensitization, fungus-associated chronic cough, allergic bronchopulmonary mycosis and allergic fungal rhinosinusitis (Chowdhary et al. 2016; Ogawa et al. 2009; Singh et al. 2013).

Thus, the problems of elucidating the mechanisms of action of antifungal preparations, highlighting ways to obtain resistance to their action and research strategies aimed at discovering new compounds with antifungal properties remain the focus of contemporary biomedicine and pharmaceutics.

## 2 Antifungal Preparations and Their Mechanisms of Action

Appropriate antifungal therapy is prescribed in dependence on the patient's immune status, site of infection, the intrinsic properties of the pathogen and the pharmacokinetic characteristics of a drug. Antifungal preparations, which can be successfully applied in the treatment of mycoses in humans, should act on the basis of differences between the fungal cells and those of the human body. At the same time, fungi like mammals are eukaryotic organisms, and therefore, the

development of preparations that attack the fungal cells without causing damage to the human body is an extremely difficult task. One of the distinctions, which can be exploited in the pharmaceutical design of antifungal drugs, is the presence of a specific sterol found in the cell membrane of yeast – namely ergosterol (with many of the same functions that cholesterol serves in animal cells) (Parks and Casey 1996). Specific targets for the antifungal action are also the components of cellular wall – mannoproteins and β-glucans. Consequently, the vast majority of active drugs against pathogenic fungi are developed in particular on the basis of these essential differences.

Preparations that are currently used to treat fungal diseases may be divided by several criteria, the main of which is the mechanism of action. After this criterion, antifungal drugs are divided into 7 groups, according to Table 1.

The mechanisms of action of substances belonging to these seven groups are well known and fairly well studied and described. At the same time, there are many antifungal agents that cannot be attributed to any of these mechanisms. Ciclopirox – a topical antimycotic agent belonging to the chemical class of hydroxypyridones is not related to azoles or any other class of antifungal drugs. Its antifungal profile targets almost all clinically important dermatophytes, yeasts and molds, and is therefore wider than the profile of most antimycotics. It is active against certain azole-resistant strains of *Candida albicans* and against some bacteria. The mechanism of action is thought to be through the chelation of polyvalent metal cations, such as $Fe^{3+}$ and $Al^{3+}$. These cations are cofactors of many enzymes, including cytochromes and their inhibition may lead to the disruption of the biosynthesis of ergosterol. Ciclopirox is also thought to act by modifying the fungal plasma membrane, resulting in the disorganization of internal structures. This unique mechanism provides a very low potential for the development of resistance to this drug (Subissi et al. 2010).

Organic acids such as caprylic acid, salicylic acid, undecylenic acid, propionic acid, and benzoic acid exhibit antifungal activity by interacting with non-specific components in the cell membrane. Reports over the years have indicated that short and medium-chain organic acids have antimicrobial properties. In addition, common aromatic acids such as benzoic acid, have found utility as preservatives in foods on the basis of their antifungal properties. Sorbic acid is an antimycotic agent which demonstrates broad spectrum activity against yeast and fungal molds. Most mentioned compounds act by targeting different stages of biotin biosynthesis, which is essential for fungal metabolism (Mazu et al. 2016).

Some natural preparations like haloprogin, the active part of which is allicin, appear to have a mode of action related to its ability to cross the cell membrane and combine with sulfur-containing groups in amino acids and proteins and interfering with cell metabolism. More often the effects of this preparation are associated with the fact that allicin blocks glutathione activity (Davis 2005).

**Table 1** Classification of antifungal drugs

| The groups, according to the mechanism of action | Examples |
|---|---|
| Inhibitors of fungal cell wall synthesis | Nikkomycins, Polyoxins, Caspofungin, Micafungin, Anidulafungin, Benanomicin, Pradimicin |
| Antifungals that operate by complexing directly with membrane ergosterol | Nystatin, Amphotericin B |
| Concomitant inhibitors of lanosterol and ergosterol biosynthesis | Terbinafine, Naftifine, Butenafine |
| Inhibitors of fungal ergosterol biosynthesis | Fluconazole, Itraconazole, Ketoconazole, Voriconazole, Clotrimazole |
| Inhibitors of nucleic acid synthesis | Flucytosine |
| Inhibitors of fungal mitosis | Griseofulvin |
| Antifungal drugs with other mechanisms of action | Ciclopirox, Haloprogin |

Some of the mechanisms of antifungal activity relate not to the direct action on the fungi, as to the modeling of host-pathogen interaction. Very often it is noted that natural antifungal agents stimulate the cellular immunity of the affected macroorganism. These effects were shown in the process of study of tea tree oil, citronella oil, orange oil, palmarosa oil, lemon and myrtle oils, coconut oil – all exhibit pronounced antifungal activity. However, it is hypothesized that they might act by altering membrane properties and compromising membrane-associated functions. Olive leaf extract directly stimulates the process of phagocytosis of fungi in the body (Uniyal et al. 2012).

