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Are the Co(III) complexes with diamine chelate ligands a response to new antifungal compounds?

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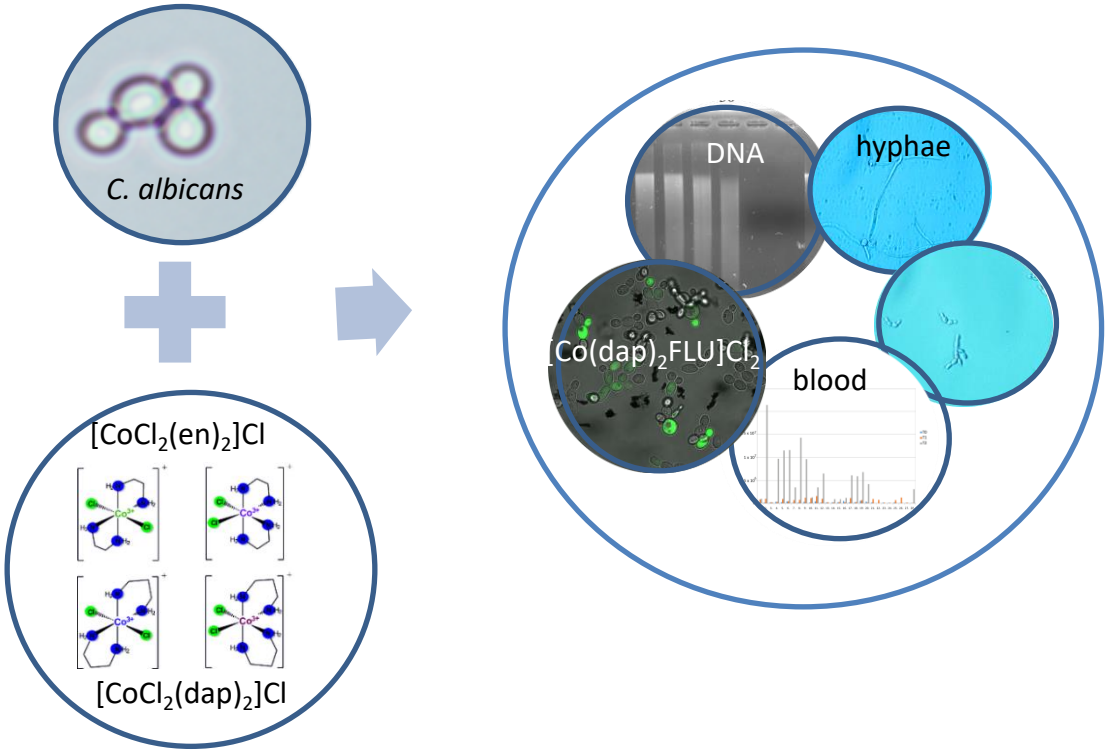
Graphical Abstract



Abstract

Increasing resistance of fungi, especially *Candida* spp., successively has reduced the already short list of effective antifungal drugs used in clinical therapy. Thus there is an urgent need for new, non-toxic antifungals with novel mode of action. A very interesting and attractive group of compounds seems to be complexes of Co(III) with diamine chelate ligands due to their therapeutic uses as antiviral, antibacterial, antifungal, antiparasitic, or antitumor agents. Two Co(III) complexes with diamine chelate ligands ($[CoCl_2(dap)_2]Cl$ (1) and $[CoCl_2(en)_2]Cl$ (2)) (where dap = 1,3-diaminopropane, en = ethylenediamine) were synthesized and characterized by elemental analysis, an ATR technique, and a scan method and sequentially tested to evaluate the mode of action. The assessment of cell damage by the newly synthetized fotosensitive fluorescein-labelled complex 1 ($[Co(dap)_2FLU]Cl_2$) was performed using fluorescent microscopy, which indicated cell membrane permeability and altered cell morphology. The study of the *C. albicans* survival in blood showed a slight reduction in the number of viable, colonizing cells in the sample compared to the results obtained for the substances with confirmed antifungal activity – ketoconazole. DNA cleavage ability of the Co(III) complexes using agarose gel electrophoresis against genomic DNA isolated from a *C. albicans* cells showed nuclease activity for both complexes. This study provides new data on potential antifungal drugs, especially against *Candida* species.

