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Antifungal activity of extracts from *Arthrospira platensis* against some pathogens, causing invasive mycoses

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Abstract

Background: The aim of this study was to investigate the effect of extracts from the biotechnologically obtained *Arthrospira platensis* (spirulina) biomass with metals, on some strains of filamentous fungi causing invasive mycoses.

Material and methods: The extracts from spirulina biomass were used as material. The agar diffusion method was used to identify antifungal activity of extracts. Five strains of filamentous fungi were used as test objects. The level of toxicity was evaluated based on the quantitative determination of the activity of extracellular lactate dehydrogenase (LDH).

Results: The ethanol extracts from biomass of spirulina containing chromium, copper, cadmium, cobalt, zinc and iron, have antifungal activity against *Aspergillus fumigatus CNM-FA-02, Mucor vulgaris CNMN-FD-07, Penicillium expansum CNMN-FD-05, Fusarium solani* and *Fusarium oxysporum.* At the same time, the extracts from standard biomass are inactive (exception: the ethanol extract slightly suppresses the growth of *Aspergillus fumigatus CNM-FA-02*). Naftifine hydrochloride was used as a control. We found four variants with higher antifungal activity than naftifine hydrochloride. In the case of biomass extracts containing copper, cobalt, chromium and cadmium the inhibition of fungal growth was associated with increased activity of extracellular LDH.

Conclusions: Extracts from biomass containing metals are characterized by various antifungal activities, inhibiting the fungal growth and increasing the release of lactate dehydrogenase into the extracellular medium.

Key words: antifungal activity, ethanol and water extracts, *Arthrospira platensis*.

Introduction

Over the last few decades, the number of patients susceptible to invasive infections caused by filamentous fungi, usually found in different natural habitats such as soil and various organic substrates, grew steadily. The list of such patients includes persons with hematologic and autoimmune diseases, subjected to organ transplant or with a compromised immune status [1]. Even in case of proper treatment, the most invasive fungal infections are associated with high rates of mortality of over 50% [2,3]. The most known agents of invasive mycoses belong to the genera *Aspergillus* and *Mucor*. In recent years, this list has been supplemented by the less common filamentous fungi, such as *Fusarium spp.* and *Penicillium spp.* There have been also expanded the limits of applicability of "fungal invasion" term from "invasive disease" to previously less recognized entities, such as severe asthma with fungal sensitization, chronic cough associated with fungal infections, allergic bronchopulmonary mycosis and allergic fungal rhinosinusitis [2,4,5].

Normally, the appropriate antifungal therapy is prescribed depending on the patient's immune status, the site of infection, the biological characteristics of the pathogen and the pharmacokinetic characteristics of the applied drug. There is utilized a limited number of antifungal medications available in the treatment of systemic fungal infections, forming four different classes [6]: (a) polyene macrolides that change the membrane functions of the pathogen; (b) azole derivatives that inhibit lanosterol 14 α-demethylase, a key enzyme in the biosynthesis of ergosterol; (c) inhibitors of DNA and RNA synthesis; and (d) inhibitors of 1,3-β-glucan synthesis.

Evolution of medical practices with the introduction of new therapies, such as the use of more aggressive chemotherapy or new immunosuppressive drugs, like tumor necrosis factor antagonists, anti-CD52 antibody (alemtuzumab), and interleukin receptor antagonists (basiliximab), contribute to increasing in the incidence of invasive mycoses [1]. The progress achieved in increasing the survival time of patients, in combination with the selection pressure generated by the use of prescribed antifungal preparations for prophylaxis or preventive treatment, also are factors which strengthen the frequency of opportunistic mycotic infections, but also cases of pronounced resistance to applied antimycotic therapy.

Microorganisms develop common mechanisms to counteract the fungicidal and fungistatic effects of antifungal preparations. Currently, it is considered that resistance to drugs of fungi is based on three main mechanisms, namely, (a) reducing the drug accumulation within the fungal cell, (b) reducing the drug affinity towards its target and (c) alteration of metabolism in order to counterbalance the effect of the drug [7].

