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Spectrophotometry As A Method To Estimate The Resistance Of Candida Albicans To Polyene Antibiotics

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Abstract

Spectrophotometry method may be useful for estimation of Candida albicans resistance to polyene antibiotics. It is quantitative, exact and rapid (3-4 hours). Assessing the possibility of using this method to estimate the sensitivity of C. albicans to polyenes was the goal of this study.

Yeast culture of C. albicans No. 927 was used. The traditional test for the sensitivity of yeast culture to polyenes was carried out using the method of two-fold microdilutions. The rapid estimation of the sensitivity of yeast cells to polyenes was carried out by the spectrophotometric method. The drug solution in saline was added to the cell suspension; the final cell concentration was 10^{10} CFU /ml. The control sample contained the saline and the yeast suspension. The sensitivity of yeast cells (i.e., drug antifungal activity) was calculated as the percentage of dye accumulated in the destroyed cells as compared to the control. The coefficient R was calculated using the formula: $R = Aa / A$.

The fungicidal activity data (sensitiv[1]ity of yeast) at different concentrations of polyene is directly proportional to the concentration of polyenes. The introduction of another parameter R – the ratio between activity values at the nearest concentrations of polyenes – helps with understanding how to choose the minimum inhibitory concentration. Resulted values are correlated to MICs, which were estimated by microdilution method.

With data obtained, it can be said that the spectrophotometric method can be used to quickly assess the values of the minimum inhibitory concentration of antifungal substances.

Keywords: *Spectrophotometry, resistance of Candida albicans, polyene antibiotics.*

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Introduction

Candida albicans is an opportunistic fungus that is associated with several causes of mortality worldwide in individuals with immunocompromised system. Morphological transformation, biofilm formation and the secretion of hydrolytic enzymes were considered the virulence factors associated with C. albicans' pathogenicity mechanism [1]. There are significant differences in the resistance of Candida to different antifungal drugs [2]. Polyenes were the first antifungal antibiotics and more than 200 of such chemical substances have been discovered, of which amphotericin B, nystatin and natamycin are most commonly used in the therapy of mycoses [3]. Polyenes are still used in medical practices because of their broad activity spectrum against pathogenic yeasts and molds, such as Candida spp., Cryptococcus spp., Aspergillus spp., Fusarium spp., Rhizopus spp. etc. [4].

As of now, four models of the polyene mechanism of antifungal action have been proposed: the pore forming model [5], the surface adsorption model [6,7], the sterol sponge model [8] and the oxidative damage model [9]. In all proposed models, the binding of the polyene with ergosterol and destruction of cell membrane is key to its antifungal effect [10].

The traditional way of estimating candidacidal effect on different substances is by the microdilution method [11], which have had some flaws and restrictions, the incubation time being about 24 h in particular [12]. Taking the microscopy/staining method as a basis [13], we developed a spectrophotometric method [14,15] based on the property of antimicrobial substances which is to disrupt the integrity of the membranes of microbial cells. This results to subsequent staining which allows the absorption of the dye from the medium. The decrease in dye concentration in the incubation medium is assessed using a spectrophotometer and expressed as a percentage relative to the control sample. This method allows to quickly (within 4 hours) assess and quantify produced by a given substance or biofluid.

Assessing the possibility of using this method to estimation of C. albicans sensitivity to polyenes was the goal of this study.

Materials and Methods

Yeast culture of C. albicans No. 927 from the Mechnikov Research Institute for Vaccines and Sera was passaged on the solid glucose-pepton-yeast extract (GPY) medium containing the antibiotic at 25°C during 19 h [16] then used in experiments.

The traditional test for the sensitivity of yeast culture to polyenes was carried out by the method of two-fold microdilutions in 96-well plates with a defined medium [11,17], and a starting drugs concentration of 100 µg/ml.. The starting content of yeast cells was about 10^4 CFU/ml. The plates were incubated at 32°C until growth appeared in the well, which contain low concentration of antifungal drug, i.e. about 24 hours.