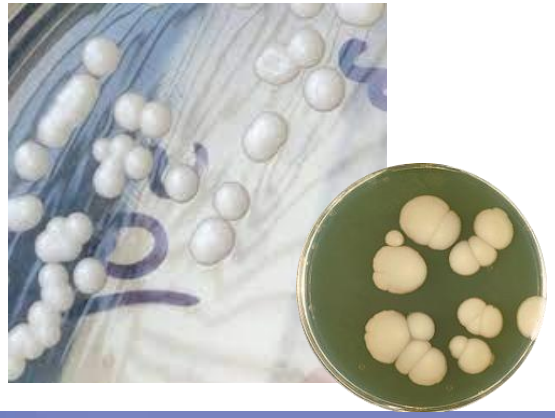
Keywords: *Candida* spp., Co(III) coordination complexes, antifungal activity, confocal microscopy, nuclease activity

Introduction

Candida spp., in particular *Candida albicans*, is one of the most important opportunistic fungal pathogens, which can harmlessly colonize the gastrointestinal tract, mouth, skin and urogenital system [1-5].

It can also cause infections, especially among people with weakened immune systems, attacking the skin, mucous membranes, getting into the blood, and attacking internal organs [6, 7].

Other species of *Candida* increasingly isolated from patients are *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei* [8].



Introduction

The list of antifungal agents used currently in clinical therapy for the treatment of infections caused by *Candida* is limited (**Polyenes, Griseofulvin, Azoles, Echinocandins, Allylamines, 5-Fluorocytosine, Cyclopirox, Amorolfine).**
Polyenes, azoles or **echinocandins**, presently considered to be the most effective in antifungal therapy.

Major targets for potential antifungal agents are:

- ergosterol (inhibiting its biosynthesis or binding to it), an essential lipid of the yeast cell membrane (not present in mammalian cells);
- chitin and β-glucan (inhibition of its synthesis), key structural components of the fungal cell wall [9-12].

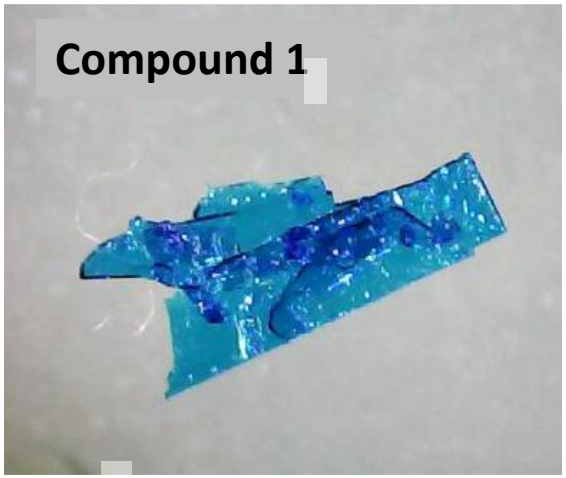
Introduction

In the search for new antifungals, due to the growing resistance to antifungal drugs, the metallopharmaceuticals is a promising alternative.

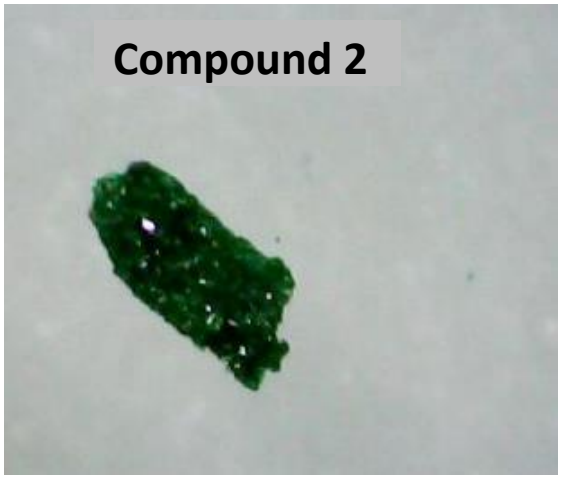
Cobalt complexes are very interesting and attractive as potential candidates with antimicrobial activity. Their therapeutic uses as antiviral, antibacterial antifungal, antiparasitic, antitumour, transferrin transporters, and anti-inflammatory agents are being intensively investigated.