Adverse effects, often severe, of the antifungal treatment in combination with high rate of multiple resistances of pathogens dictate the necessity of new preparations intended for treatment of invasive infections caused by fungi. Preparations of natural origin are in the center of attention

for both pronounced biological effects and the fact that these are more easily accepted by patients. Biomass of various plants and microorganisms is considered as a raw material for the extraction of compounds with the potential fungicidal and fungistatic effect. *Arthrospira platensis* (*Spirulina*) is a cyanobacterium used extensively as a source of protein, but also of substances with high biological activity, including antiviral, antibacterial and antifungal activity [8–10].

Phenolic extract from *Spirulina platensis* has pronounced influence on the production of structural components of *Aspergillus flavus*. At a concentration 1.15 mg of phenols extracted from 1 g of *Spirulina platensis* biomass the amount of glucosamine in the fungal biomass decreases by 56%. Thus, the biomass and alcoholic extract from *Spirulina* possess antifungal action towards *Aspergillus flavus* [11]. Purified water extracts and concentrates from spirulina showed pronounced antifungal action towards *Penicillium oxalicum* (91% inhibition) and *Fusarium solani* (65% inhibition) [8]. Methanolic extract from dry spirulina biomass possesses antifungal activity towards *Aspergillus flavus* and *Aspergillus niger* [10]. The above mentioned authors stress that the mechanisms of action of spirulina extracts on filamentous fungi are based on inhibiting the synthesis of ergosterol, glucosamine and proteins.

Many biologically active compounds used as drugs exhibit different pharmacological properties and toxic potential, when administered in the form of metal-based compounds [12]. Thus, biomass enriched with various metals could serve as a perspective source in order to obtain efficient preparations for treatment of different diseases, including invasive mycoses.

The aim of the researches presented in this article is to highlight the antifungal properties of extracts obtained from *Spirulina* biomass enriched with metals (Zn, Fe, Cu, Cd, Co și Cr) towards some representatives of the genera *Aspergillus, Penicillium, Fusarium* și *Mucor.*

Material and methods

Aspergillus fumigatus CNM-FA-02, Mucor vulgaris CNMN-FD-07, Penicillium expansum CNMN-FD-05 from National Collection of Microorganisms of the Institute of Microbiology and Biotechnology of the Academy of Sciences of Moldova, have been used as reference strains. Two strains of fungi that represent spontaneous flora, which were isolated from soil and identified as *Fusarium solani* and *Fusarium oxysporum* were also included in this study. Fungi were grown on malt agar medium at a temperature of 30 °C.

Sensitivity to the action of extracts from spirulina biomass was determined by the agar-well diffusion method. There was poured agar medium populated by test-culture in Petri dishes with a diameter of 100 mm. Culture (in the form of spores) was introduced into the medium when it has reached a temperature of 65-70 °C. The inoculum had a concentration of about 20 mln spores to 1 ml. Wells were drilled into the agar using sterile drill. The diameter of the wells was 8 mm. There were introduced equal volumes of the standard solution and the tested solutions in wells. Petri dishes were left at room temperature for 2 hours, after which were incubated at 30±1°C for 96 hours. The inhibition zone diameter of test-microorganisms growth has been measured using caliper with accuracy of 0.1 mm.

There were used two types of extracts from biomass of *Arthrospira platensis CNMN-CB-11* as preparations with antifungal effect. The cyanobacterium was grown on nutrient medium SP -1 with added metal compounds (Zn(II), Fe(III), Cu(II), Cd(II), Co(II) și Cr(III)). The first type of extracts (EE) has been obtained by extraction with ethyl alcohol of 96% from biomass (2 parts of alcohol volume to 1 part biomass 100 mg/ml) at room temperature for 2 hours on the mechanical stirrer. The second type of extracts (HE) was obtained by extraction in hot water (90°C) for 1 hour, observing the same report – 2 parts of purified water volume and 1 part biomass 100 mg/ml. The extracts have been standardized after the dry substance so that 1 ml of extract contains 10 mg dry substance. Content of metals in the extracts was the following: EE_{Zn} – 0,15%; HE_{Zn} – 0,38%; EE_{Fe} – 0,09%; HE_{Fe} – 0,08%; EE_{Cu} – 0,35%; HE_{Cu} – 0,42%; EE_{Cd} – 0,09%; HE_{Cd} – 0.03%; EE_{Co} – 0,09%; HE_{Co} – 0,09%; EE_{Cr} – 0,11%; HE_{Cr} – 0, 15%. Corresponding extracts from standard biomass were taken as control: EE-ethanolic extract, HE-hydric extract. There was also taken naftifine hydrochloride (NH), solution, 10 mg/ml, as reference antifungal compound.