There are also a lot of preparations in this category, which act on membrane ATPases, on signal-transduction pathways, electron transport chains and other metabolic processes of vital importance. However, further research is required, as all these affect common elements of fungal and human metabolism, and therefore, do not meet the requirements for an ideal antifungal agent.

## 3 Antifungal Natural Products

The adverse antifungal therapy reactions, in association with the high rate of multi-resistant pathogens, require the development of new remedies for the treatment of fungal infections. Furthermore, the intensive use of fungicides in agriculture, food industry, etc. causes essential damage to both the environment and human health. For that reason, the natural compounds with antifungal activity have become an attractive alternative over the past two decades. There are several classes of phytochemicals with antifungal activity: terpenes, tannins, flavonoids, alkaloids, lecithin, various polypeptides and others (Castillo et al. 2012).

Plants produce a variety of medicinal components that can inhibit pathogen growth. A considerable number of studies of medicinal plants and alternative compounds, such as secondary metabolites, phenolic compounds, essential oils and extracts, have been performed (Negri et al. 2014). Most of the antifungal compounds of plant origin belong to the phenol category. This group is extremely heterogeneous, including over 10 thousand compounds, a large part of them present in leaf extracts, tree bark, wood, fruits and other tissues of fern, gymnosperm and angiosperm plants. Phenols perform important functions, like resistance to microorganisms (including fungi), insects and herbivorous animals that can affect them. In particular, polyphenols have strong antioxidant properties, making them active in the fight against stress and free radicals, maintaining plant integrity in the process of continuous exposure to ultraviolet radiation, relatively high temperatures and dehydration.

Plant phenolic compounds display high antifungal activity against various species (*Penicillium italicum, Penicillium expansum, Monilinia laxa, Aspergillus carbonarius, Botrytis cinerea, Monilinia fructicola* and others) (Gatto et al. 2013). Phenol action on yeasts differs from the mode of action on filamentous fungi (Ansari et al. 2013). Thus, *Candida* dimorphic species are affected by phenolic compounds in the process of transition from one form to another. For example, phenolic compounds of *Baseonema acuminatum* leaves, *Lycium chinense*, *Sida urens, and Curcuma longa* root bark have shown strong antifungal activity against various *Candida albicans* strains. Their effect is associated with an increase in the amount of reactive species of oxygen and nitrogen in fungal cells, with predominant location in membrane, which induces early apoptosis. It is very important that the effects are irreversible and cannot be removed by the action of antioxidants (Ansari et al. 2013; Zerbo et al. 2014).

The extract from *Curcuma longa* is extremely effective against *C. albicans* strains. The mechanism of antifungal effect is based on inhibition of desaturase expression (ERG3), leading to a significant reduction of ergosterol in fungal cells. Accumulation of biosynthetic precursors of ergosterol in cells leads to cell death by the generation of reactive oxygen species (ROSs). Thus, the curcumin, bisbibenzyl, carvacrol and thymol compounds have been successfully used in the candidiasis treatment, through their effects on dimorphic transformation, biofilm formation and ROS generation (Ansari et al. 2013).

Among the phenolic compounds, hydrolysable tannins are also recognized as antifungal agents. In particular, by their effect on filamentous fungi *Epidermophyton floccosum, Microsporum canis, Microsporum gypseum, Trichophyton mentagrophytes, Trichophyton rubrum, Trichophyton tonsurans, Trichophyton terrestre, Penicillium italicum, Aspergillus fumigatus, Mucor racemosus, Rhizopus nigricans,* as well as on pathogenic opportunistic yeasts *Candida albicans, Candida glabrata, Candidata krusei, Cryptococcus neoformans* (Latté and Kolodziej 2000). The biological action of these compounds on fungi is associated with the formation of stable bonds between tannins and fungal cell proteins, leading to inactivation of enzymes and degradation of structural proteins (Marichal et al. 1997). Condensed tannins (also called proanthocyanidins) are polymers of 3-flavanol and 3–4 flavandiol (catechin and leucoanthocyanidins). These low solubility substances exhibit biological properties, including antifungal action, with a particularly pronounced efficacy in disaggregation of biofilm matrix.

Terpenoids are another group of natural compounds that are known for multiple biological effects, including the antifungal one. They make up the largest class of plant secondary metabolites, most of which are insoluble in water. Different substances such as geraniol, nerol, citral, neral and geranial have been shown to be very active against dermatophytes and opportunistic pathogenic yeasts, the maximum activity level being expressed on *Trichophyton rubrum.* The mechanism of action of these substances is explained by their affinity for ergosterol, which leads to the modification of fungal membrane fluidity (Arif et al. 2009; Miron et al. 2014).