The rapid estimation of the sensitivity of yeast cells to polyenes was carried out by the spectrophotometric method [14,18]. For this, 300 µl of drug solution in saline (concentration see in Table) was added to 50 µl of cell suspension; the final cell concentration was 10^{10} CFU /ml. The control sample contained 300 µl of saline and 50 µl of the yeast suspension. The samples were incubated/ stirred at 32°C and for 2 hours, centrifuged for 5 min at 16000 rpm, the supernatant was removed, and 300 µl of the dye bromocresol purple 1 mM solution in 1.25 M phosphate buffer (pH 4.6) was added to the pellets. The samples were incubated again for 45 min at 32°C and centrifuged. Microscopy of sediments was performed at a total magnification of 1750 ("LOMO", Russia); photographed with a "Sony" digital camera (Japan), after this the percent of dead cells was calculated. 50 µl of supernatants were added to 2.5 ml of phosphate buffer pH 4.6 and the optical density of the solutions was measured by "Genesys 10SUV-Vis" spectrophotometer (USA) at 440 nm. These procedures were carried out three times. For each sample, the average value was calculated from three measurements. The sensitivity of yeast cells (i.e., drug antifungal activity) was calculated as the percentage of dye accumulated in the destroyed cells as compared to the control, i.e. the ratio of the difference between the optical density of the control and experimental samples to the optical density of the control sample, expressed as a percentage.

Statistical data processing was carried out using Microsoft Excel.

The coefficient R was calculated using the formula:

$R = Aa / An$,

R – step-by-step rate of activity loss;

Aa – fungicidal activity corresponding to actual concentration of polyene;

An – fungicidal activity corresponding to next concentration of polyene (i.e. actual concentration divided by 2).

Results

The result of assessing the minimum inhibitory concentration of polyenes towards C. albicans by microdilution method is presented in the

Figure **1**. The MIC values are: 0.195 µg/ml for amphotericin B, 1.56 µg/ml for natamycin and 0,098 µg/ml for nystatin.

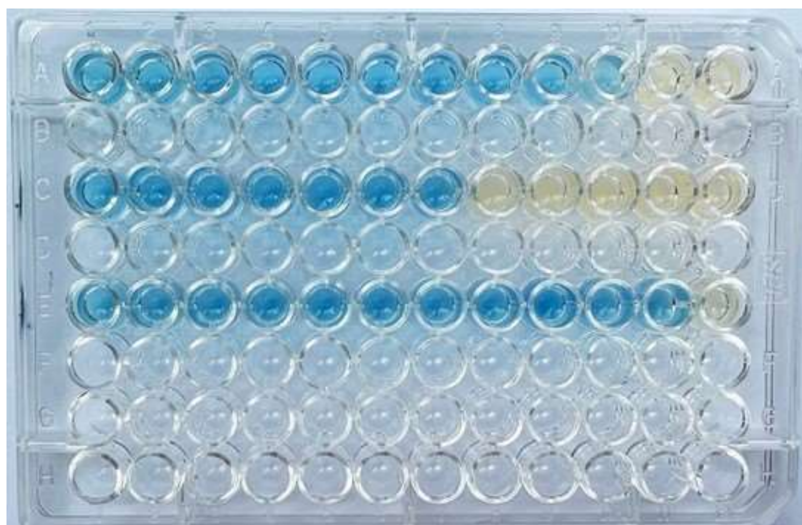

Figure 1: Estimation of minimal inhibitory concentration of polyenes towards C. albicans by microdilution method: line A – amphotericin, line C – natamycin, line E – nystatin. Initial concentration (left cell) – 100 µg/ml.

The same culture was used in the experiments of sensitivity estimation by microscopy and spectrophotometry methods. As can be seen from the presented microscopy data, amphotericin B (ATB) showed the maximal antifungal effect towards the yeast cells in both concentrations, nystatin (NST) – the medium effect, but natamycin (NAT) - the minimal effect (see Figure 2, Figure 3).