In the presented study, we evaluated the mode of action of Co(III) complexes with simple bidentate inorganic ligands (en = ethylenediamine; dap = 1,3diaminopropane).

Results and discussion



Compound (1): *trans*-$[CoCl_2(dap)_2]Cl$



Compound (2): *trans*-$[CoCl_2(en)_2]Cl$

Figure 1. Microscopic images of the compounds crystals studied: *trans*-$[Co(dap)_2Cl_2]Cl$ and *trans*-$[Co(en)_2Cl_2]Cl$, twenty times zoom [13].

Fluorescent Microscopy Assay

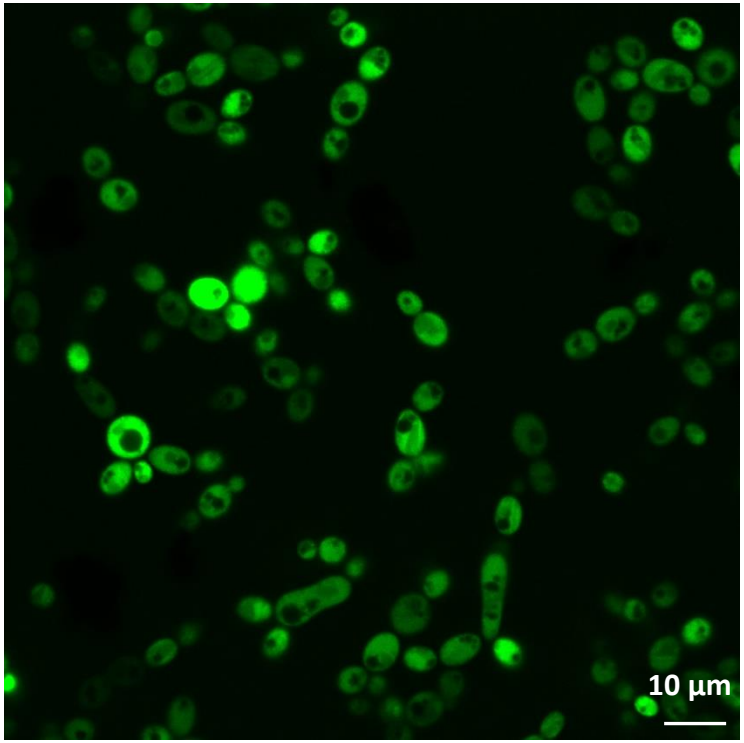


Figure 2. *Candida albicans* ATCC 10231 with FITC-labeled $[CoCl_2(dap)_2]Cl$.

C. albicans ATCC 10231 was incubated with newly synthetized fotosensitive complex FITC-labelled $[Co(dap)_2]Cl_2$ for 1 h at 37°C. Localization of the fluorescence complex in the fungal cell was visualized using a confocal microscope from Leica Microsystems. Fluorescein conjugated with compound 1 ($[Co(dap)_2FLU]Cl_2$) in subinhibitory concentrations (subMIC) penetrated the fungal cell membrane and has been found to be distributed in the cytosol.

Cells of *C. albicans* in the presence of fluorescein alone did not show any fluorescence.