Many plant species produce alkaloids characterized by broad-spectrum antifungal activity. For example, the medicinal plant *Kopsia hainanensis* produces 15 indole alkaloids, which exhibit high antifungal activity against *Erwinia carotovora* and *Fusarium oxysporum* (Chandrasekar 2011). From the aerial parts of the *Waltheria* plant, 11 quinolone alkaloids with antifungal activity against *Candida albicans* (CMI <32 μg/ml) were obtained by extraction in dichloromethane. The microscopic examination of yeasts treated with these compounds revealed multiple deviations from the normal morphological structure (Cretton et al. 2016). Oxygen-containing alkaloids of microbial origin possess high antifungal activity against phytopathogenic fungi, making them attractive for development in agrochemistry (Wang et al. 2016).

Natural antimicrobial peptides (AMP) are probably one of the first-line forms in the chemical defense of eukaryotic cells against bacteria, protozoa, fungi and viruses (Silva et al. 2009). Antimicrobial peptides, including antifungal agents, are part of the innate immune system and practically all organisms – from bacteria to humans – produce these peptides that ensure the integrity of the body.

Defensins are the most common antimicrobial peptides present in all types of eukaryotic organisms. However, there are some practical challenges in new defensin-based drug development. First of all, it is about the amphiphilic cationic character and the protease lability, which leads to the rapid elimination of the drug from the circulating body fluids and limits its systemic administration. However, defensin combines antimicrobial, including antifungal capacity with the ability to positively modulate immune system activity, and has been found to be efficient during biological evolution, which make this peptide extremely attractive for anti-infective strategies (Silva et al. 2009).

## 4 Cyanobacteria as a Source of Antifungal Compounds

The research on the causative agents of mycoses, especially invasive ones, as well as effective preparations for treating mycoses and applied treatment strategies have marked a noticeable revival over the last two decades. The treatment of fungal infections has improved significantly in the few short years. New antifungal agents have been licensed for use, and indications for the use of various antifungal agents have changed considerably. The introduction of new antifungals from the echinocandin and azole class drugs and the

growing use of lipid formulations of amphotericin B have allowed safer and more effective therapy for severe fungal infections, especially in immunosuppressed patients (Baghi et al. 2016; Blyth 2012; Grover 2010; Kauffman et al. 2011; Perlin 2015; Rodríguez-Leguizamón et al. 2015). The scientific basis of antifungal therapy has been improved with new studies on the pharmacodynamics and pharmacokinetics of these drugs. However, these positive developments have been tempered by increasing resistance of causal agents to several classes of antifungal drugs. Recently, multiple forms of pathogenic fungi have been discovered, for which multidrug resistance to antifungal agents is an inherent capacity fixed at the genetic level (Sanglard et al. 2009).

Thus, the discovery of new substances with antifungal properties remains one of the most important tasks in this field.

Cyanobacteria, the most widely distributed group of photosynthetic prokaryotes, are a prolific source for bioactive compounds, which could be toxic or potentially new drug leads. Over 400 cyanobacterial strains were screened for the production of antifungal compounds, which belong to structural classes, such as peptides, polyketides and alkaloids. There are currently numerous studies that have highlighted the compounds extracted from biomass of various cyanobacterial strains responsible for antifungal effects. The macrolide scytophycin was identified from *Anabaena sp*. HAN21/1, *Anabaena cylindrica* PH133, *Nostoc sp*. HAN11/1 and *Scytonema sp*. HAN3/2, which exhibits antifungal activity against *Candida albicans* and *Aspergillus flavus*. *Anabaena spp*. BIR JV1, *Anabaena spp*. HAN 7/1 and *Nostoc spp*. CENA 219 produced glycolipopeptide hassallidins with pronounced antifungal activity (Kshetrimayum and Kant 2016; Shishido et al. 2015). Among the substances with antifungal effects detected in cyanobacterial extracts, the following compounds have been identified: fisherellin A, hapalindole, hassallidin/balticidins, *carazostatin,* phytoalexin, tolytoxin, scytophycin, toyocamycin, t*jipanazole*, nostocyclamide, nostodione and nostofungicidine (Abed et al. 2009; Burja et al. 2001; Vestola et al. 2014).

Aqueous extracts from biomass of several strains of *Nostoc commune* and *Spirulina platensis* are active against *Aspergillus flavus*, their activity being largely determined by the conditions under which biomass was obtained. The change in nitrogen content of a medium is a factor that determines the antifungal activity of the extracts from these two species (Shaieb et al. 2014). In *Nostoc insulare*, two metabolites were extracted and characterized: norharmane (9H-pyrido(3,4-b)indole) and 4,4-′-dihydroxybiphenyl, which exhibited antifungal activity against strain *Candida albicans ATCC 10231* in concentrations from 32 to 40 μg/ml (Volk and Franz 2006). Many cytotoxic metabolites of different *Nostoc* species possess antifungal activity. For instance, such cytotoxins include nostocyclophanes and borophycin, a boron-containing polyketide, which are associated with blue-green alga *Nostoc linckia* (Rawat and Bhargava 2011).