Antifungal effect of preparations had been also appreciated after the activity of lactate dehydrogenase (LDH). The release of this cytoplasmic enzyme, which shows instability of cell membranes, was determined by applying the procedure described by Arokiyaraj and coauthors with application to the strains of filamentous fungi [13]. Summarily, the determination has been made in the following manner: the strains were grown through submerged cultivation on Sabouraud medium. At the transition of fungi into stationary phase of the vital cycle, biomass was separated from the nutrient medium, washed and resuspended in purified water, to which in the experimental variants were added extracts from *Spirulina* (200 mg dry substance/L). Incubation was carried out at room temperature for 4 hours. 100 µL of supernatant of each control cultures and those treated with extracts from spirulina, were added to the reaction mixture containing 0.5 ml pyruvate of 100 mM, 5 mg NADH in 20 ml buffer solution of potassium phosphate of 500 mM, pH 7.5. Absorbance (A) was read for 5 min at intervals of 30 seconds at the wavelength of 340 nm at the spectrophotometer UV-VIS PG Instrument T-80. LDH activity in international units (U/L), which expresses the amount of enzyme which reduces 1 µM of NAD per minute, was calculated according to the formula:

$$U/L = \frac{\Delta A/_{min} \times TV \times 1000}{d} \times d \times \varepsilon \times SV$$

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Where ΔA/min represents the relative change of absorbance at 340 nm, TV is the total volume of reaction, 1000 is the passage coefficient from U/ml to U/L, *d* is the luminous flux in cm, ε represents the molar extinction coefficient, SV is the volume of sample in ml [14].

All experiments were performed in three replicates. There was calculated the average value and the standard deviation. The veracity of the differences between the experimental and control variants was assessed on the basis of Student criterion.

Results and discussion

The results obtained in case of testing the antifungal action of extracts from spirulina standard biomass and containing metals through diffusion method are presented in Table 1. It includes the diameters of the inhibition zones of fungal growth and P values only for cases excluded the null hypothesis and indicates the difference between the variants in the direction of increasing the effects of the tested preparations. Ethanolic extract from standard biomass of *Spirulina* had an inhibitory effect on the growth of only one of the tested strains – *Aspergillus fumigatus CNM-FA-02*. Extracts from other types of biomass also had an inhibitory effect on the growth of *Aspergillus fumigatus*. In the case of extracts from spirulina biomass containing copper and cadmium antifungal effect is more pronounced than in the case of the ethanolic extract from standard biomass. The diameters of the inhibition zones are higher (P=0.005) than in case of standard extract. However, even these two extracts remain significantly weaker than antifungal preparation of reference naftifine hydrochloride. While the growth of other tested fungal strains was not inhibited by the ethanolic extracts from standard biomass, the extracts from biomass with metals have manifested an antifungal effect of different intensity against all investigated fungi. Antifungal effect towards *Penicillium expansum CNMN-FD-05*, *Fusarium oxysporum* and *Fusarium solani* of extracts from spirulina containing metals is small compared to that of naftifine hydrochloride in all the experimental variants. There have been highlighted the results recorded for the strain *Mucor vulgaris CNMN-FD-07*. In this case 4 of 6 extracts from biomass with metals showed a more pronounced antifungal effect than the reference substance naftifine hydrochloride. With the exception of the extract from *Spirulina* biomass containing copper, which produces no inhibition effect, and from biomass containing iron,

Table 1

Antifungal effect of extracts from *Spirulina* biomass with metals towards some strains of filamentous fungi

