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In vitro ACTIVITY OF THE ANTIBIOTIC BATUMIN AGAINST *Candida albicans* BIOFILM

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The aim of this work was to study action of batumin on the strains of *Candida albicans* and *Candida krusei* in planktonic and biofilm form and also to obtain more detailed insights into the influence of batumin on biofilm formation by using atomic-force microscopy. The Minimum Inhibitory Concentration (MIC) of batumin was studied according to CLSI standards. Formation of a biofilm was studied by the photometric O'Toole method by means of a plate photometer ELx808 (BioTek, USA) at wavelength of 630 nanometers.

The batumin has a high selective activity against staphylococci (MIC ≥ 0,25 μg/ml), at the same time, antibiotic, being not active concerning yeast of the genus *Candida* (MIC ≥ 512 μg/ml) showed the inhibiting action on biofilm formation of these microorganisms. Batumin influence on biofilm formation was studied in type, collection strains *C. albicans*, *C. krusei* and clinical isolates. Presence 0,125 μg/ml of batumin in the broth (1/2 MIC for staphylococci) reduced the biofilm formation at 55.6% of the studied strains. Their biofilm formation values varied for *C. albicans* from 1.5–3.9 CU (conventional unit: OD_{630} in experimental samples/OD_{630} in control samples), for *C. krusei* of 2.3–3.0. Batumin was more effective against *Candida* strains with strong biofilm formation.

Atomic force microscopy revealed qualitative changes in the exopolymeric matrix due to batumin treatment, as well as a significant reduction in the number of cells adhered to the coverslip, preventing formation of *C. albicans* 127 biofilm, However, *C. albicans* ATCC 24433 a significant reduction in the number of cells adhered to the coverslip weren't observed.

The data obtained by an Atomic force microscopy confirm ability of a batumin to prevent formation of a biofilm at the studied strains that allows to consider it as the preventive agent at treatment of the infections caused by yeast-like fungi of the genus *Candida*.

Key words: batumin, *Candida*, biofilm, atomic force microscopy.

Candida spp. are serious causes of hospital-acquired blood and urinary tract infections, and the most of these infections are associated with implanted medical devices such as central venous and bladder catheters with biofilm formation within these devices [1].

A relevant characteristic of *Candida* biofilms is resistance to antifungal agents, which can be intrinsic or acquired by transfer of genetic material between biofilm cells [2].

It has already been shown that antibacterial drugs can affect *Candida* biofilm formation. Tigecycline, for instance, is highly active against growing and mature biofilms of *Candida albicans* [3], whilst rifampicin can induce biofilm formation by this *Candida* species [4].

The polyketide antibiotic batumin synthesized by the producer strain *Pseudomonas batumici* has a high selective activity against staphylococci. At the same time strains of the yeast species *C. tropicalis*, *C. utilis* and *C. albicans* are resistant to batumin (MIC ≥ 512 μg/ml) [5, 6]. However, our data indicate that the addition of antibiotic in concentration of 0.125 μg/ml reduces the formation of biofilm not only in staphylococci strains, but in *C. albicans* as well [7].

The objective of this work was to study action of batumin on the strains of *C. albicans* and *C. krusei* in planktonic and biofilm form and also to obtain more detailed insights into the influence of batumin on biofilm formation by using atomic-force microscopy.

Materials and Methods

Batumin, obtained by fermentation of *Pseudomonas batumici*, was purified by silica gel preparative chromatography to 85% of purity.

Batumin is commercially available from Santa Cruz Biotechnology (Santa Cruz, CA) or Enzo Life Sciences Antwerp, Belgium).

The object of the study were type and collection strains of *C. albicans* (n = 33) and *C. krusei* (n = 12) (Table 1), isolated from patients after examination for intestinal dysbiosis, from skin microbial dysbiosis (microbial collection of the Institute of Cellular and Intracellular Symbiosis, Ural Branch of Russian Academy of Sciences, Orenburg, Russia).

Identification of *C. albicans* and *C. krusei* was carried out on the basis of morphological, cultural and biochemical properties with the use of the commercial test system API20CAUX (bioMerieux, France).

The Minimal inhibitory concentration (MIC) of batumin was studied according to CLSI Standards in Mueller-Hinton agar [8]. The microbial load of *C. albicans* was 0.5×10^7 cfu/ml and the Petri dishes were incubated at 37 °C for 24 hours.