Different types of spirulina extracts exhibit a certain level of activity towards various strains of fungi. Thus, fractions of terpenoids and sterols extracted from *Spirulina platensis* biomass demonstrated antifungal activity against *Candida albicans* (Uyisenga et al. 2010). Spirulina extracts have also proved effective against fungi *Aspergillus fumigatus* and *Aspergillus niger*. In particular, methanol extracts from spirulina biomass are effective against species of filamentous fungi mentioned above, but also on pathogenic yeast species *Candida albicans* (Kumar et al. 2009).

Extracts in organic solvents from *Spirulina platensis* biomass, the major active components of which are phenolic compounds, possess antifungal action against *Aspergillus flavus* and *Aspergillus niger* (Moraes De Souza et al. 2011; Pugazhendhi et al. 2015). Purified and concentrated hydric extracts from spirulina showed antifungal activity against *Penicillium oxalicum* and *Fusarium solani* (Battah et al. 2014). It is considered that mechanisms of action of spirulina extracts on filamentous fungi are based on the inhibition of the synthesis of ergosterol, glucosamine and proteins.

Thus, there is now sufficient evidence, which confirms that the search for new compounds with

antifungal action must cover as many groups of substances – both chemical and natural synthesis. Such an approach allows the selection of new remedies with high activity and multiple mechanisms of action on fungal cells, which is a guarantee of success in contemporary research.

Effects of hydro-ethanol extracts (20 (Extr.20%), 50 (Extr.50%) and 70 (Extr.70% ethanol) from biomass of *Arthrospira platensis* and *Nostoc linckia* were tested. Hydro-ethanol extracts from spirulina biomass showed antifungal activity against *Trichophyton, Microsporum* and *Candida* (Fig. 1). The most significant antifungal activity was recorded for 20% hydro-ethanol extract, which in the case of *Candida albicans* ATCC®10231™ was more active than reference antifungal preparations included in the study, and in the case of *Microsporum gypseum* ATCC®24102™ was as active as ketoconazole. The 50% hydro-ethanol extract showed activity equivalent to that of ketoconazole against the reference strain *Trichophyton mentagrophytes* ATCC®9533™, and 70% extract demonstrated antifungal activity equal to that of itraconazole and naftifine hydrochloride (NHCh) against *Candida albicans* ATCC®10231™.

Hydro-ethanol extracts from *Nostoc linckia* CNM-CB-03 have shown high biological activity (Fig. 2). The most significant antifungal activity had 20% ethanolic extract, the obtained values being significantly higher than the activity of ketoconazole against *M. canis ATCC®36299™, M. gypseum ATCC®24102™* and *C. albicans ATCC®10231™;* itraconazole against *M. gypseum ATCC®24102™* and *C. albicans ATCC®10231™;* naftifine hydrochloride against *C. albicans ATCC®10231™. Both* 50% and 70% hydro-ethanol extracts also possess antifungal activity, but less pronounced. At the same time, 50% hydro-ethanol extract had antifungal activity equivalent to that of ketoconazole against *Trichophyton mentagrophytes ATCC®9533™,* and 70% hydro-ethanol extract – against *Trichophyton rubrum ATCC®28188™.*

Another series of extracts were obtained from spirulina biomass grown under biotechnological conditions of metal bioaccumulation (Zn(II), Fe (II), Co(II), Cr(II), Cd(II)). During the vital cycle, metals were accumulated in spirulina biomass, and some of them are incorporated into biomass components (proteins, oligopeptide *fraction*, polysaccharides, lipids). In the process of extraction with ethanol and purified water, the organically bound metals pass into respective extracts. *Hydric extracts* ($HE_{Me}$) and ethanol extracts ($EE_{Me}$) were standardized after dry mass, and then these were used in research. The most performing results were obtained in the case of applying the ethanolic extract from spirulina biomass with cobalt content. Thus, in three of tested strains, *Microsporum canis* ATCC®36,299™, *Microsporum gypseum* ATCC®24,102™ and *Trichophyton mentagrophytes* ATCC®9533™,

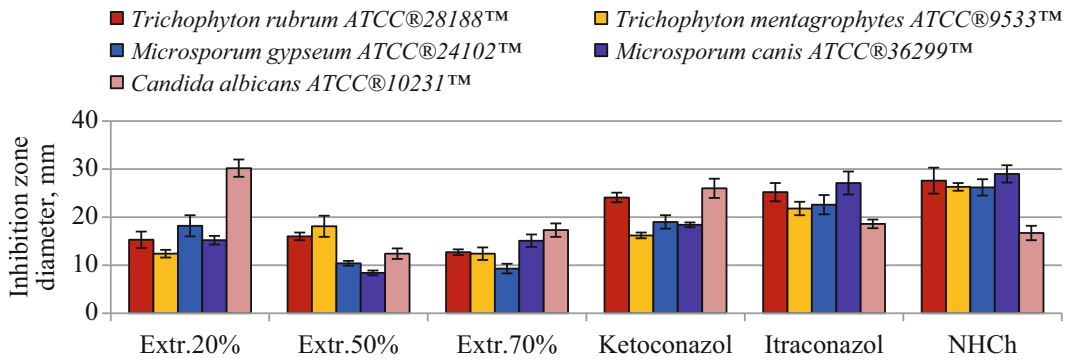


**Fig. 1** Antifungal action of hydro-ethanol extracts from biomass of *Arthrospira plantesis.* Extr.20% – hidro-ethanol extract 20% of ethanol; Extr.50% – hidro-ethanol extract 50% of ethanol; Extr.70% – hidro-ethanol extract 70% of ethanol; NHCh – naftifine hydrochloride