Antifungal activity of Co(III) complexes against *C. albicans* strains in blood

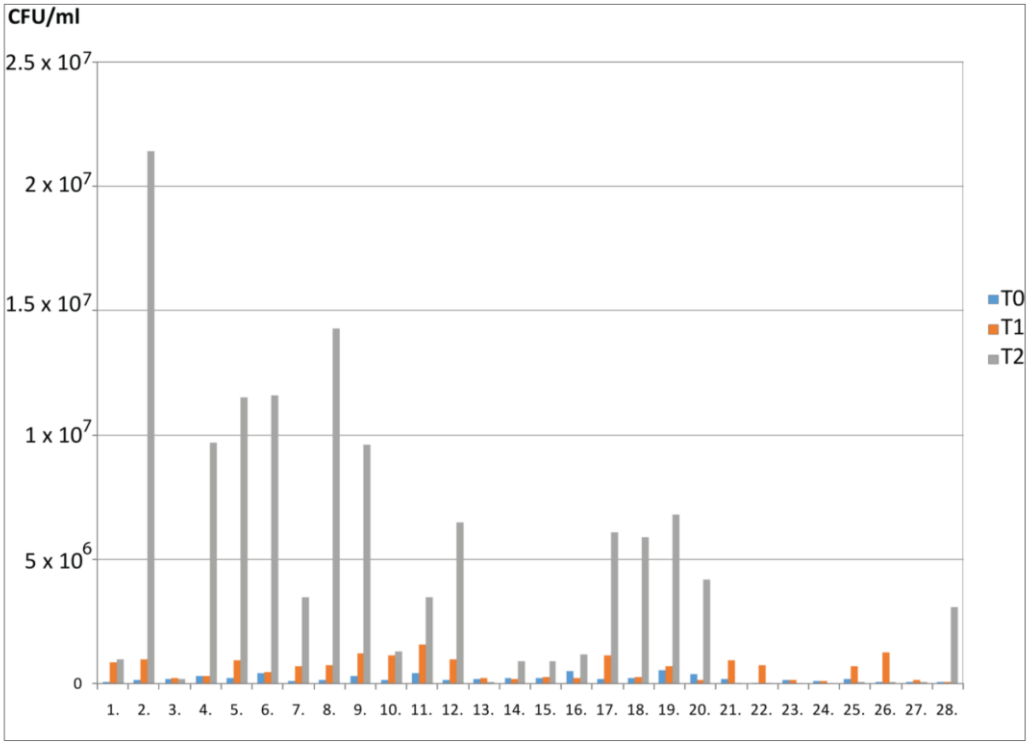


Figure 3. Number of viable cells capable of forming new *C. albicans* colonies in samples after incubation in the presence of test compounds. T0, T1 (3h), T2 (24h)

In order to study the antifungal activity of Co(III) complexes against *C. albicans* strains in blood the fungus suspension was incubated in the presence of sheep blood and Co (III) complexes. Controls were also carried out - without compounds (1. *C. albicans* ATCC 10231., 2. *C. albicans* 12823) and with ketoconazole (21.- 28.).

The growth inhibition effect was observed only in the case of *C. albicans* ATCC 10231 suspension in defibrinated sheep blood + $[CoCl_2(en)_2]Cl$ at the concentration of 63 µg/mL (13.) and at almost all concentrations of ketoconazole (21.-27.) (*C. albicans* 12823 cells increased in the presence of ketoconazole at a concentration of 241 µg / mL) (28.).

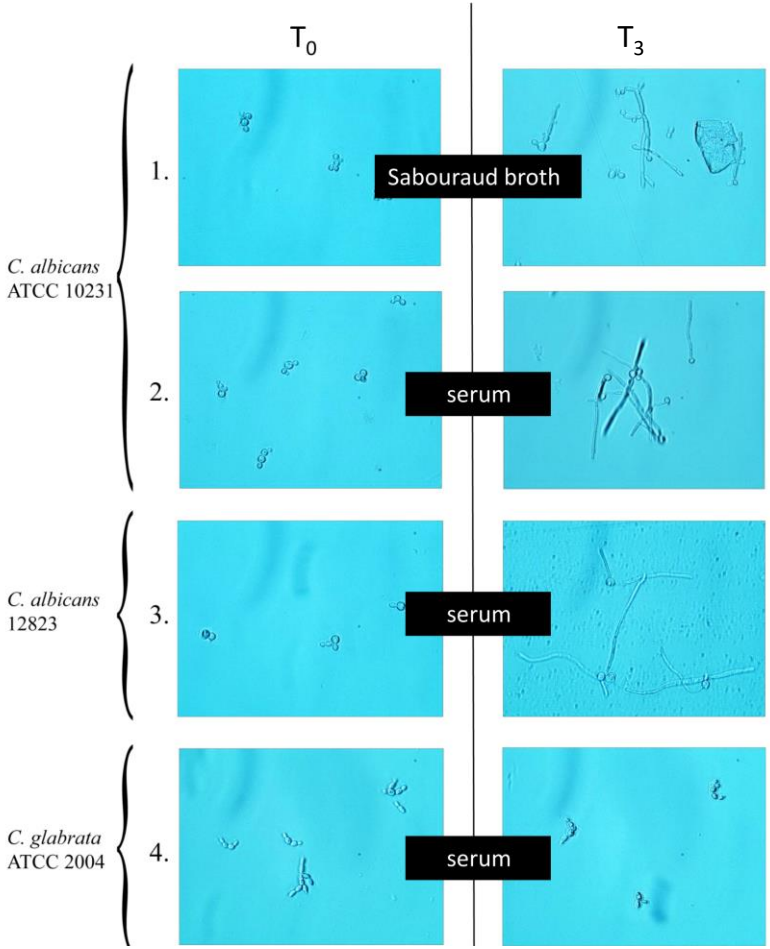
Filamentation Test (Germ Tube test)