Concentration of batumin (0.125 μg/ml) was used to study its effect upon biofilm formation in *Candida*. Biofilm formation was studied by a photometric method determining the bacterial capacity to adhere to the 96-well polystyrole plane-table surface (Thermo Scientific, USA) with subsequent crystal violet staining [9]. An antibiotic was added into culture medium simultaneous with the culture of fungi and cultivated 24 hours. At the study of influence of batumin (0.125 μg /ml) on the different stages of biofilm formation by *C. albicans* strains the antibiotic was added into culture medium simultaneously with yeast-like fungi in 90 minutes, 24 and 48 hours from the beginning of incubation.

Optical density measurement was done using a photometer ELx808 (BioTek, USA) at a wavelength of 630 nm. Degree of biofilm formation was presented in conditional units (CU) which was the optical density of the broth after growth of the strain relative to the nutrient broth optical density.

For the study of batumin effect on biofilm formation by atomic force microscopy, we used *C. albicans* ATCC 24433 as test-culture and *C. albicans* 127 (clinical isolate). For testing the influence of batumin on biofilm production, glass coverslips were immersed into Luria-Bertani broth with 0.125 μg/ml of batumin and incubated for 48 h at 37 °C.

Visualization of the biofilms was done by atomic force microscopy using the SMM-2000 microscope (Proton-MIET Closed JOINT Stock Company, Russia), in contact mode in an air environment [10] [11].

Statistic analysis was performed by non-parametric method using Mann Whitney U-test [12].

Results and Discussion

All the studied 45 strains of *C. albicans* and *C. krusei* were highly resistant to batumin (MIC of 512 μg/ml), in correspondence with earlier data [6]. The resistance of strains to batumin was studied according to CLSI Standards in Mueller-Hinton agar [8]. At the same time, our preliminary research showed effectiveness of the antibiotic on inhibition of formation of a biofilm in the cultures of the genus *Candida* [7, 13].

In experiments on batumin effect on biofilm formation by strains of *Candida* we used concentration 0.125 μg/ml, which causes only modification of biological properties, including biofilm formation and did not influence on grows properties of cultures.

The obtained results showed that the effect of batumin on the formed biofilm in the representatives of the genus *Candida* was variable for different strains and species (Table 1).

The biofilm formation values varied for *C. albicans* from 1.5 to 3.9 CU, for *C. krusei* from 2.3 to 3.0. Presence of batumin (0.125 μg/ml) in the broth reduced the biofilm formation for 55.6% in the studied strains of fungi, whereas for staphylococci this was 85% [14].

Of special interest is the fact that the change in biofilm in the presence of batumin differs for different strains of *Candida*, and that these differences are caused at a concentration of only 0.125 μg/ml, for strains resistant to the studied antibiotic (Table 1).

Apparently, the changes we have detected in the biofilms of yeast-like fungi under the influence of batumin made them unstable, incapable of persistence and dissemination in the human body.

It should be noted that batumin is more effective against *C. albicans* strains with

Table 1. **Batumin effect against biofilm formation by *Candida albicans* and *Candida krusei***