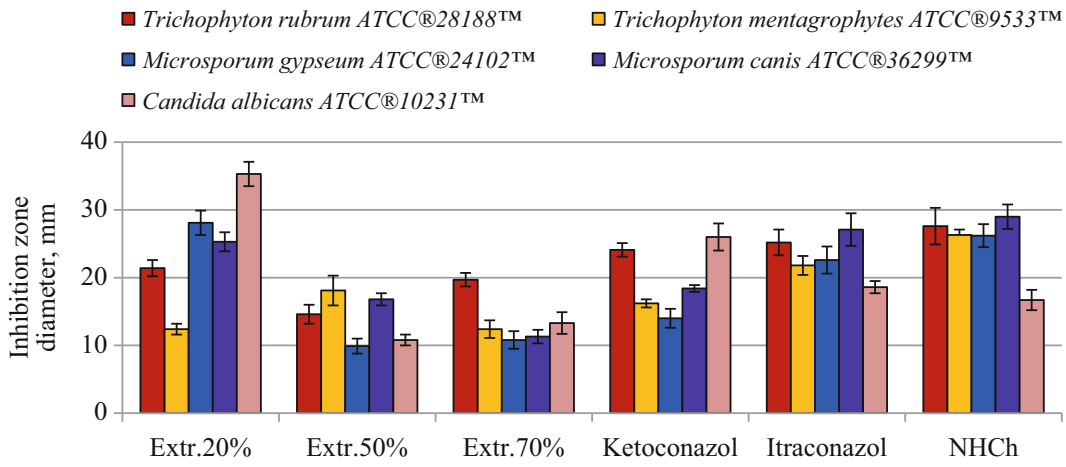


**Fig. 2** Antifungal action of hydro-ethanol extracts from biomass of *Nostoc linckia*. Extr.20% – hidro-ethanol extract 20% of ethanol; Extr.50% – hidro-ethanol extract 50% of ethanol; Extr.70% – hidro-ethanol extract 70% of ethanol; NHCh – naftifine hydrochloride

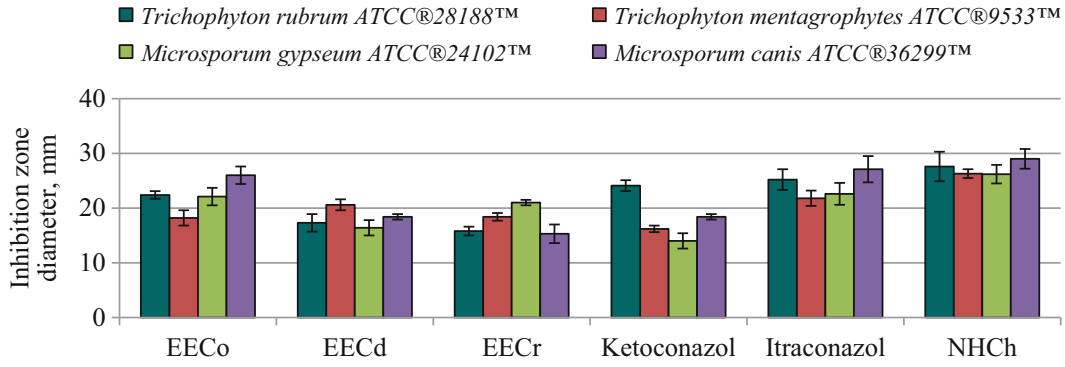


**Fig. 3** Antifungal action of extracts from *Arthrospira platensis* biomass with metal content. EECo – ethanol extract from biomass with bioaccumulated cobalt, EECd – ethanol extract from biomass with bioaccumulated cadmium, EECr – ethanol extract from biomass with bioaccumulated chromium, NHCh – naftifine hydrochloride

the diameters of inhibition zones obtained under the influence of this extract were actually larger in comparison with those obtained under the influence of ketoconazole ($P < 0.001$) (Fig. 3).

For all substances with antifungal effect, minimum inhibitory concentration and minimum fungicidal concentration were *assessed by* standard method EUCAST 7.1. Table 2 contains a selection of the best results obtained for tested substances depending on the used strain.

