https://doi.org/10.31925/farmacia.2022.3.19 **ORIGINAL ARTICLE**

ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF SOME 2- AND 1,2-SUBSTITUTED BENZIMIDAZOLE DERIVATIVES

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Manuscript received: November 2021

Abstract

Benzimidazoles are a large class of compounds with many clinical and pharmacological applications. At present, there is an intense and renewed effort to synthetize new derivatives starting from the well-known benzimidazole moiety and to explore their properties. Due to the increasing antibiotic resistance of pathogens, a major objective is to find new efficient anti-bacterial agents. In this context, the study was carried out in order to estimate the antimicrobial potential of four 2- and 1,2- substituted benzimidazole derivatives already known to have photoprotective properties. Their antimicrobial capacity was assessed by diffusimetric tests by using 13 bacterial and 7 *Candida* strains and further completed by Minimum Inhibitory Concentration (MIC), and time-kill assay. The mean values of MIC varied between a minimum of 87.5 µg/mL and a maximum of 200 µg/mL in the *Staphylococcus* group, while for *Candida* values between 104.6 µg and 151.78 µg/mL were recorded. The results demonstrate a significant inhibitory activity against Gram-positive bacteria and *Candida* strains. Experiments of time-kill carried out on *Candida* showed a concentration-dependent activity pattern.

Rezumat

Benzimidazolii sunt o clasă mare de compuși cu numeroase aplicații în practica clinică și farmacologie datorită proprietăților lor bioactive valoroase. În prezent, există un efort intens de a sintetiza noi derivați plecând de binecunoscutul nucleu benzimidazolic și de a le explora proprietățile. În acest context, studiul a fost realizat cu scopul de a estima capacitatea antimicrobiană a patru derivați substituiți 2- și 1,2- ai benzimidazolului, cunoscuți pentru proprietățile lor fotoprotectoare. Potențialul antimicrobian a fost evaluat prin testul difuzimetric, determinarea concentrației minime inhibitorii și prin evaluarea mortalității microorganismelor în funcție de timp. Valoarea medie a concentrației minime inhibitorii (CMI) a variat între 87,5 µg/mL și 200 µg/mL la *Staphylococcus*, în timp ce la *Candida* s-au înregistrat valori cuprinse între 104,6 µg/mL și 151,78 µg/mL. Rezultatele demonstrează o activitate inhibitorie semnificativă față de bacteriile Gram-pozitive și față de tulpinile de *Candida*. Experimentele asupra mortalității celulare în timp, realizate pe tulpinile de *Candida*, au evidențiat un model de acțiune dependent de concentrație.

Keywords: benzimidazoles derivatives, antimicrobial activity, *Staphylococcus, Candida*

Introduction

Substituted benzimidazoles 2- and 1,2- have a wide potential for biological activities. For this reason, the benzimidazole scaffold is used extensively in chemistry synthesis to obtain new derivatives with various properties. Numerous properties of benzimidazole substituted derivatives 1- and 1,2- have been described over time. Biological properties of these benzimidazole derivatives include: modulators of cardiovascular activity [11], anticancer potential [4, 21], antiparasitic effect [3], antioxidant [20], antibacterial [3, 10, 17], antifungal activity [1] and antitubercular activities [5]. Benzimidazole derivatives are used successfully in the drug industry. Proton pump inhibitors are derived from benzimidazole, having an asymmetric sulphur atom in their structure, which provides chiral properties to these molecules. Proton pump inhibitors are among the most widely used drug classes in therapy today, being prescribed in pathologies related to gastric hyperacidity. Among the most used compounds with antiparasitic action are benzimidazole derivatives: albendazole, mebendazole, thiabendazole.

Pathogenic microorganisms are a real danger especially for people with a weakened or even compromised immune system, for children and new-borns, for diabetics, for people with a certain type of diet, with various chronic diseases or who have undergone

antibiotic therapy for a long time. Many of the infections caused by them can have a serious evolution until death or are particularly contagious, posing a great danger to people who come in contact with infected people [23, 24]. For these reasons, the range of antimicrobial and antifungal medicinal products is constantly expanding so that there are viable alternatives to combat the harmful effects of pathogenic micro-organisms.