Strains		Source	Biofilm formation before batumin		Biofilm formation after 0.125 μg/ml batumin	
			optical density (OD_{630})	CU*	optical density (OD_{630})	CU*
1	*C. albicans* 24433	Reference ATCC 24433^T	**0.12**	**2.4**	**0.13**	**2.6**
2	*C. albicans* 3	Vaginal	0.2	4.0	0.105	2.1
3	*C. albicans* 4	Vaginal	0.155	3.1	0.135	2.7
4	*C. albicans* 8	Vaginal	**0.075**	**1.5**	**0.085**	**1.7**
5	*C. albicans* 13-2	Vaginal	0.195	3.9	0.075	**1.5**
6	*C. albicans* 27	Vaginal	**0.136**	**2.7**	**0.135**	**2.7**
7	*C. albicans* 18s	Skin	0.185	3.7	0.13	2.6
8	*C. albicans* 118	Colon	0.145	2.9	0.085	1.7
9	*C. albicans* 53	Skin	**0.115**	**2.3**	**0.115**	**2.3**
10	*C. albicans* 54b	Skin	**0.105**	**2.1**	**0.11**	**2.0**
11	*C. albicans* 1-n	Nose	**0.075**	**1.5**	**0.086**	**1.7**
12	*C. albicans* 123	Colon	**0.09**	**1.8**	**0.1**	**2.0**
13	*C. albicans* 127	Colon	0.145	2.9	0.09	1.8
14	*C. albicans* 128 c	Colon	0.215	4.3	0.125	2.5
15	*C. albicans* 139	Colon	0.256	5.1	0.195	3.9
16	*C. albicans* 145-1	Colon	0.25	5.0	0.135	2.7
17	*C. albicans* 146	Colon	0.47	3.4	0.106	2.1
18	*C. albicans* 147	Colon	0.16	3.2	0.101	2.0
19	*C. albicans* 172	Colon	0.225	4.5	0.14	2.8
20	*C. albicans* 173	Colon	**0.105**	**2.1**	**0.105**	**2.1**
21	*C. albicans*174	Colon	**0.09**	**1.8**	**0.1**	**2.1**
22	*C. albicans* 175-2	Colon	**0.1**	**2.0**	**0.105**	**2.1**
23	*C. albicans*176	Colon	**0.09**	**1.8**	**0.09**	**1.8**
24	*C. albicans* 177	Colon	**0.11**	**2.2**	**0.1**	**2.0**
25	*C. albicans* 178c	Colon	**0.105**	**2.1**	**0.105**	**2.1**
26	*C. albicans* 183	Colon	**0.075**	**1.5**	**0.085**	**1.7**
27	*C. albicans* 28	Vaginal	**0.115**	**2.3**	**0.105**	**2.1**
28	*C. albicans* 29	Vaginal	**0.096**	**1.9**	**0.095**	**1.9**
29	*C. albicans* 30	Vaginal	**0.125**	**2.5**	**0.0125**	**2.5**
30	*C. albicans* 23s	Skin	0.171	3.4	0.075	1.5
31	*C. albicans* 212	Colon	0.145	2.9	0.09	1.8
32	*C. albicans* 213	Colon	0.135	2.7	0.105	2.1
33	*C. albicans* 215-1	Colon	**0.09**	**1.8**	**0.1**	**2.0**
34	*C. krusei* 9	Colon	**0.075**	**1.5**	**0.085**	**1.7**
35	*C. krusei* 10	Colon	0.16	3.2	0.115	2.3
36	*C. krusei* 21	Colon	**0.1**	**2.0**	**0.095**	**1.9**
37	*C. krusei* 22	Colon	**0.12**	**2.4**	**0.135**	**2.7**
38	*C. krusei* 23	Colon	0.24	4.8	0.13	2.6
39	*C. krusei* 2-n	Nose	0.175	3.5	0.145	2.9
40	*C. krusei* 2	Vaginal	**0.135**	**2.7**	**0.125**	**2.5**

Table 1. End

Strains		Source	Biofilm formation before batumin		Biofilm formation after 0.125 μg/ml batumin	
			optical density (OD_{630})	CU*	optical density (OD_{630})	CU*
40	*C. krusei* 2	Vaginal	**0.135**	**2.7**	**0.125**	**2.5**
41	*C. krusei* 5	Vaginal	0.19	3.8	0.13	2.6
42	*C. krusei* 6	Vaginal	0.215	4.3	0.151	3.0
43	*C. krusei* 7	Vaginal	**0.075**	**1.5**	**0.065**	**1.3**
44	*C. krusei* 25	Colon	**0.075**	**1.5**	**0.09**	**1.8**
45	*C. krusei* 26	Colon	**0.11**	**2.2**	**0.11**	**2.2**

Note: * — CU: conventional unit: OD_{630} in experimental samples / OD_{630} in control samples. The optic density of control samples is 0.05, which is the nutrient broth density; ** — the results are representative on three separate experiments. $P < 0.05$ (Mann-Whitney U-test).

strong biofilm formation (CU values between 2.6 and 3.1).

The analysis of the experimental data on batumin at the stage of biofilm formation for *C. albicans* showed dependence of batumin effectiveness at the stage of biofilm formation (Table 2).

Addition of batumin to the cultivation medium simultaneously with *C. albicans* did not influence biofilm formation of fungi in 40% of cases, and promoted reduction of biofilm formation values in 60% of cases for 63.1 ± 3.4% of the initial level ($P < 0.05$).

Addition of batumin in 90 min after incubation of *C. albicans* (an initial stage of biofilm formation) reduced values of biofilm formation in 70% of cases on average by 48.8 ± 4.4% versus control ($P < 0.05$). Addition after 24 hours of growth, the antibiotic reduced formation of biofilm in culture in 50% of cases versus 24.8% of control ($P < 0.05$), and in 50% of cases — stimulated this parameter by 32.4%.