Ethanolic extracts from spirulina biomass containing cobalt and cadmium exhibited high antifungal activity against dermatophyte strains *Trichophyton mentagrophytes* ATCC®9533™, *Trichophyton rubrum* ATCC®28188™ and *Microsporum canis* ATCC®36299™. The ethanol extract from chromium-containing biomass was active against strain *Microsporum gypseum* ATCC®24102™. In the case of filamentous fungi *Aspergillus fumigatus* CNM-FA-02, *Mucor vulgaris* CNMN-FD-07, *Penicillium expansum* CNMN-FD-05, low minimum inhibitory concentrations were obtained for aqueous and ethanol extracts from spirulina biomass

**Table 2** Minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of selected substances against tested fungal strains

| Substance | MICs | MFCs | Substance | MICs | MFCs |
|---|---|---|---|---|---|
| *Trichophyton mentagrophytes* ATCC®9533™ | | | *Trichophyton rubrum* ATCC®28188™ | | |
| $EE_{Co}$ | 0.125 mg/ml | 1 mg/ml | $EE_{Co}$ | 0.125 mg/ml | 1 mg/ml |
| $EE_{Cd}$ | 0.065 mg/ml | 0.5 mg/ml | $EE_{Cd}$ | 0.065 mg/ml | 0.5 mg/ml |
| Intraconazole | 0.25 μg/ml | 1.0 μg/ml | Intraconazole | 0.125 μg/ml | 0.5 μg/ml |
| *Microsporum canis* ATCC®36,299™ | | | *Microsporum gypseum* ATCC®24,102™ | | |
| $EE_{Co}$ | 0.125 mg/ml | 1 mg/ml | $EE_{Co}$ | 0.125 mg/ml | 1 mg/ml |
| $EE_{Cd}$ | 0.065 mg/ml | 0.5 mg/ml | $EE_{Cr}$ | 0.065 mg/ml | 0.5 mg/ml |
| Intraconazole | 0.125 μg/ml | 0.5 μg/ml | Intraconazole | 0.065 μg/ml | 0.5 μg/ml |

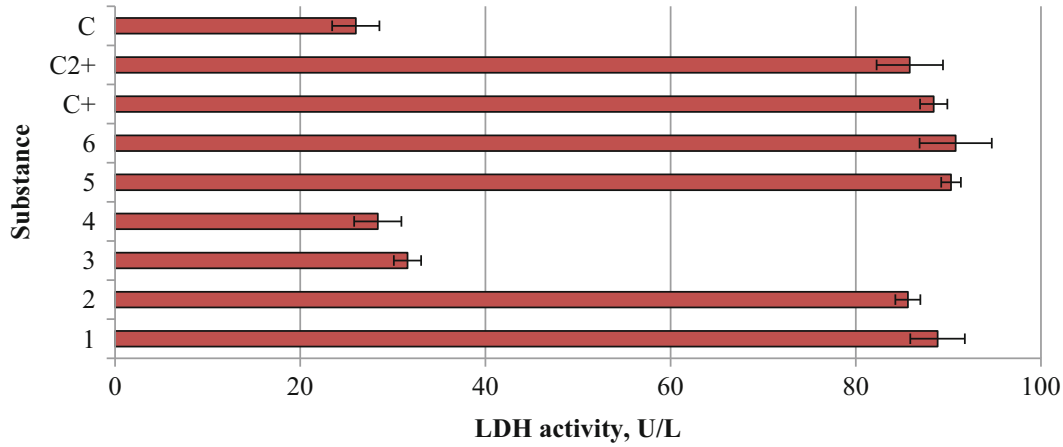


**Fig. 4** *Lactate dehydrogenase activity released by Microsporum canis* ATCC®36,299™ under the action of tested substances. 1. *J. Regia* leaf extract (ethanol); 2. 70% hydro-ethanolic extract of *S. platensis* biomass; 3. 20% hydro-ethanolic extract of *N. linckia*; 4. 70% hydro-ethanolic extract of *N. linckia*; 5. $EE_{Co}$; 6. $EE_{Cd}$; $C_+$ – itraconazole; $C_{2+}$ – naftifine hydrochloride; C – untreated control

containing cadmium and chromium. Ethanolic extracts from spirulina biomass containing cadmium and chromium also acted on both natural isolates of the genus *Fusarium.*

In order to identify the mechanisms of action of substances with antifungal effect on target cells, several biochemical tests were carried out. The first used test reffered to measuring the amount of released lactate dehydrogenase (LDH). Thus, increased lactate dehydrogenase activity in the extracellular environment was an indicator for toxic effects on cells and disruption of membrane permeability and structure. The effect of tested remedies was compared with that of the reference antifungal preparations.

In the case of dermatophyte strains *Trichophyton mentagrophytes* ATCC®9533™, *Trichophyton rubrum* ATCC®28188™, *Microsporum canis* ATCC®36299™ and *Microsporum gypseum* ATCC®24102™, the results are similar: substances with antifungal effect caused the loss of intracellular LDH and its release into the culture medium. Hydro-ethanol extracts from spirulina and nostoc biomass did not substantially increase the activity of released LDH. Figure 4 contains the results obtained for culture *Microsporum canis* ATCC®36299™.

The antioxidant status of fungal cultures was *assessed* on the basis of 2 parameters – the ability to reduce free radicals and the level of lipid peroxidation end products.