The *Candida albicans* cells incubated in serum at 37°C for 2-4 hours, produce short, slender, tube-like structures called germ tubes. The formation of germ tubes is associated with increased synthesis of protein and ribonucleic acid. Various media like fetal bovine serum may be used as a substitute for human pooled serum.

The aim of the study was to evaluate of the filamentation inhibition activity Co (III) compounds against *Candida albicans* strains. For comparative purposes *Candida glabrata* strains and ketoconazole were also examined.

Samples containing yeast suspensions, cobalt compounds or ketoconazole were incubated in the presence of bovine serum albumin or Sabouroud broth at 37°C for 3 hours. Additionally, non-incubated samples were prepared. The filamentation inhibitory activity was assessed using an optical microscope. The obtained results are shown in Figures 3 and 4.

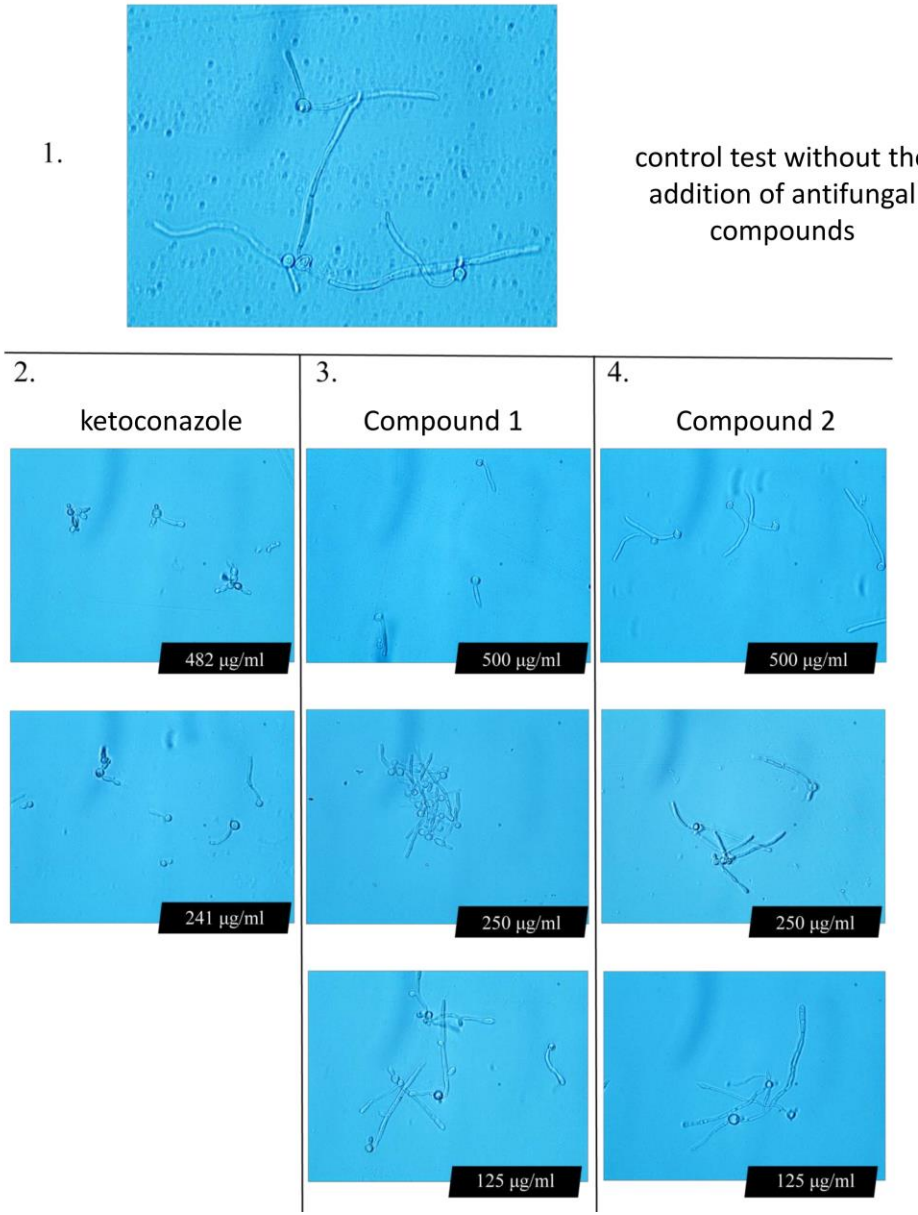
Filamentation Test (Germ Tube test)