Extract/Substance	Diameter of inhibition zone, mm				
	Aspergillus fumigatus CNM-FA-02,	Mucor vulgaris CNMN-FD-07	Penicillium expansum CNMN-FD-05	Fusarium oxysporum	Fusarium solani
EE_{Zn}	11,66±0,58	14,23±1,12 P_2=0,019**	10,63±0,72	10,73±0,64	9,70±0,31
EE_{Fe}	0	10,27±0,67	10,37±0,57	11,70±0,79	11,40±0,62
EE_{Cu}	19,33±0,81 P_1=0,005*	0	10,20±0,91	11,67±0,60	10,03±0,35
EE_{Cd}	20,33±1,00 P_1=0,005	13,63±0,74 P_2=0,013	19,13±1,15	14,23±0,59	13,23±0,89
EE_{Co}	11,2±0,79	23,30±1,05 P_2=0,001	11,37±0,25	11,70±0,30	11,40±0,90
EE_{Cr}	15,87±0,83 P_1=0,008	18,43±0,61 P_2=0,003	16,70±0,55	12,67±1,52	10,83±0,56
EE	12,76±0,74	0	0	0	0
HE_{Zn}	0	0	0	0	0
HH_{Fe}	0	0	0	10,0±0,26	0
HE_{Cu}	0	0	0	0	0
HE_{Cd}	28,67±0,58	0	27,33±0,64	16,67±0,64	21,6±0,53
HE_{Co}	0	19,2±0,72	16,07±0,90	29,67±0,577 P_2=0,001	0
HE_{Cr}	15,1±0,17	0	21,33±0,58	24,67±0,57	0
HH	0	0	0	0	0
Naftifine hydrochloride	34,5±1,08	11,63±0,35	34,67±0,31	24,17±0,76	31,10±0,85

P_1* - veracity of differences between the variants EE_{Me} and variant EE.

P_2** - veracity of differences between the variants with extracts and naftifine hydrochloride.

inhibition zones in experimental variants were more extensive compared to those obtained in the case of the reference substance ($0.003 \leq P \leq 0.019$).

Water extracts from *Spirulina* biomass were less active as preparations with antifungal activity. Extract from standard biomass has not inhibited the growth of studied filamentous fungi. The same thing can be said about water extracts from *Spirulina* biomass containing zinc and copper – inhibition zones of fungal growth have not been certified. Water extract with iron showed weak antifungal action only towards strain isolated from the spontaneous edaphic microflora – *Fusarium oxysporum*.

Experimental variant HE_{Cd} containing cadmium showed antifungal effect towards 4 strains of fungi (with the exception of *Mucor vulgaris CNMN-FD-07*). Inhibition zones of growth of fungi *Penicillium expansum CNMN-FD-05* and *Aspergillus fumigatus CNM-FA-02* were similar to naftifine hydrochloride zones, but nonetheless smaller in diameter. Water extract from *Spirulina* biomass containing cobalt has inhibited the growth of three strains of fungi – *Mucor vulgaris CNMN-FD-07*, *Penicillium expansum CNMN-FD-05* and *Fusarium oxysporum*. In the last case, the antifungal effect of the extract was more pronounced than that of naftifine hydrochloride, the inhibition zone being higher by 22.76%.

Hydric extract with chromium was manifested by growth inhibition of *Aspergillus fumigatus CNM-FA-02, Penicillium expansum CNMN-FD-05* and *Fusarium oxysporum*. In this case, inhibition zone of growth of *Fusarium oxysporum* is equal to that measured in the variant with naftifine hydrochloride.

Then, it was determined the level of release into the medium of the enzyme lactate dehydrogenase in experimental variants in which antifungal activity was detected. In the case of the integrity of cell sheaths it remains seized in the cytoplasm of cells and only in the event of disturbing the structure and functions of the membrane and cell wall the enzyme is released into the medium. Increasing the activity of lactate dehydrogenase is an indicator of toxicity of substances, which have acted upon the studied cultures. Figure 1 represents the activity of extracellular lactate dehydrogenase at the action of spirulina extracts on *Aspergillus fumigatus CNM-FA-02*.