Both the used positive controls and the selected substances caused the *decrease of antiradical*

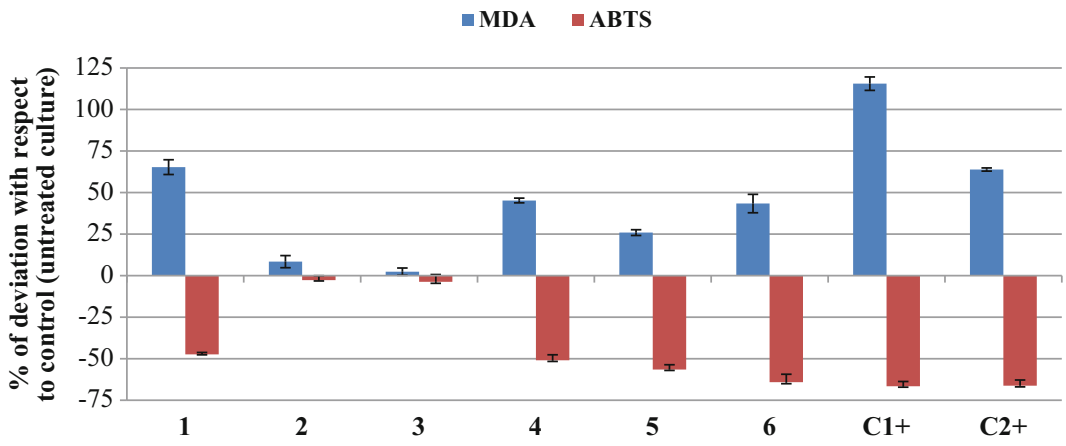


**Fig. 5** Antioxidant activity (ABTS *radical* scavenging assay) and MDA levels at *Trichophyton mentagrophytes* ATCC®9533™ under the action of tested substances. 1. *J. Regia* leaf extract (ethyl acetate); 2. 50% hydro-ethanolic extract of *S. platensis* biomass; 3. 20% hydro-ethanolic extract of *N. linckia*; 4. $EE_{Co}$; 5. $EE_{Cd}$; 6. $EE_{Cr}$; $C+_1$ – itraconazole; $C+_2$ – naftifine hydrochloride

*capacity* and an overaccumulation of lipid peroxidation products. In Fig. 5, as an example, the results for the action of antifungal remedies on culture *Trichophyton mentagrophytes* ATCC®9533™ are presented. The concentration of malonic dialdehyde in control was 2.50 ± 0.12 nmol/g, and total antioxidant capacity was 35.05± % inhibition of $ABTS^{+\cdot}$ radical. The most pronounced effect of intensifying lipid peroxidation had itraconazole (MDA increase by 115.6% compared to untreated control). Close value effects showed naftifine hydrochloride and ethyl acetate extract of walnut leaves, which produced an increase in MDA levels by 62.6 and 65.3%, respectively. The ethanolic extracts from spirulina biomass containing cadmium, cobalt and chromium produced an increase of MDA levels with 25.9–45.2% compared to untreated control. Hydro-ethanol extracts from spirulina and nostoc biomass did not produce modification of MDA levels. Similarly, these extracts did not alter total antioxidant capacity of biomass of *Trichophyton mentagrophytes* ATCC®9533™. Both positive controls and ethanolic extract from chromium-containing spirulina biomass had similar effects on total antioxidant capacity of biomass, reducing it by 64.2–66.7% compared to positive control.

One of the fungal virulence factors that provide defence against the host immunity are the primary antioxidant enzymes – superoxidismutase (SOD), catalase (CT) and peroxidases, that remove reactive oxygen species generated by the host cells and ensure the spread of infection. Reduced activity of these enzymes leads to the inability of fungi to protect themselves from the consequences of oxidative stress and reduce their viability. Therefore, further research aimed at assessing the modification of SOD, CT and glutathione peroxidase (GPx) activity in the tested fungi cells under the influence of antifungal compounds. The activity of the three major antioxidant enzymes was evaluated in fungal cultures reaching the exponential growth phase after 24 h contact with the selected compounds in the concentrations corresponding to CMI. The obtained results showed substantial changes in the activity of the primary antioxidant enzymes in almost all experimental variants.

In the case of dermatophytes, the antifungals caused the decrease of the activity of the first line antioxidant enzymes, again, except the hydro-ethanolic extracts of the standard biomass of cyanobacteria, in which only a decrease in CT activity and an increase in SOD activity was observed. As example, Fig. 6 shows the results for the *Trichophyton mentagrophytes* ATCC®9533 ™ strain.