In a series of previous papers, it has been reported the obtaining and characterization of four benzimidazole derivatives, 1-benzyl-2-phenyl-1H-1,3-benzimidazole (C1), 1-(4-methylbenzyl)-2-(4-methylphenyl)-1H-1,3-benzimidazole (C2), 1-(4-carboxy)-phenyl-2-(4-methoxyphenyl)-1H-1,3-benzimidazole (C3), 1-(4-dimethylamino) benzyl-2-(4-dimethylamino-phenyl)-1H-1,3-benzimidazole (C4). The compounds showed a low degree of toxicity to animal organisms and they were the basis for obtaining pharmaceutical products with photoprotective activity [13, 14]. Herein we report on the antibacterial and antifungal properties of the four derivatives.

Materials and Methods

To test the effect of C1, C2, C3 and C4 compounds we used 13 bacterial strains and 7 fungal strains (Table I). C1 - C4 compounds were dissolved in 5% (w/v) dimethylsulphoxide (DMSO) to a final concentration of 10 mg/mL. 25 µL of each compound were pipetted in wells and plates were incubated for 48 h at 35ºC. Inhibitory activity of C1 - C4 was evaluated as mm inhibition zones.

Table I

Bacterial and fungal strains tested against C1 - C4 benzimidazole derivatives

No. crt.	Strain	Observation
1	*Citrobacter sp* IC1	Isolated from clinical specimens
2	*Escherichia coli* IC1	Isolated from clinical specimens
3	*E. coli* ATCC 25922	Reference strain
4	*Proteus* IC1	Isolated from clinical specimens
5	*Proteus* IC2	Isolated from clinical specimens
6	*Pseudomonas* IC1	Isolated from clinical specimens
7	*Pseudomonas* IC2	Isolated from clinical specimens
8	*Pseudomonas aeruginosa* ATCC 27853	Reference strain
9	*Staphylococcus* IC1	Isolated from clinical specimens
10	*Staphylococcus* IC2	Isolated from clinical specimens
11	*Staphylococcus* IC3	Isolated from clinical specimens
12	*Staphylococcus* IC4	Isolated from clinical specimens
13	*Staphylococcus aureus* ATCC 25923	Reference strain
14	*Candida albicans* IC1	Isolated from clinical specimens
15	*Candida albicans* IC2	Isolated from clinical specimens
16	*Candida albicans* IC3	Isolated from clinical specimens
17	*Candida albicans* IC4	Isolated from clinical specimens
18	*Candida albicans* IC5	Isolated from clinical specimens
19	*Candida albicans* IC6	Isolated from clinical specimens
20	*Candida albicans* ATCC 10231	Reference strain

Difusimetric test assay

Candida strains. *Candida* susceptibility to C1 - C4 derivatives was assessed by the well diffusion method [12, 22]. *Candida* strains were sub-cultured on SDA (Sabouraud Dextrose Agar) plates for 48 h at 35ºC. Inoculum was prepared by suspending five colonies in sterile saline solution. Turbidity of suspension was adjusted to 0.5 Mc Farland and SDA plates were inoculated by flooding the agar surface with 0.5 mL of cell suspension. The plates were allowed to dry for ½ h and wells were cut in agar plates (4 mm in diameter).

Bacterial strains. A similar procedure was used to estimate the inhibitory activity against bacteria. Bacterial strains were sub-cultured 24 h prior experimentation on Mueller Hinton Agar (MHA) plates and incubated at 37ºC. In case of *Staphylococcus*, after overnight incubation on Tryptone Soy Agar (TSA), 1 - 2 colonies were selected and diluted in sterile saline solution to achieve a density equal to 0.5 McFarland standard. Other bacterial strains were inoculated in Mueller Hinton Broth (MHB) and incubated overnight at 37ºC, diluted to a density equivalent 0.5 Mc Farland and bacterial suspensions were inoculated onto MHA plates, the excess was removed and inoculated agar plates were kept inverted 30 min to allow the surface media to dry. Then, we cut holes in agar by using a sterile test tube (8 - 9 mm in diameter) and 25 µL of each compound were pipetted into the wells. After compound diffusion, the plates were incubated at 37ºC and they were checked at 24 and again at 48 h. The effect of bezimidazole derivatives was assessed as mm of inhibition zones. Triplicate determinations were performed for each sample. Results are expressed as mean ± S.D (standard deviation) of triplicate analysis.