After incubation of *C. albicans* with the antibiotic during the 48 hours in 70% of cases the lack of the preparation influence was noted and only in 30% of strains decrease in biofilm formation was noted only by 5.6%.

Thus, the biofilm of the studied strains of *C. albicans* is sensitive to batumin at early stages of its formation whereas the well-established biofilm was more resistant to studied preparation.

On the contrary, batumin in 22.2% of cases stimulated low level of biofilm formation in yeast-like fungi. Possibly, the obtained data reflect the developed relationship between species of microorganisms in a microbiocenosis, as it is known that in interaction of bacteria of the genus *Pseudomonas* and fungi of the genus *Candida* there is a mutual depression of biofilm formation of microorganisms at all stages of development [15, 16].

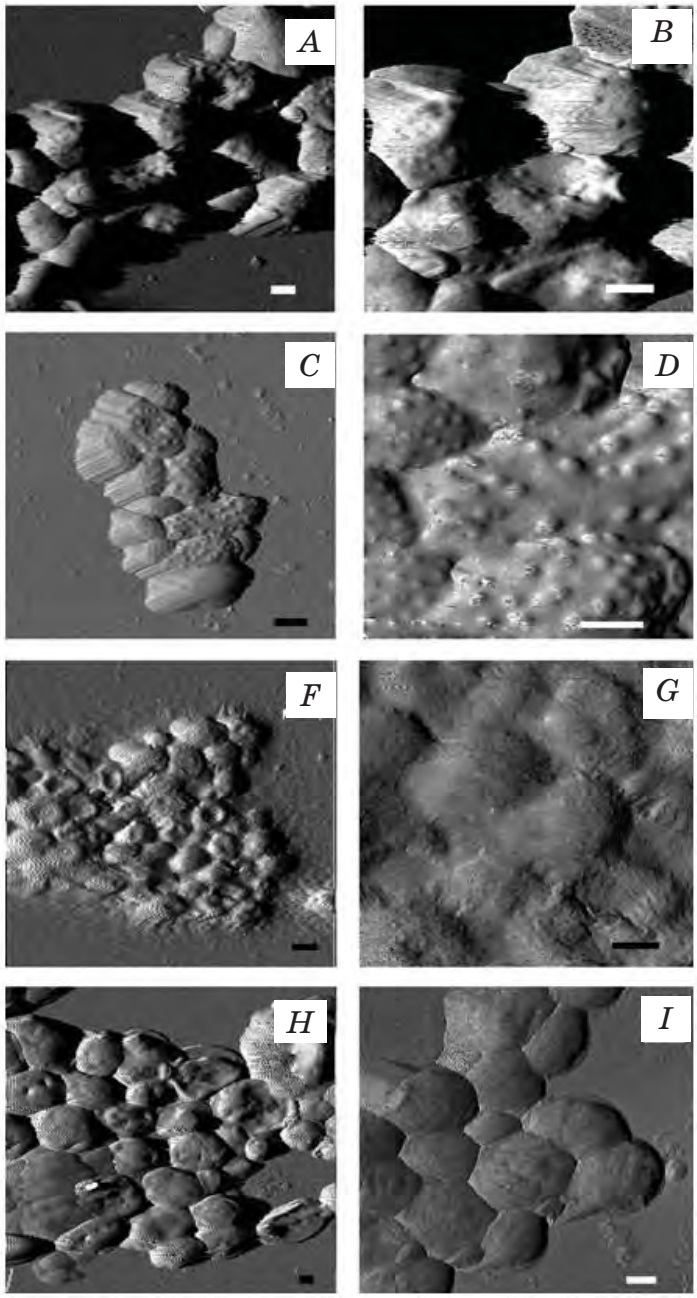
By its action on biofilms of cultures of *Candida* batumin is similar to action on biofilms of *Staphylococci* [13]. Sidrim et al. (2015) reported a similar effect when studying β-lactam antibiotics and vancomycin effects on formation of biofilms of *Candida* spp.

A more detailed study of batumin action upon *C. albicans* biofilm formation was carried out using atomic force microscopy for *C. albicans* ATCC 24433^T and *C. albicans* 127 (clinical isolate).