After a 3-hour incubation, the *C. albicans* strains in the blood serum produced hyphae, thus giving a positive result in the filamentation test. Whereas, *C. glabrata* cells did not produce hyphae after incubation, so they were negative in the filamentation test.

Figure 3. Photographs of microscopic slides constituting controls in the filamentation test: 1. samples containing *C. albicans* ATCC 10231 suspension in Sabouraud medium, 2. samples containing *C. albicans* ATCC 10231 in bovine serum, 3. tests containing a suspension of *C. al*bicans 12823 in bovine serum, 4. tests containing a suspension of *C. glabrata* ATCC 2004 in bovine serum.

Filamentation Test



At the tests with the addition of compound 1, and to a lesser degree 2, the appearance of clearly smaller hyphae compared to the control sample at the concentration of 500 µg/mL was observed.

In case of the *C. albicans* in bovine serum and in the presence of ketoconazole, it can be seen that for both the ketoconazole concentrations used - 482 µg/mL and 241 µg/mL, the production of hyphae by fungal cells is limited. The resulting hyphae are very small or fungal cells do not produce them at all.

Figure 4. Microscopic slides prepared from samples containing the suspension of *C. albicans* 12823 in bovine serum with the addition: 1. Control sample without the addition of antifungal compounds, 2. Ketoconazole, 3. $[CoCl_2(dap)_2]$ Cl, 4. $[CoCl_2(en)_2]$ Cl.