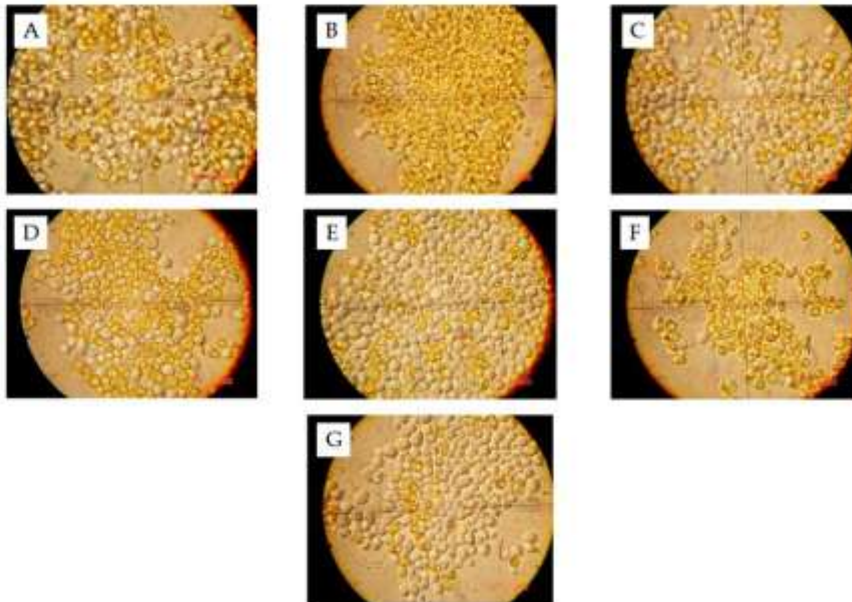

Figure 2: Microscopy of C. albicans cells incubated with polyenes: A – amphotericin, 0.25 µg/ml; B - amphotericin, 10 µg/ml; C - natamycin, 0.25 µg/ml; D - natamycin, 10 µg/ml; E - nystatin, 0.25 µg/ml; F - nystatin, 10 µg/ml; G – control – native cells. Living cells are colored yellow, dead cells are white.