On the coverslip incubated with the cells *C. albicans* ATCC 24433^T without batumin the set of cells adhered to its surface (Figure, *A*). The glass coverslips were immersed into Luria-Bertani broth with 0.125 μg/ml of batumin and incubated for 48 h at 37 °C. At visualization of the surface of microorganisms, the spherical formations which are presumably gemmating daughter cells (Figure, *B*) are clearly visible. However, a significant decrease of the number of adherent cells in the presence of the antibiotic was not observed (Figure, *C*, *D*).

A detailed study of the surface of bacterial cells treated with batumin allows establishing significant reduction of their roughness values (Table 3).

The cells of *C. albicans* 127 were observed in the form of a monolayer of separate islets (Figure, *F*). This strain formed biofilm on the



Atomic force microscopy — topography images of *C. albicans* ATCC 24433^T ((*A*) (*B*)) without batumin; ((*C*) (*D*)) — in the presence of 0.125 μg/ml of batumin and *C. albicans* 127 without batumin (*F*, *G*), with antibiotic (*H*, *I*)

Scale bars: 1 μm.

surface of the glass, as can be seen. Surface biofilm was formed by an exopolymeric matrix with cells of round shape immersed in it (Figure, *G*). The addition in the medium of batumin caused, on the one hand, the lack of signs of an exopolymeric matrix (Figure, *H*, *I*), and on the other hand, the change of cell morphology. So the mean diameter of cells was equal to 1.88 ± 0.43 μm, which was significantly less than control values 2.29 ± 0.20 (Table 3).

Atomic force microscopy revealed qualitative changes in the exopolymeric matrix due to batumin treatment, as well as a significant reduction in the number of cells adhered to the coverslip, preventing formation of *C. albicans* 127 biofilm. In *C. albicans* ATCC 24433^T, a significant reduction in the number of cells adhered to the coverslip was not observed. In this case a nonspecific interaction of batumin and a surface ligand of *C. albicans* ATCC 24433^T is probable.

Table 2. **Influence of batumin (0.125 μg /ml), expressed as CU*, after addition at different stages of biofilm formation by *Candida albicans* strains**

C. albicans strains	Before	At 0 h	After 90 min	After 24 h	After 48 h
ATCC 24433^T	2.4	**2.6**	1.7	1.7	2.4
118	2.9	1.7	1.8	2.9	2.8
123	1.8	**2.0**	**1.8**	**2.3**	**2.0**
173	2.1	**2.1**	2.0	**2.8**	2.1
174	1.8	**2.1**	1.8	**2.6**	1.8
127	2.9	1.8	1.8	2.6	2.9
128c	4.3	2.5	3.0	3.5	4.0
146	3.4	2.1	2.2	2.4	3.4
147	3.2	2.1	2.5	3.0	3.2
112	2.9	1.8	1.9	**3.6**	2.9

Note: * — CU: conventional unit: OD630 in experimental samples / OD_{630} in control samples; ** — the results are representative on three separate experiments. $P < 0.05$ (Mann-Whitney U-test).

Table 3. **Morphological characteristics of *C. albicans* ATCC 24433^T and *C. albicans* 127 in the presence of batumin 0.125 μg/ml**

C. albicans strain	Batumin concentration (μg/ml)	Adherent cells,%	Length (μm)	Width (μm)	Height (μm)	Roughness values (nm)
ATCC 24433	0	100.0 ± 11.0	3.50 ± 1.17	2.09 ± 0.50	1.86 ± 0.53	49.4 ± 12.3
	0.125	47.0 ± 4.0*	3.89 ± 1.38	1.91 ± 0.61	1.09 ± 0.35*	41.0 ±16.0
127	0	100.0 ± 11.0	2.66 ± 0.45	2.29 ± 0.20	1.85 ± 0.25	45.6 ± 6.5
	0.125	15.0 ± 5.5	2.77 ± 0.42	1.88 ± 0.43*	1.62 ± 0.15	44.2 ± 14.4

Note: * — $P < 0.05$ (Mann-Whitney U-test).

The results presented in this work showed that all studied *C. albicans* and *C. krusei* strains were highly resistant against batumin (MIC ≥ 512 μg/ml). However, the antibiotic showed inhibition of biofilm formation of these microorganisms.

The biofilm of the studied strains of *C. albicans* and *C. krusei* was sensitive to batumin at early stages of its formation, whereas the well-established biofilm was more resistant to studied preparation.

The data obtained by an atomic-force microscopy confirm the ability of batumin to prevent formation of biofilm in the studied strains that allows to consider it as the preventive agent for treatment of yeast-like fungi of the genus *Candida*.