DNA Cleavage Study

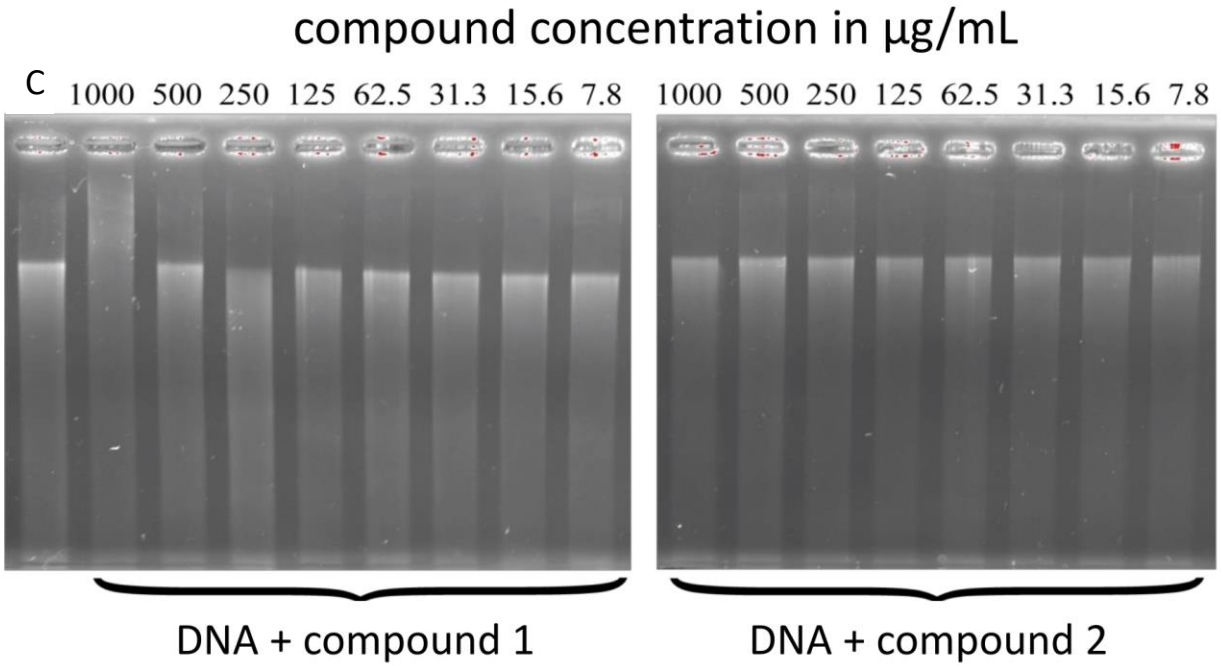


Figure 5. The cleavage of genomic DNA (62.5 µg/mL) by the complexes (in concentration range of 1000 - 7.8 µg/mL) in 5 mM Tris-HCl, 50mM NaCl buffer. DNA was isolated from *C. albicans* ATCC 10231 cells. C - control - trial without addition of compounds.

DNA Cleavage Study

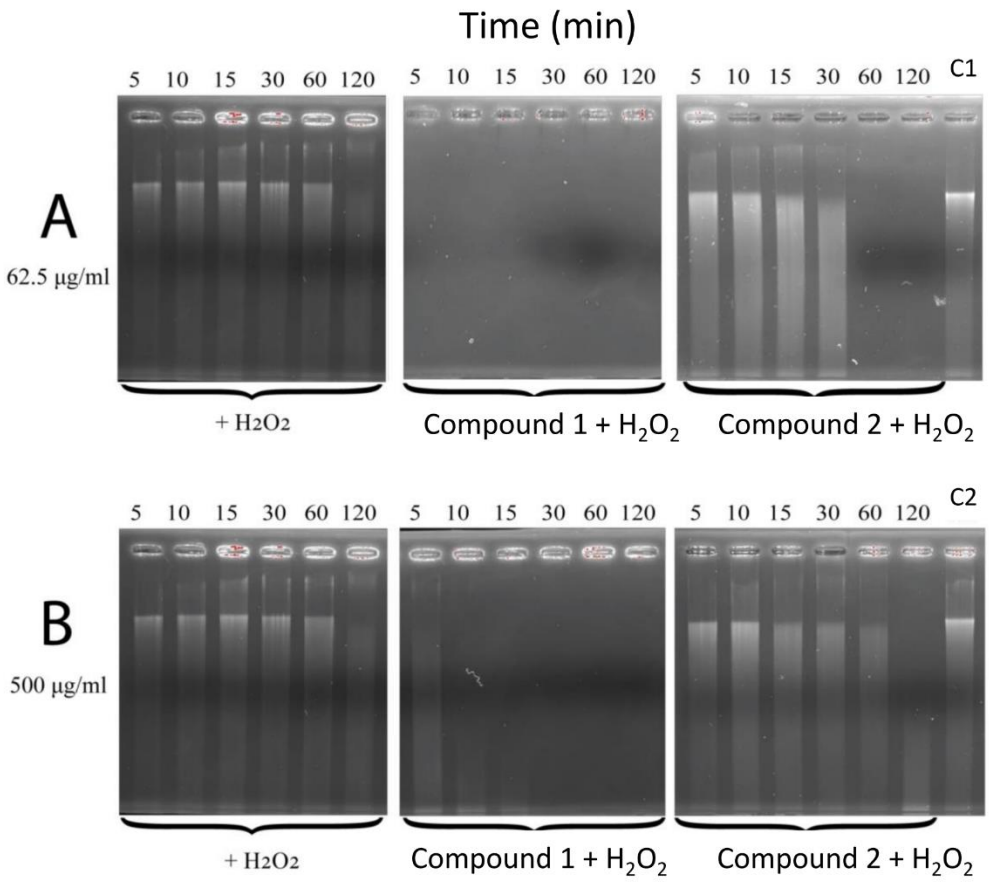


Figure 6. The cleavage pattern of genomic DNA (62.5 µg/mL) by the complexes (at the concentration of 62.5 and 500 µg/mL) in 5 mM Tris-HCl, 50mM NaCl buffer. DNA was isolated from *C. albicans* ATCC 10231 cells. C1, C2 - controls - trials without addition of compounds. **A** - DNA incubated with H_2O_2 (100 µM), compound 1 (62.5 µg/mL) + H_2O_2 (100 µM), and 2 (62.5 µg/mL) + H_2O_2 (100 µM), respectively; c, control line — DNA in buffer (5 mM Tris HCl, 50 mM NaCl) (1:1), 5–120 min, time of incubation. **B** - DNA incubated with H_2O_2 (100 µM), compound 1 (500 µg/mL) + H_2O_2 (100 µM) and 2 (500 µg/mL) + H_2O_2 (100 µM), respectively; c, control line—DNA in buffer (5 mM Tris HCl, 50 mM NaCl) (1:1), 5–120 min, time of incubation.