Under normal conditions the activity of extracellular LDL from *Aspergillus fumigatus CNM-FA-02* constitutes 34 international units per liter. The same level of activity can be also observed in supernatant of culture treated with hydric extract obtained from *Spirulina* standard biomass. In the case of the ethanolic extract from standard biomass we can observe an increase of LDH activity by 23.5% compared to the control, which is also associated with the described above inhibitory effect of culture growth. Extracts from biomass containing cadmium, both ethanolic and hydric one, leading to duplication of LDH activity, the values are practically equal with those obtained for naftifine hydrochloride. In the case of extracts from biomass containing copper, cobalt and chromium, the effect of disturbance of the permeability of cell sheaths is much higher when applying the ethanolic extracts than water ones, but the effect is present in all these variants. In all these cases, it has been also observed the inhibition of fungi growth under the influence of mentioned extracts. The results presented in this figure emphasize the ethanolic extract obtained from *Spirulina* biomass containing zinc. Though, it was previously described moderate effect of inhibition of *Aspergillus* growth to the action of this extract, LDH activity is quite low – 14.7% compared to the control.

Figure 2 represents the activity of extracellular lactate dehydrogenase to the action of spirulina extracts on *Mucor vulgaris CNMN-FD-07* and *Penicillium expansum CNMN-FD-05*.

A very pronounced increase of LDH activity at *Mucor vulgaris CNMN-FD-07* was registered in the experimental variants with ethanolic extract from biomass containing cadmium, cobalt, chromium, the values being 2.7-3.2 times higher than in the case of the control. Moreover, these ex-

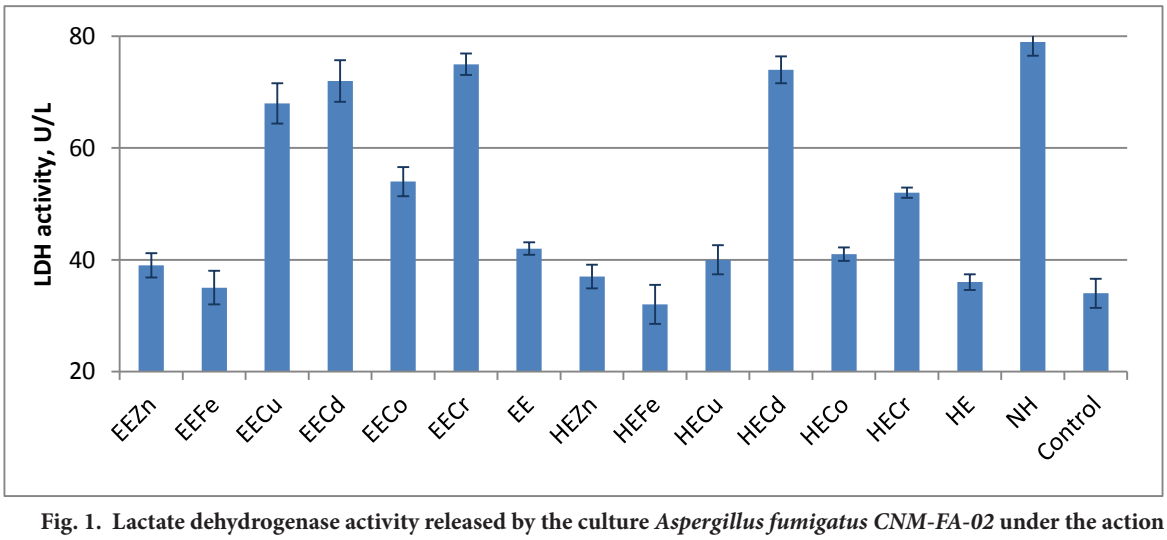

Fig. 1. Lactate dehydrogenase activity released by the culture *Aspergillus fumigatus CNM-FA-02* under the action of spirulina extracts (NH - naftifine hydrochloride).