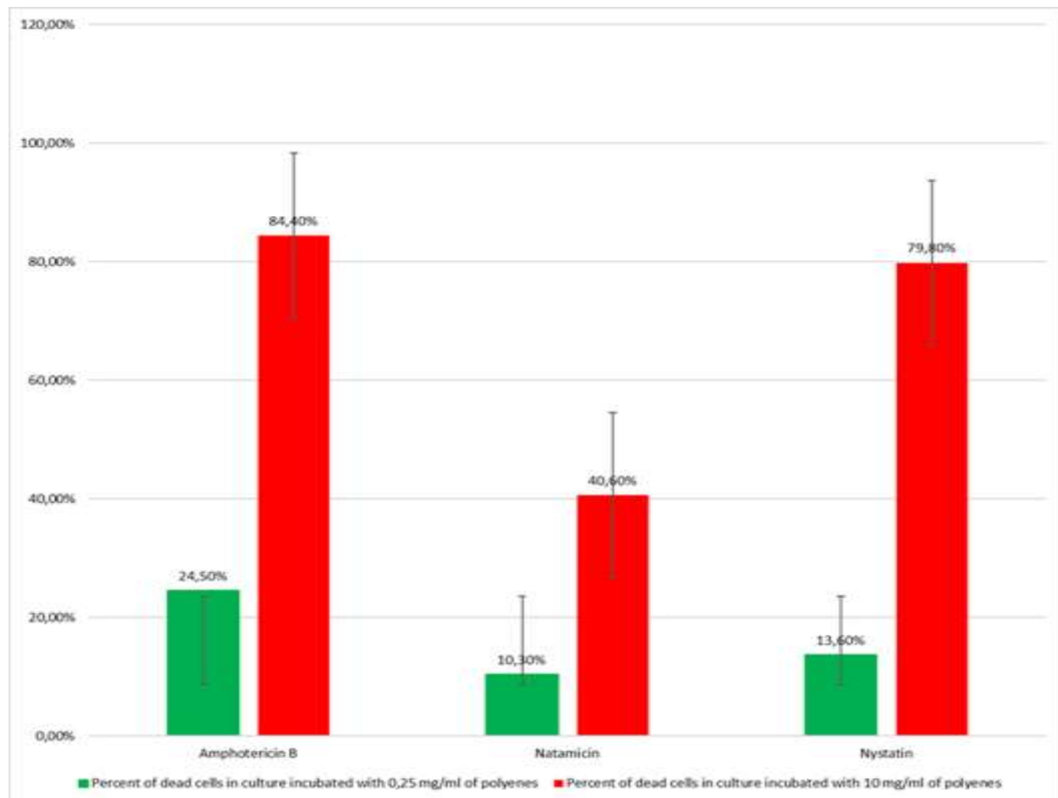

Figure 3: Percent of dead cells in C. albicans culture incubated with different concentrations of polyenes – calculation of microscopy data compare to control (%): left column - 0.25 µg/ml; right column - 10 µg/ml.

The fungicidal activity data (sensitivity of yeast) at different concentrations of polyenes are presented in the Table 1. It is clear that the activity is directly proportional to the concentration of polyenes. Microscopy data are correlated with spectrophotometry data: Pirson`s coefficient which characterized correlation between percent of dead cells and activity of three polyenes is equal 0.930 for 10 µg/ml of antifungals and 0.989 for 0.25 µg/ml of antifungals.

Table 1: Sensitivity of C. albicans cells to polyenes (i.e. fungicidal activity of polyenes towards C. albicans) estimated by spectrophotometry method.

Polyenes	Concentration, µg/ml	Fungicidal activity, %, M ±m	R*
Amphotericin B	10	65,8 ± 1,0	1,0
	5	64,2 ± 0,1	1,1
	2,5	60,4 ± 0,5	1,4
	1	43,5 ± 0,1	1,3
	0,5	32,2 ± 0,9	2,1
	0,25	15,0 ± 0,9	**7,5**
	0,125	2,0 ± 1,0	-

Natamicin	10	41,1 ± 0,6	1,3
	5	31,1 ± 0,4	1,8
	2,5	17,3 ± 0,2	**2,6**
	1	6,7 ± 0,6	2,3
	0,5	2,9 ± 0,6	1,5
	0,25	2,0 ± 1,0	1,0
	0,125	2,0 ± 1,0	-
Nystatin	10	54,5 ± 0,7	0,9
	5	61,6 ± 0,5	0,9
	2,5	65,2 ± 0,6	1,8
	1	36,9 ± 0,4	1,8
	0,5	20,0 ± 0,7	**9,3**
	0,25	2,2 ± 1,4	1,1
	0,125	2,0 ± 1,0	-

The introduction of another parameter R – the ratio between activity values at the nearest concentrations of polyenes – helps to understand how to choose the minimum inhibitory concentration (Table 1). The maximum value of R must correspond to the MIC, since it reflects a sharp decrease in cell sensitivity to polyene (see Figure 4, Figure 5, Figure 6). With this in mind it can be seen that MIC of amphotericin B is equal to 0.25 µg/ml, natamycin – 2.5 µg/ml and nystatin - 0.5 µg/ml. These values are correlated to MICs, which were estimated by microdilution method (r = 0.987).