DNA Cleavage Study

Agarose gel electrophoresis was used to evaluate the DNA cleavage ability (metallonuclease activity) of compounds 1 and 2. Different amounts of 1 and 2 complexes were mixed with a fixed amount of genomic DNA isolated from *C. albicans* cells in the medium of 5 mM Tris-HCl, 50 mM NaCl buffer, pH 7.4, in the presence and absence of H_2O_2 and incubated for 5, 10, 15, 30, 60, and 120 min at 37 ℃. The experiment was carried out in the presence and absence of hydrogen peroxide as an oxidant.

- Control line (DNA in buffer, C) showed a non-cleaved DNA (Figure 5, 6).
- The active cleavage of the genomic DNA was observed at higher concentrations (1000 µg/mL) and only in case of the compound 1. At lower concentrations of this compound, no changes in the picture of the DNA molecule were observed compared to the control sample (C) (Figure 5). The same effect was obtained by Thamilarasan et al. (2016) with a higher concentration of the complexes ([Co(acac)(bpy)$(N_3)_2H_2O$, ([Co(acac)(en)$(N_3)_2$ and ([Co(acac)(2-pic)$(N_3)_2$, where acac = acetylacetone, bpy = 2,2'-bipyridine, en = ethylenediamine, 2-pic = picolylamine, and NaN_3 = sodium azide), and the cleavage (plasmid) was found to be much more efficient (14).
- No changes were also observed for the DNA samples treated with the compound 2 over the entire concentration range. This means that the structure of the DNA has not changed.

DNA Cleavage Study

- *C. albicans* has a diploid genome (8 pairs of chromosomes) which was first sequenced in 2004 [15]. It contains 6,100 genes and is approximately 14 million base pairs [16-18]. Perhaps the size and complexity of the DNA molecule isolated from the yeast's cells do not allow the complexes, even of such small sizes as the studied complexes of Co(III) to have an effect on DNA by digesting it.
- The results obtained for the tests containing only DNA of the *C. albicans* and H_2O_2 showed that hydrogen peroxide alone does not have such an effect as its combination with Co (III) complexes. Complete degradation of DNA only occurs after 120 minutes of incubation.
- The addition of hydrogen peroxide to the DNA + complex 1 mixture contributed to the degradation of the DNA molecule after 5 min, at both 62.5 and 500 µg/mL.
- In the case of compound 2 at a concentration of 62.5 µg/mL, DNA degradation occurred after 60 minutes of incubation, while at a concentration of 500 µg/mL after 120 minutes of incubation.
- This may be attributed to the formation of hydroxyl free radicals.

Conclusions

1. Analysis using a confocal microscope with the newly synthetized photosensitive complex of the type $[Co(dap)_2FLU]Cl_2$, showed the luminescence of the cells suggesting cell membrane permeability.
2. Ketoconazole in the blood tests presented a stronger antifungal activity than the Co(III) complexes - it reduced the number of cells in both the reference and clinical strains. The Co (III) complexes, in the studied concentration range and in the presence of blood, showed a fungistatic rather than fungicidal effect.
3. The filamentation test showed that the abilities of $[CoCl_2(dap)_2]Cl$ and $[CoCl_2(en)_2]Cl$ to inhibit hyphal morphogenesis by *C. albicans* is dependent on the concentration of the compounds tested. Only the clinical strain of *C. albicans* 12823 showed sensitivity to the tested compounds, with the compound $[CoCl_2(dap)_2]Cl$ being more active. The limitation of the hyphae production is visible only at high concentrations of the tested compounds: 500 µg/mL.
4. The DNA cleavage ability of the compounds showed, by agarose gel electrophoresis methods, nuclease activity, especially in the presence of oxidant H_2O_2 for both complexes. This phenomenon may be attributed to the formation of hydroxyl free radicals, but we did not rule out the hydrolytic mechanism of cleavage, highlighting that more tests are needed.

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