MIC testing. To estimate MIC we used the macro-broth dilution procedure. C1 - C4 compounds were dissolved in DMSO then were diluted two fold in MHB and Sabouraud Dextrose broth (SDB) to reach a concentration between 31.25 μg/mL and 500 μg/mL. *Candida* strains were incubated in SDB for 48 h at 35ºC and 100 μL were pipetted in test tubes containing C1 - C4 at the above concentrations. Inoculated test tubes were incubated at 37ºC and growth was checked up at 24 and again at 48 h. MIC was recorded as the highest concentration of each compound with no visible turbidity.

Reference strains (ATCC) are used to compare and recognize strains isolated from various pathological samples.

Time-kill assay. The test was performed with two concentration of compounds (125 and 500 μg/mL). Test tubes with broths and compounds were inoculated with fungal suspension so that the final density was between 8 x 10^5 and 9 x 10^5 CFU/mL. The time-kill assay was carried out by sampling 100 μL and inoculating broths by plating out onto SDA over at different time period (0, 2, 4, 8 and 24 h). Inoculation was done in triplicate and incubated for 48 h. We used both negative controls (un-inoculated broths) and positive controls (inoculated broths with respective strain without compounds). After incubation, the number of colonies developed was counted and the average value was plotted against time.

Statistical analysis

Statistical analysis was implemented using the open-source software R (R version 4.1.3.) [19]. When the number of observations is relatively small ($n < 20$), results from some traditional methods can differ notably from reality so we have to perform a computational approach meaning a Bootstrap version of two-way ANOVA. Because the interaction between levels of the two factors consists of one value, we considered that there was no variability. From *Post-hoc* comparisons we figured out which groups in your sample differ. Statistical significance level was considered at alpha 5% ($p < 0.05$).

Results and Discussion

Difusimetric assessment of inhibitory activity

Antibacterial activity of C1-C4 was in general weak, showing small inhibition zones ranging from 2.16 mm to 9.5 mm (Table II). *Staphylococcus* strains seemed to be a little more sensitive than Gram negative bacteria (Table IV). A higher inhibitory activity was recorded against *Candida*. In general, inhibition zones diameters varied from 5.16 to 9.16 mm. C3 and C4 compounds were more efficient in inhibiting *Candida* growth than C1 and C2 derivatives (Table III). Since we noticed a difference between Gram-negative on the one hand and Gram-positive bacteria and *Candida* on the other, we tested only the latter groups to establish MIC value (Table IV and Table V).

As we earlier stated, benzimidazole derivatives are very useful considering their biological potential and clinical applications. The biological potential is very wide ranging from antioxidant, antidiabetic and anti-cancer to antimicrobial activity (antimalarial, anti-bacterial, antifungal and antiviral) [18]. Kumar *et al.* synthesized and tested 2-substituted benzimidazole derivatives against bacteria and fungi [9]. They found that derivatives had a pronounced inhibitory activity against bacteria such as *Bacillus subtilis*, *P. aeruginosa* and *S. aureus*. Also, these derivatives were active against several species of fungi (*C. albicans*, *A. niger*, *Saccharomyces sp*). The inhibition zones ranged from 7 to 11 mm, data very similar to our findings.