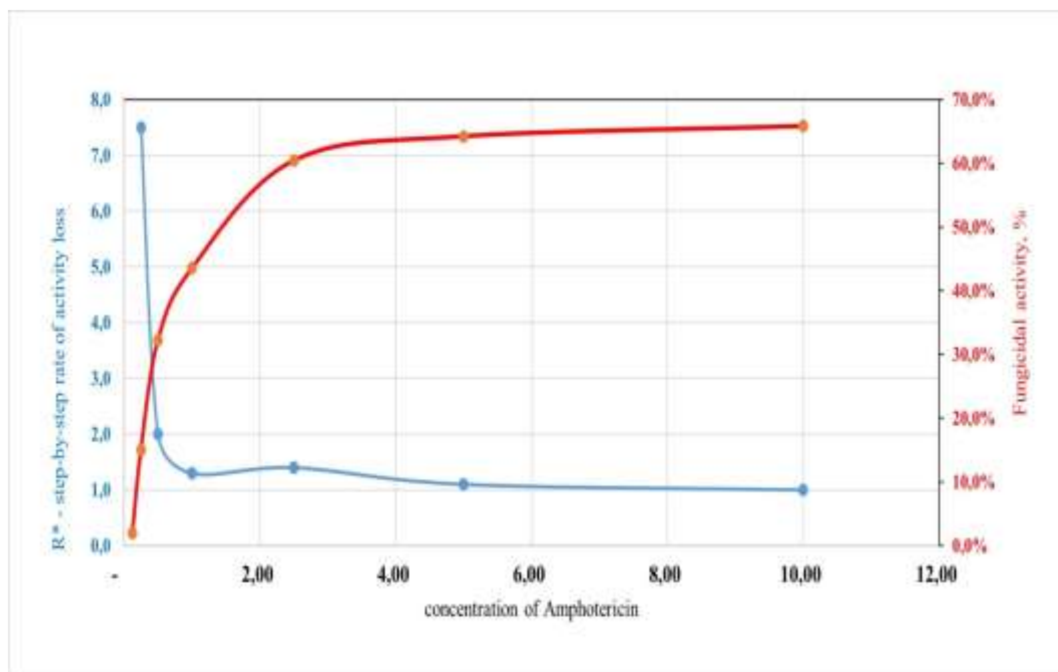

Figure 4: Dependence of the amphotericin`s fungicidal activity on its concentration

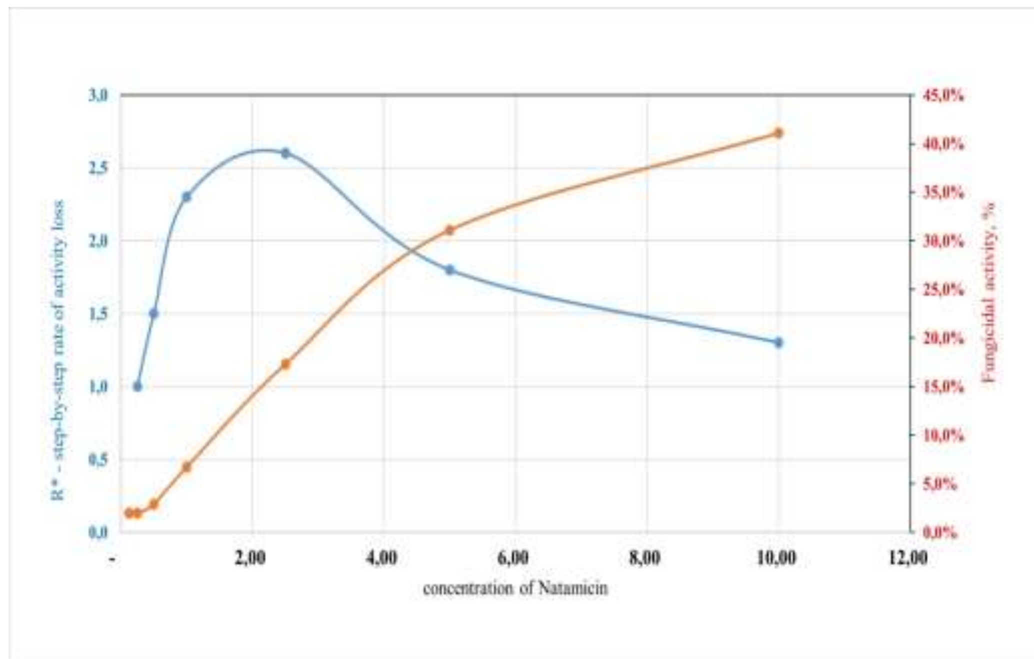

Figure 5: Dependence of the natamicin`s fungicidal activity on its concentration.