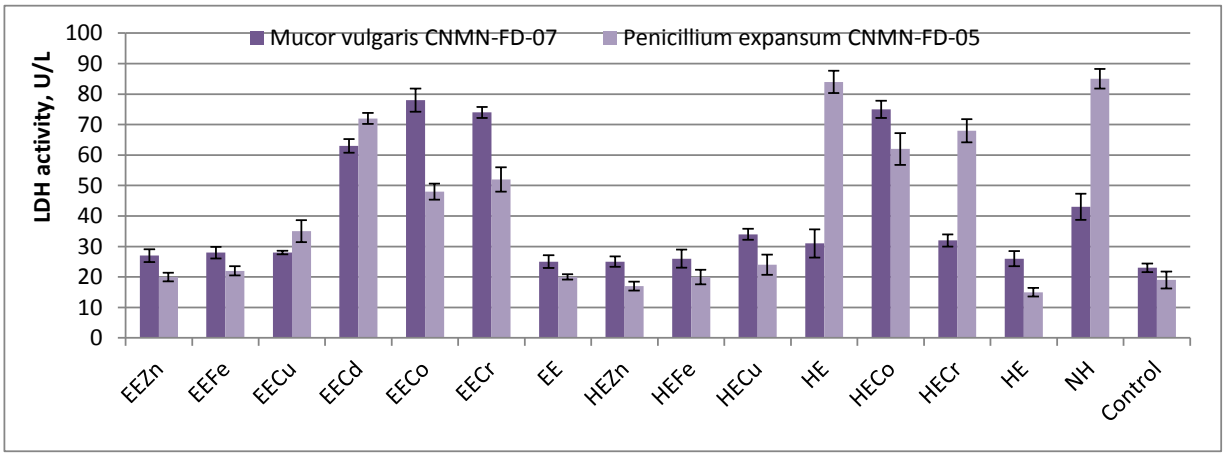

Fig. 2. Lactate dehydrogenase activity released by the culture *Mucor vulgaris CNMN-FD-07* and *Penicillium expansum CNMN-FD-05* under the action of spirulina extracts (NH-naftifine hydrochloride).

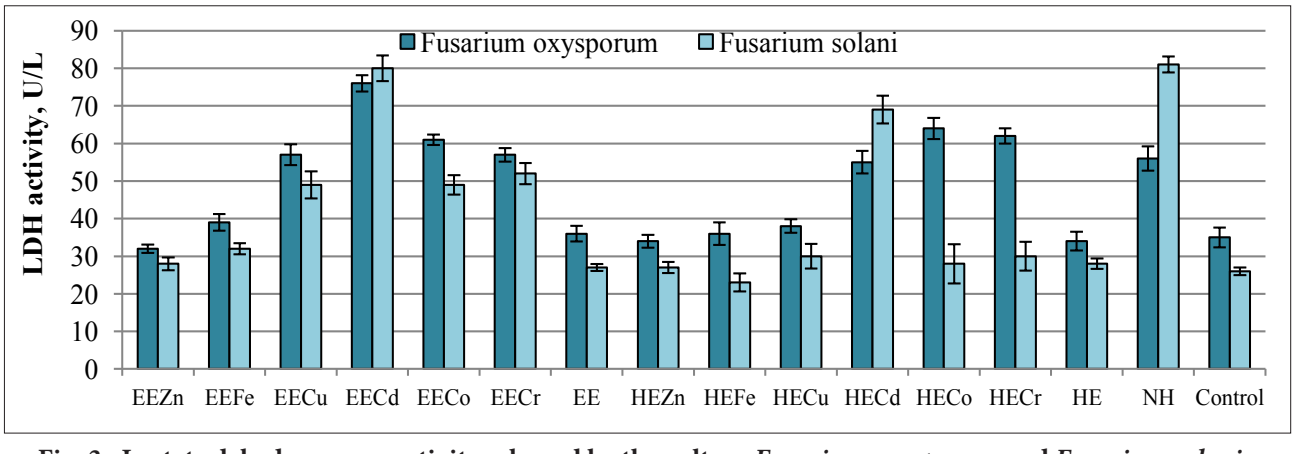

Fig. 3. Lactate dehydrogenase activity released by the culture *Fusarium oxysporum* and *Fusarium solani*

ceed the LDH activity by 1.47-1.81 times in the variant with naftifine hydrochloride. Of all the water extracts, a pronounced toxic effect was registered only for the variant containing cobalt, in which LDH activity is 3.26 times higher than in the case of control and 1.74 times higher than in the case of application of naftifine hydrochloride.