Table II

Inhibitory effect of benzimidazole derivatives (C1 - C4) against the tested bacterial strains

No. crt.	Strain	Compound Inhibition zones (mm) (Mean ± SD)			
		C1	C2	C3	C4
1	*Citrobacter sp.* 1	7.33 ± 0.57	6.33 ± 0.28	5.5 ± 0.50	4.16 ± 0.28
2	*E. coli* IC1	2.33 ± 0.57	2.5 ± 0.50	2.5 ± 0.50	2.33 ± 0.57
3	*E. coli* ATCC 25922	4.16 ± 0.28	4.16 ± 0.28	5.33 ± 0.28	5.66 ± 0.28
4	*Proteus* IC1	2.16 ± 0.28	4.33 ± 0.28	4.5 ± 0.50	4.33 ± 0.57
5	*Proteus* IC2	2.33 ± 0.57	2.5 ± 0.50	2.16 ± 0.28	4.16 ± 0.28
6	*Pseudomonas* IC1	2.16 ± 0.28	2.66 ± 0.28	2.33 ± 0.57	2.5 ± 0.50
7	*Pseudomonas* IC2	2.16 ± 0.28	2.16 ± 0.28	2.16 ± 0.28	2.33 ± 0.57
8	*Pseudomonas* ATCC 27853	4.33 ± 0.57	2.16 ± 0.28	4.16 ± 0.28	4.33 ± 0.28
9	*Staphylococcus* IC1	4.33 ± 0.57	8.33 ± 0.57	4.5 ± 0.50	6.16 ± 0.28
10	*Staphylococcus* IC2	2.33 ± 0.57	8.16 ± 0.28	2.66 ± 0.28	2.5 ± 0.50
11	*Staphylococcus* IC3	7.16 ± 0.28	9.16 ± 0.28	5.66 ± 0.28	2.33 ± 0.28
12	*Staphylococcus* IC4	4.33 ± 0.57	8.33 ± 0.28	7.33 ± 0.57	5.16 ± 0.28
13	*Staphylococcus* ATCC 25923	7.16 ± 0.28	9.5 ± 0.50	6.33 ± 0.57	6.33 ± 0.57

SD - standard deviation

At the 95% level of confidence, we conclude there is significant difference in the inhibition zones of the four strains of *Staphylococcus* ($p < 0.05$). At the 95% level of confidence we pointed out that there is

significant difference in the inhibition zones produced by the four compounds ($p = 0.001$). From Tukey-adjusted comparisons we found a significant difference between the mean inhibition zone of *Staphylococcus* ATCC 25923 *vs*. *Staphylococcus* IC2 and relative to factor Compound there is a significant difference for C2 *vs*. the others.

For *Pseudomonas* strain, we concluded there is significant difference in the inhibition zones of the three strains ($p < 0.05$). From Tukey-adjusted comparisons we found a significant difference between the mean inhibition zone of *Pseudomonas* ATCC 27853 and *Pseudomonas* IC2.

In the case of *E. coli*, we revealed a significant difference in the inhibition zones of the tested strains ($p < 0.001$).

A highly significant difference in the inhibition zones of the seven strains of *Candida albicans* ($p < 0.001$) was identified, and at the 95% level of confidence we registered a highly significant difference in the inhibition zones produced by the four Compounds ($p < 0.001$). From Tukey-adjusted comparisons we found a significant difference between the mean inhibition zones of *Candida albicans* IC4 *vs. Candida albicans* ATCC 10231 and *Candida albicans* IC1, respectively. Also, there is a significant difference between the mean inhibition zones of *C. albicans* IC6 and the rest of the tested strains. Relative to factor Compound there is a significant difference between all levels except for the pair C1 - C2.

Table III

Inhibitory effect of benzimidazole derivatives against the tested *Candida* strains

No. crt.	Strain	Compound Inhibition zones (mm) (Mean ± SD)			
		C1	C2	C3	C4
1	*Candida albicans* IC1	6.16 ± 0.28	6.33 ± 0.57	7.66 ± 0.28	11.5 ± 0.50
2	*Candida albicans* IC2	5.33 ± 0.57	5.5 ± 0.50	7.16 ± 0.28	8.5 ± 0.50
3	*Candida albicans* IC3	5.16 ± 0.28	5.33 ± 0.57	7.33 ± 0.57	8.16 ± 0.28
4	*Candida albicans* IC4	2.66 ± 0.28	4.16 ± 0.28	7.16 ± 0.28	8.33 ± 0.57
5	*Candida albicans* IC5	5.66 ± 0.28	6.33 ± 0.57	7.5 ± 0.50	8.66 ± 0.28
6	*Candida albicans* IC6	7.33 ± 0.57	7.16 ± 0.28	10.16 ± 0.28	12.16 ± 0.28
7	*Candida albicans* ATCC 10231	6.5 ± 0.50	6.16 ± 0.28	9.16 ± 0.28	10.33 ± 0.57