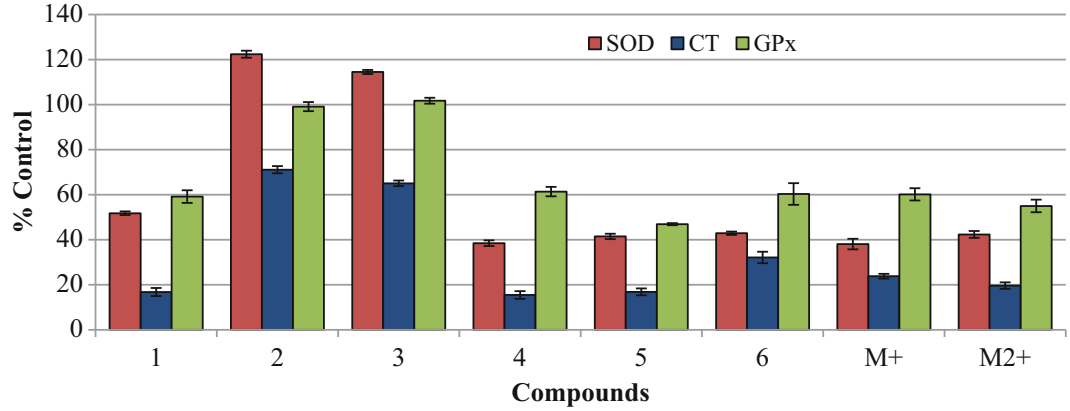


**Fig. 6** The activity of antioxidant enzymes in *Trichophyton mentagrophytes* ATCC®9533 ™ under the influence of the studied compounds. 1. Leaf extract *J. regia* (ethyl acetat); 2. Hydro ethanolic etract 50% *S. platensis*; 3. Hydro ethanolic extract 20% *N. linckia*; 4. EE $_{Co}$; 5. EE $_{Cd}$; 6. EE $_{Cr}$; $M_+$ – Itraconazol; $M_{2+}$ – Naftifine hydrochloride

Thus, the extracts from spirulina biomass obtained under biotechnological conditions of metal accumulation significantly reduced the activity of antioxidant enzymes. Hydro-ethanolic extracts from spirulina and nostoc biomass did not substantially influence GPx activity, decreased CT activity and increased SOD activity, which is explained abowe all by the lack of toxic potential in these cultures. At the same time, these extracts exerted an antifungal activity against studied dermatophytes. Cyanobacterial phycobiliproteins are considered as active elements of the water extracts. These compounds, in particular phycocyanin and phycoerythrin, are present in nostoc hydro ethanolic extracts, and phycocyanin and allophycocyanin – in spirulina extracts.

The tested in this study 50 and 70% hydro ethanolic cianobacterial extracts are also rich in such active components as phenols. Thus, it is certain that the extracts from the standard biomass of spirulina and nostoc, due to the safety for human consumption, but also due to the specific effects on some causative agents of the mycoses, deserve to be the subject of separate studies.

The results of our study suggest that one of the mechanisms of antifungal action of the tested natural extracts is the decrease of the primary antioxidant enzymes activity of the fungi, considered as a virulence factor of the fungal cells.

## 5 Final Remarks

Pathogenic fungi possess an impressive arsenal of intrinsic and acquired resistance mechanisms to the action of the most effective antifungal agents. Hence the need to develop and implement new remedies with a noticeable effect on pathogens with minimal adverse effects on the host organism. In the current concept there are several perspectives for the development of new antifungal agents, including the detection of natural antifungal compounds and the application of multivalent complex preparations acting on the various levels against multiresistant pathogens. The biomass of cyanobacteria *Arthrospira platensis* and *Nostoc linckia* is a suitable raw material for extracting compounds with potential for the development of new generation of antifungal drugs.

The hydro-ethanolic extracts from spirulina biomass obtained under biotechnological conditions of metal accumulation displayed an antifungal activity against all studied dermatophytes. The best results were recorded for water and ethanolic extracts from spirulina biomass containing cobalt, chromium and cadmium (Oltu and Rudic 2016). The following response reactions of fungicultures were observed at the application of these extracts: extracellular lactate dehydrogenase release; decreased activity

of first line defense antioxidant enzymes – superoxide dismutase, catalase and glutation peroxidase; decreased total antioxidant activity of the fungal biomass and increased quantity of the lipid peroxidation products. These reactions suggest the possible mechanism of antifungal action of tested extracts, based on the generation of oxidative stress, which coincides with the intense degradation of cellular components, primarily membrane ones. As a result, the normal permeability and physical integrity of the fungal wall and cell membrane is compromised (Oltu and Rudic 2016). Reduced activity of primary antioxidant enzymes, considered as virulence factor, and of total antioxidant capacity of causative agents of mycoses under the influence of studied extracts, leads to the inability of fungi to protect themselves from the consequences of oxidative stress and reduce their viability.

**Compliance with Ethical Standards**

Conflict of Interest: Author Iulian Oltu declares that he has no conflict of interest. Author Liliana Cepoi declares that she has no conflict of interest. Author Valeriu Rudic declares that he has no conflict of interest. Author Ludmila Rudi declares that she has no conflict of interest. Author Tatiana Chiriac declares that she has no conflict of interest. Author Ana Valuta declares that she has no conflict of interest. Author Svetlana Codreanu declares that she has no conflict of interest.

Ethical Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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