Water and ethanolic extracts from biomass containing cadmium, cobalt and chromium have manifested antifungal effects towards *Penicillium expansum CNMN-FD-05*. In the case of applying the ethanolic extracts, the activity of LDH released into the extracellular medium is 2.7-3.8 times higher than control, but none of the tested variants showed higher values than those obtained from the treatment of culture with naftifine hydrochloride. Ethanolic extract containing copper has also produced the release of LDH in the medium, thereby increasing its activity by 1.84 times. Water extracts containing cobalt, chromium and cadmium also led to the disruption of membrane permeability of *Penicillium*, expressed through intense elimination of LDH. In the case of water extract with cadmium, LDH activity is at the level of the sample with naftifine hydrochloride. And for these two strains, although ethanolic extracts containing iron and zinc have inhibited growth on agar medium, especially *Mucor*, where inhibition was at the level of naftifine hydrochloride, treating the fungal biomass does not lead to increase of LDH activity.

Figure 3 represents the activity of extracellular lactate dehydrogenase to the action of spirulina extracts on the strains isolated from spontaneous microflora *Fusarium oxysporum* and *Fusarium solani*.

We can see from the figure, that in the case of these two fungi strains, the ethanolic extracts containing copper, cadmium, cobalt and chromium possess higher toxic potential, which causes the active elimination of LDH in extracellular medium. All the experimental variants showed values of LDH activity of 2.5-3.3 times higher than control. Ethanolic extract containing cadmium has a higher toxicity towards *Fusarium oxysporum* than naftifine hydrochloride, LDH activity released by the culture being 1.36 times higher. Water extracts from these types of biomass have led to an increase in LDH activity, but only in the case of *Fusarium oxysporum*. Only water extract from biomass containing cadmium had toxic effect against strain *Fusarium solani*. Ethanolic extracts from biomass containing iron and zinc produced inhibition of *Fusarium* growth, but excessive elimination of LDH under their influence has not been observed.

Conclusions

Ethanolic and hydric extracts from biotechnologically obtained biomass of *Arthrospira platensis*, under conditions of bioaccumulation of metals, are characterized by antifungal activity of different intensity against filamentous fungi taken in study, while the extracts obtained from standard biomass are lacking this capacity (with the exception of the ethanolic extract towards *Aspergillus fumigatus CNM-FA-02*). The majority of ethanolic extracts are toxic to fungal cultures, which is expressed both by the development of the inhibition zones of growth at the application of agar diffusion method and by the release of lactate dehydrogenase in the extracellular medium. Ethanolic extracts from *Spirulina* biomass containing zinc, cobalt, cadmium and chromium have been manifested as preparations with antifungal properties towards strain *Mucor vulgaris CNMN-FD-07*, their action being superior to naftifine hydrochloride.

There were highlighted water extracts obtained from biomass containing cadmium, cobalt and chromium, that showed growth inhibitory effect on 3-4 tested strains. Water extracts containing cobalt and chromium showed pronounced antifungal effect towards *Fusarium oxysporum*, equal (HE_{Cr}) or higher (HE_{Co}) than naftifine hydrochloride.

In the case of treating the fungal cultures with *Spirulina* extracts containing copper, cadmium, chromium and cobalt, the growth inhibitory effect of the mycelium is associated with the release of lactate dehydrogenase in the extracellular medium, which denotes disruption of permeability of cell sheaths. In the case of biomass extracts containing iron or zinc, inhibition of growth is not followed by the release of LDH, which denotes a different mechanism of action that will be researched further.

Extracts from *Spirulina* biomass with metals linked in structure of organic compounds are promising in order to obtain preparations with antifungal action towards causative agents of invasive mycoses.

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