SD - standard deviation

MIC estimation

On average, MIC fluctuated between a minimum of 87.5 µg/mL (C4) to a maximum of 200 µg/mL (C1) in *Staphylococcus* (Table IV). However, the variability of individual strains was larger and ranged from 62.5 µg/mL to 250.66 µg/mL (Table IV). Instead, for *Candida* group, MIC fluctuations were less variable. Most efficient was C4 while C1 had a lower potential to inhibit *Candida* strains.

Even if the MIC values are relatively close for the two groups, some differences can be observed. Thus, *Staphylococcus* as a whole seems more sensitive to the action of these benzimidazole derivatives compared to the other bacterial strains and *Candida*. Moreover, while *Staphylococcus* is mainly inhibited by C2 and C4, *Candida* is preferentially inhibited by C3 and C4 (Table IV and Table V).

Table IV

MIC value of tested compounds against *Staphylococcus* strains (µg/mL)

No. crt.	Strain	Compound MIC value (µg/mL) (Mean ± SD)			
		C1	C2	C3	C4
1	*Staphylococcus* IC1	125.5 ± 0.50	62.5 ± 0.50	62.5 ± 0.50	62.5 ± 0.50
2	*Staphylococcus* IC2	250.16 ± 0.28	125.16 ± 0.28	250.33 ± 0.57	125.16 ± 0.28
3	*Staphylococcus* IC3	250.16 ± 0.28	250.66 ± 0.28	250.33 ± 0.57	62.5 ± 0.50
4	*Staphylococcus* IC4	250.66 ± 0.28	62.5 ± 0.50	62.5 ± 0.50	62.5 ± 0.50
5	*Staphylococcus aureus* ATCC 25923	125.33 ± 0.57	125.33 ± 0.57	125.66 ± 0.28	125.16 ± 0.28

SD - standard deviation

Very similar results of the inhibitory activity of some new benzimidazole derivatives were reported by Özkay *et al*. with MIC able to inhibit *Candida* that ranged from 50 to 200 µg/mL [16]. Ansari *et al*. reported that the antimicrobial activity of new synthesized derivatives of benzimidazole was also variable as potency [2]. They were more active against Gram-negative bacteria (*E. coli*, *P. aeruginosa* and *S. typhimurium*) than against Gram-positive bacteria (*S. aureus*, *B. subtilis* and *S. mutans*).

Also their derivatives were more active against *Candida* strains than against *Aspergillus* (*A. niger* and *A. flavus*). Mavrova *et al*. reported on the synthesis and anti-bacterial activity of some new benzimidazoles. These authors found that the inhibitory potential against

Gram-negative and Gram-positive bacteria ranged from 0.016 mg/mL to 1.0 mg/mL [15].

Table V

MIC value of tested compounds against *Candida* strains (μg/mL)

No. crt.	Strain	Compound MIC value (μg/mL) (Mean ± SD)			
		C1	C2	C3	C4
1	*Candida albicans* IC1	125.16 ± 0.28	125.33 ± 0.57	125.66 ± 0.28	62.5 ± 0.50
2	*Candida albicans* IC2	62.5 ± 0.50	125.16 ± 0.28	62.5 ± 0.50	125.33 ± 0.57
3	*Candida albicans* IC3	250.16 ± 0.28	125.33 ± 0.57	125.66 ± 0.28	125.5 ± 0.50
4	*Candida albicans* IC4	125.33 ± 0.57	62.5 ± 0.50	62.5 ± 0.50	125.16 ± 0.28
5	*Candida albicans* IC5	125.66 ± 0.28	125.33 ± 0.57	125.16 ± 0.28	62.5 ± 0.50
6	*Candida albicans* IC6	125.33 ± 0.57	250.16 ± 0.28	125.33 ± 0.57	62.5 ± 0.50
7	*Candida albicans* ATCC 10231	250.33 ± 0.57	125.66 ± 0.28	125.16 ± 0.28	125.33 ± 0.57

SD - standard deviation

Time-kill

When exposed to 500 μg/mL, *Candida albicans* ATCC showed a similar pattern of mortality against all compounds. All derivatives were able to kill the whole population cell in 8 h (Figure 1).