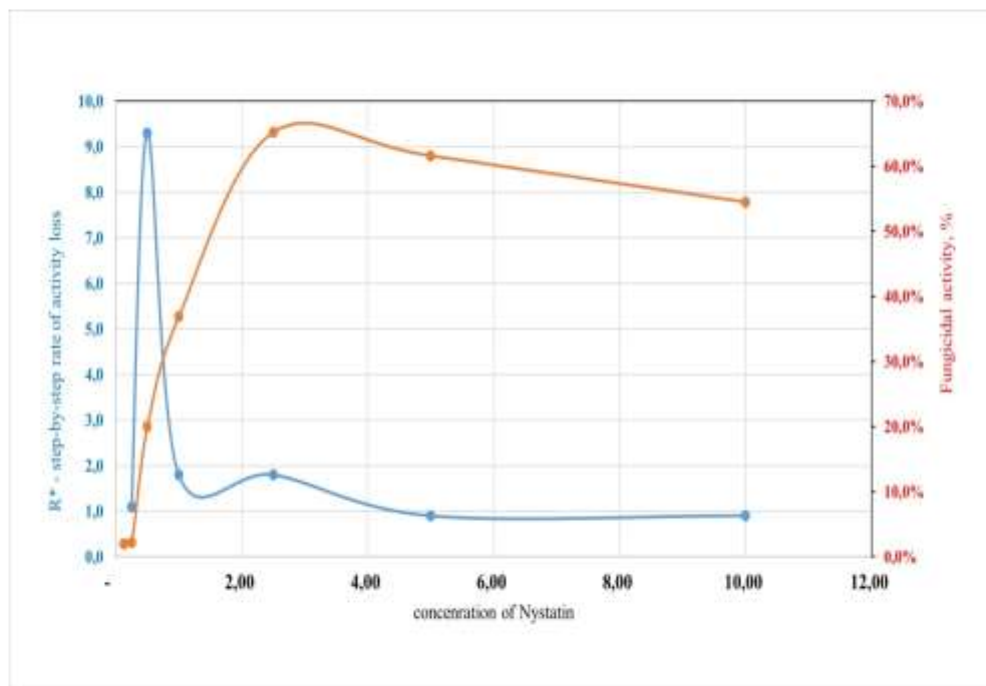

Figure 6: Dependence of the nystatin`s fungicidal activity on its concentration

Discussion

As a concurrent infection with other diseases, C. albicans is associated with high morbidity, prolonged hospital stays, high relapse rates, and substantial healthcare costs. The transition, which depends on its environment, is important for C. albicans infection, colonization, and the evasion of the host immune system [19]. The imperfect pharmacological properties of current antifungal drugs, along with resistance, make it a necessary and pressing mission to develop new antifungal agents [20]. Antifungal resistance is based on different mechanisms, namely, reduced drug intracellular accumulation, decreased target affinity/processivity for the drug, and counteraction of the drug effect. Particularly, the mechanism of resistance will be different depending on the mode of action of antifungal

compounds [21]. Ergosterol is the major sterol found in fungal membranes. It was first isolated in 1889 by Charles Tanret from ergot. Some of the physiological functions of ergosterol include maintaining membrane integrity and fluidity. Fungal cells also require ergosterol for the completion of their cell cycle, a process known as the sparkling function". In fungal cells, ergosterol also plays a role in mating. A study by Aguilar et al. showed that ergosterol had an effect on the shape of the cell and that disruption of the cell figure affected the fusion of fungal cells during mating. The importance of this pathway reasons as a target for antifungal drugs due to the fact that the biosynthesis of ergosterol occurs exclusively in fungal membranes [22]. Yeast of the Candida genus is widely distributed in nature, and is also a human symbiont. Some specia of this genus are normal mucosal habitats, while in immunocompromised persons may cause superficial and deep mycoses and mycoallergoses [23]. In clinical practice the antifungal substances of different chemical groups have wide application, exactly azoles, polyenes, allylamines etc. However, the resistance of the opportunistic fungi to antifungals is still a serious problem, which needs to be solved by the creation of new drugs and by the estimation of sensitivity of each clinical fungal isolate to available in stock drugs.

In our laboratory, since 1999 to the present, the sensitivity of yeast fungi isolates to preparations of different chemical groups has been monitored by microdilution method. For example, it has been shown that patients with diseases of the bronchopulmonary system had a significant decrease in sensitivity to azole–type drugs - ketoconazole, clotrimazole, fluconazole and itraconazole for 10 years [24]. At the same time, the sensitivity of yeast cultures to the polyene preparation - natamycin has significantly increased. Similar results were obtained in groups of patients with vulvovaginal candidiasis and atopic dermatitis diagnosed for 10 years[25]. It is known that the active efflux of drugs, a common mechanism of resistance for azole drugs, does not appear to play a role for polyene resistance [10]. Apparently, the selection of more azole-resistant yeast strains paired with changes in the cytoplasmic membrane are accompanied by increased vulnerability to natamycin. Thus, polyenes represent an interesting model for studies of the sensitivity/ resistance of the yeast microbiota.