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АКТИВНІСТЬ *in vitro* АНТИБІОТИКА БАТУМІНУ ЩОДО БІОПЛІВОК *Candida albicans*

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Метою роботи було вивчити дію батуміну на штами *Candida albicans* і *Candida krusei* в планктонній та біоплівковій формі, а також одержати більш детальні відомості щодо впливу батуміну на формування біоплівки з використанням атомно-силової мікроскопії. Мінімальну інгібувальну концентрацію (МІК) батуміну досліджували відповідно до стандартів CLSI. Формування біоплівки вивчали фотометричним методом O'Toole за допомогою планшетного фотометра ELx808 (BioTek, USA) за довжини хвилі 630 нм.

Полікетидний антибіотик батумін має високу селективну активність стосовно стафілококів. Водночас антибіотик, до є неактивний стосовно дріжджів роду *Candida* (МІК 512 мкг/мл), показав інгібувальну дію на формування біоплівок у цих мікроорганізмів. Вплив батуміну на утворення біоплівки вивчали на типових і колекційних штамах *C. albicans*, *C. krusei* та клінічних ізолятах. Присутність у середовищі 0,125 мкг/мл батуміну (1/2 МІК для стафілококів) знижувало утворення біоплівки у 55,6% досліджуваних штамів. Їхні значення варіювали для *C. albicans* від 1,5 до 3,9 УО, для *C. krusei* від 2,3 до 3,0. Батумін був ефективніший щодо штамів *Candida* з високими значеннями біоплівкоутворення.

Атомно-силова мікроскопія виявила якісні зміни в екзополімерному матриксі за дії батуміну, а також значне зменшення кількості адгезованих клітин, запобігаючи утворенню біоплівки *C. albicans* 127. Однак у *C. albicans* ATCC 24433 суттєвого зменшення числа адгезованих клітин у присутності антибіотика не спостерігалося.

Отримані за допомогою атомно-силової мікроскопії дані підтверджують здатність батуміну запобігати утворенню біоплівки у досліджуваних штамів, що дає змогу розглядати його як превентивний засіб при лікуванні інфекцій, які спричинені дріжджоподібними грибами роду *Candida*.

Ключові слова: батумін, *Candida*, біоплівка, атомно-силова мікроскопія.

АКТИВНОСТЬ *in vitro* АНТИБИОТИКА БАТУМИНА В ОТНОШЕНИИ БИОПЛЕНОК *Candida albicans*

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Целью работы было изучение действия батумина на штаммы *Candida albicans* и *Candida krusei* в планктонной и биопленочной форме, а также получение более подробных сведений о влиянии батумина на формирование биопленки с использованием атомно-силовой микроскопии. Минимальную ингибирующую концентрацию (МИК) батумина исследовали в соответствии со стандартами CLSI. Формирование биопленки изучали фотометрическим методом O'Toole с помощью планшетного фотометра ELx808 (BioTek, USA) при длине волны 630 нм.

Поликетидный антибиотик батумин обладает высокой селективной активностью в отношении стафилококков, в то же время антибиотик, будучи неактивным в отношении дрожжей рода *Candida* (МИК 512 мкг/мл), показал ингибирующее действие на формирование биопленок у этих микроорганизмов. Влияние батумина на образование биопленки изучали на типовых и коллекционных штаммах *C. albicans, C. krusei*, а также клинических изолятах. Присутствие в среде 0,125 мкг/мл батумина (1/2 МИК для стафилококков) снижало образование биопленки у 55,6% исследуемых штаммов. Их значения варьировали для *C. albicans* от 1,5 до 3,9 УЕ, для *C. krusei* от 2,3 до 3,0. Батумин был более эффективен в отношении штаммов *Candida* с высокими значениями биопленкообразования.

Атомно-силовая микроскопия выявила качественные изменения в экзополимерном матриксе при действии батумина, а также значительное уменьшение числа адгезированных клеток, предотвращая образование биопленки C. albicans 127. Однако у *C. albicans* ATCC 24433 существенного уменьшения числа адгезированных клеток в присутствии антибиотика не наблюдалось.

Полученные с помощью атомно-силовой микроскопии данные подтверждают способность батумина предотвращать образование биопленки у исследуемых штаммов, что позволяет рассматривать его как превентивное средство при лечении инфекций, вызванных дрожжеподобными грибами рода *Candida*.

Ключевые слова: батумин, *Candida*, биопленка, атомно-силовая микроскопия.