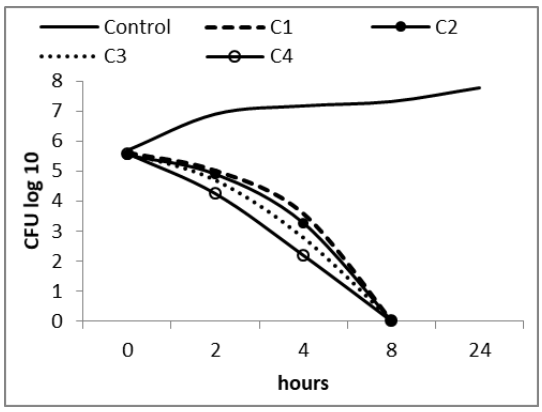

Figure 1.

Time-kill activity of C3-C4 benzimidazole derivatives in *Candida albicans* ATCC 10231 (compound concentration 500 μg/mL)

However, C3 and C4 seemed to be more active with a sharp slope of killing. We tested also two clinical strains IC3 and IC4 at the same concentration. One strains, IC3, showed a slower mortality trend, the entire cell population being killed after 24 h (Figure 2). At the lower tested concentration (125 μg/mL) the mortality of IC3 against C4 was even slower (Figure 3). Cell density decreased at 24 h from 5.2 $\log_{10}$ to 2.85 $\log_{10}$ (Figure 3).

The same strain showed a similar trend in the case of C3, all populations decreasing from 5.73 $\log_{10}$ to 3.69 $\log_{10}$ after 24 h of exposure (Figure 4). We also tested three clinical isolates against the C2 derivative. Cell mortality was very similar for IC1 and IC5 and viable counts showed a minimal variability between 3.12 $\log_{10}$ and 3.0 $\log_{10}$ after 24 h of incubation. The third strain, IC2, proved to be a little more resistant with a higher level of population still alive at the end of observation period (Figure 5).

Time-kill dynamics showed a dose-dependent behaviour of *Candida* in the presence of C2, C3 and C4 derivatives. At lower concentration than 500 μg/mL, these compounds had fungistatic properties only.

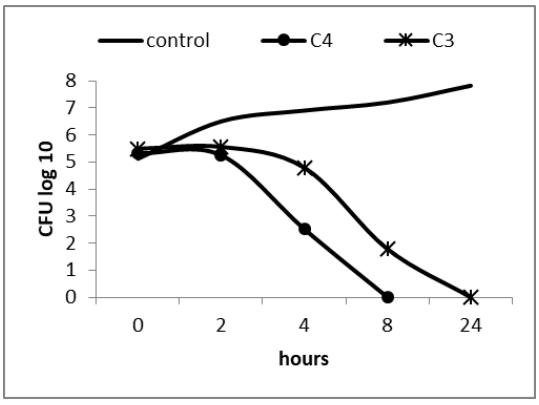

Figure 2.

Time-kill activity of C3-C4 benzimidazole derivatives in *Candida albicans* IC3 and *Candida albicans* IC4 (compound concentration 500 μg/mL)

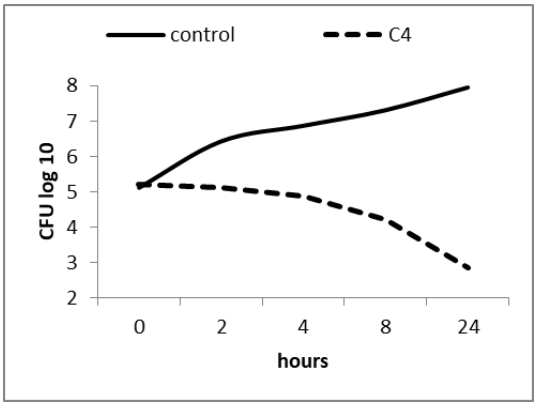

Figure 3.