To assess the sensitivity of clinical yeast strains, a very convenient microdilution method has been used for a long time [11,17]. Despite all its advantages, the result obtained depends on a number of factors, such as the composition of the nutrient medium, the composition of the buffer solution, the concentration of the yeast inoculum and the incubation time [12]. Even if all the proposed conditions are met, this type of analysis in a clinical laboratory takes at least 24 hours, whereas speeding up this process could be useful. In this regard, it is possible to use the mechanism of action of polyenes on fungal cells, which consists in the rapid destruction of cell membranes [10].

We have previously developed and are using the spectrophotometry method for assessing the integrity of microbial membranes [14], which made it possible to quantify and qualitatively assess the effect of various bioliquids and substances on microorganisms [15,18,26]. The mechanism of action of polyenes on fungi allows us to use this method to study the possibility of using it for a quick assessment of the sensitivity of fungi to drugs of this chemical group.

The object of this study was C. albicans yeast as the most commonly used fungal model. Their sensitivity to polyene drugs – amphotericin B, natamycin, and nystatin – was evaluated by three methods: microdilution, microscopy, and spectrophotometry. The microdilution method involves the effect of a substance in a certain concentration on a yeast culture, in which part of the population dies, and the rest either grows further, or its growth stops due to a small number of viable cells ("quorum sensing"). In the microscopy method, followed by spectrophotometry, we can give an answer to the question of which part of the

population died or survived. The concentration of polyene, below which the most significant sharp drop in the activity of the drug occurs and after which this activity ceases to decrease, is obviously the minimum inhibitory concentration (MIC). In this method, we pass the stage of waiting for culture growth, but immediately evaluate the cytotoxic effect. At the same time, it can be seen that the MIC values obtained by the microdilution method correlate to a large extent with those obtained by spectrophotometry method, however, somewhat lower than them. This fact can be explained by the higher density of the yeast suspension, which is used in the latter method. In any case, the task of this study has been completed – the principal possibility of using the spectrophotometry method to assess the sensitivity of C. albicans yeast to polyene preparations has been shown.

Further improvement of this method, in our opinion, can realize in the following directions: 1) expanding the list of studied strains / species of fungi; 2) improving equipment, for example, conducting analysis in 96-wells plates instead of Eppendorf-type test tubes and using appropriate centrifuges, which should further simplify and speed up the analysis; 3) using the method to determine sensitivity to other antimycotics with the similar mechanism of action.

Conclusions

From the data obtained it follows that the spectrophotometric method can be used to quickly assess the values of the minimum inhibitory concentration of antifungal substances, the mechanism of action of which is due to disruption of the integrity of the fungal cell membrane. The method differs from analogues in its speed of implementation, which may be important in clinical practice. The next stage in the development of the method should be to assess the possibility of its use on other strains and specia of clinically significant yeasts, other antymycotics and better equipment.

Data Availability

The relevant data generated and (or) analyzed in the current study is available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declares that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

V. Arzumanian contributed to the conceptualisation, software, writing—original draft preparation, writing—review and editing, project administration. A. Dzhadaeva contributed to the formal analysis and investigation. E. Chebotareva contributed to the writing—review and editing and visualization. V. Lim contributed to the validation, resources, writing—original draft preparation, funding acquisition. T. Avdeeva contributed to the conceptualisation, methodology, software, validation, formal analysis, investigation and funding acquisition. N. Gorbunov contributed to the resources, visualization and funding acquisition. E. Budanova contributed to the conceptualisation, validation, formal analysis and supervision. V. Rybakov contributed to the data curation and visualization.V. Zaborova contributed to the methodology, data curation, writing—review and editing, supervision and funding acquisition.

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Supplementary Materials
There is no supplementary materials.

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