Time-kill activity of C4 benzimidazole derivative in *Candida albicans* IC3 (compound concentration 125 μg/mL)

In a paper exploring the structure-activity relationship (SAR), Krisnanjaneyulu *et al.* showed that benzimidazole derivatives containing electron-withdrawing

groups had a better inhibitory potential against micro-organisms than electron-donating compounds [7]. Another study regarding SAR reported that major differences in antimicrobial activity of some derivatives relied on the distribution of negative potential around the nitrogen and oxygen atoms [6]. It was also observed that benzimidazole derivatives containing N,N-di-methyl group had a significant activity against Gram positive bacteria (*B. subtilis*, *S. aureus*) [8].

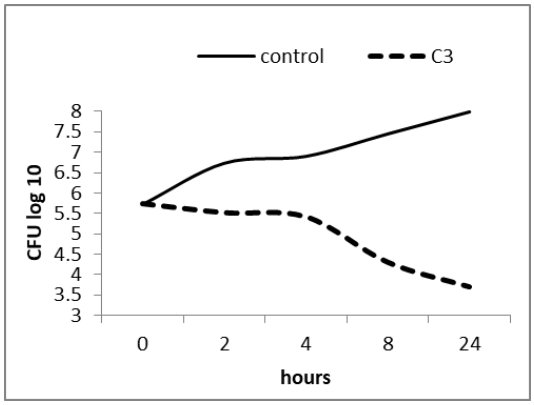

Figure 4.
Time-kill activity of C3 benzimidazole derivative in *Candida albicans* IC3 (compound concentration 125 μg/mL)

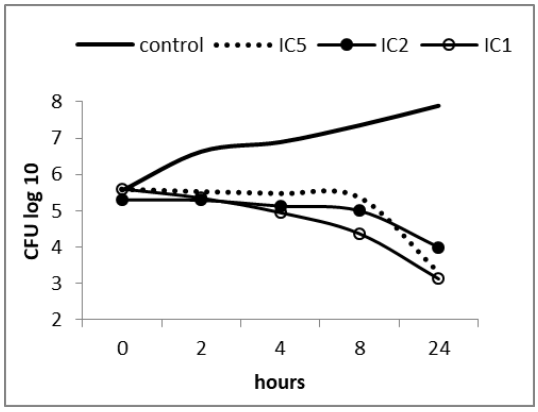

Figure 5.
Time-kill activity of C2 benzimidazole derivative in *Candida albicans* IC5, IC2, and IC1 (compound concentration 125 μg/mL)

The activity of these compounds should be considered as an inhibitory potential under laboratory conditions. Therefore, it is likely that their antimicrobial activity in different ointments will undergo some changes due to interference with conditioning agents (lanolin, beeswax, etc.). This could decrease the diffusion of benzimidazoles so that they can reach lower concentration, insufficient to inhibit the growth of microorganisms.

Conclusions

The results obtained after testing the antimicrobial action of bezimidazole derivatives taken in the study indicate a significant inhibitory potential and encourage the possibility of developing pharmaceutical products. Our results showed that 2- and 1,2- benzimidazole derivatives have moderate antibacterial activity along with significant antifungal activity. Inhibitory activity against *Candida* was mainly shown by compounds C3 and C4, with a MIC value around 100 μg/mL. Cell killing rate over time manifested in *Candida* strains a concentration-dependent pattern. The obvious anti-microbial capacity, along with its photoprotective properties and the relatively low toxicity to animal organisms, support the idea that these compounds are pharmacologically and biologically valuable. For this reason, further studies are needed to test cosmetics or pharmaceutical products containing these imidazole derivatives and evaluate their antimicrobial efficacy.

Conflict of interest

The authors declare no conflict of interest.

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